Editor:
C Moldoveanu, MD

Colchicine – 100 Years of Research

Proceedings of a Symposium on Spindle Toxins

Editura Academiei Republicii Socialiste Romania
Bucharest, 1982

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Lucrările unui Simpozion pe Tema
Toxine Fusiforme

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Abbreviations

3T3	Mouse fibroblast cell line
3T6	Cells of a mouse embryo fibroblastoma
Angstrom	10^{10} m
ATP	Adenosine Triphosphate
BHK 21	Baby hamster kidney cells
BrCN	cynogenbromide
C58	Cellosaurus 58
C5	Complement Component 5
C3H	Mouse fibroblast embryo cell line , a general purpose mouse strain
CB	Cytochalasin B
CCRFCEM	Human leukemic lymphoblasts
CM	Conditioned medium
CNBr	Cyanogen bromide
COL	Colchicine
Con A	Concanavalin A
CPM	Counts Per Minute
CPV	Cells per vision
DBA/2 mice	Commonly used inbred strain
DNA	Deoxyribonucleic Acid
DMEM	Dulbecco's Minimal Essential Medium
E. coli	Escherichia coli
EDTA	Ethylenediaminetetraacetic acid
ESC	Embryonic stem cells
FCS	Foetal calf serum
FMF	Familial Mediterranean Fever
GTP	Guanosine triphosphate
HeLa	A cancerous cell stain named after the patient Henrietta Lacks
HESF	Humane embryo skin fibroblasts
HSA	Human Serum Albumin
HT 1080	Cells of an epitheloid human fibrosarcoma
ICM	Inner cell mass
L929	A cell line established by Earl 1948, cells of a mouse fibroblastoma from an explant of subcutaneous connective tissue taken from strain C3H mouse; doubling time: 28 hours, from cell counting

L6Y1	Mouse tumour cells
LDCF-F	Lymphocyte-derived chemotactic factor for fibroblasts
MA	Mitotic apparatus
mcg	Microgram, 10^{-6} gram
mcm	Micrometre, 10^{-6} meter
MO cells	Epithelioid-type C3H mouse embryo cell line
P-1534	Acute lymphocytic leukemia
PHA	Phytohemagglutinin
PMN	Polymorphonuclear leukocytes
PPD	Purified protein derivative
PWM	Pokeweed Mitogen
RNA	Ribonucleic Acid
RER	Rough Endoplasmic Reticulum
S. aureus	Staphylococcus aureus
S. aurew	Staphylococcus aurew
SEM	Scanning Electron Microscope
SIM	Sandos inbred mice
SKSD	Streptokinase-streptodornase
SSEA-1	Stage-specific embryonic antigen-1
VBLS	Vinblastine sulphate
VCR	Vincristine
VDS	Vindesine
Velban	Vinblastine
VLBS	Vinblastine sulphate

Preface

The Bucharest Symposium from 1981 on colchicine organized by Elena Ceausescu was significant in that it provided the first text that summarized diverse studies on colchicine covering the last 100 years. Since that time, although there have been excellent workshops on microtubules and anti tubulins to provide a medium for information exchange between workers in the field, there has been no general summary of the state-of-the-art for scientists whose research interests were not directly involved with this unique pharmaceutical.

It is for this reason that the present Symposium was planned, and this book is the outcome. During the last 100 years, the colchicine field has taken creative advantage of new physical and chemical techniques to provide a wealth of new information on its interaction with microtubules and biosynthesis and its function in cell movement.

These advances have been obtained by imaginative use of many techniques, including chemical synthesis of stereoisomers of colchicine, protein sequencing of tubulin proteins, microscopy to provide insights into anti tubulin-tubulin protein interaction and the roles that they play in cell movements. There always has been interest in such subjects as the anti tubulin pharmaceuticals, and considerable success in the application of these various methods to the study of eukaryotic cell movement.

The editors have used this symposium as a basis and have tried to build on this text to bring the reader up-to-date on anti tubulin action in cell movement. Although the book presents very substantial progress, it should be patently obvious that many puzzles remain to be unravelled.

In spite of the large volume of work, we still lack details of the pharmacology, chemistry and physics of the influence of anti tubulin pharmaceuticals in cell movement as the base of metastasis of fibrosarcoma cells. The search for the key features of cellular movement will be with us for some time. However, we are confident that, as structural and functional details become collated, the pieces of the puzzle will surely fall into place.

The organizers of the symposium wish to thank Dr Elena Ceausescu for the support of the proceedings and *Publishing House VEB VERLAG VOLK UND GESUNDHEIT BERLIN, GERMAN DEMOCRATIC REPUBLIC* for their helpful encouragement. The participants in the symposium and the authors of chapters were tolerant of our organization and of our regulations for manuscripts. We thank them for their helpful contributions.

Editor: *C Moldoveanu, MD*

1. Chapter: Introduction

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Traditional medicine is becoming increasingly important in the prevention and treatment of cancer and its metastasis. Medicinal plants such as Colchicum autumnale and Periwinkle plants (vinca rosea Linn) have been known for a long time in the treatment of common diseases such as gout, liver cirrhosis, diabetes mellitus or Familial Mediterranean Fever. Both medicinal plants have in common that they produce substances which impair cell division in eukaryotes, in particular leukocytes and mesenchymal cells such as fibrosarcoma cells. They are also called spindle poisons or antitubulin agents, because they are regarded as cell-cycle-specific agents, which block mitosis. Mitosis is the only phase of the cell cycle that is visible with the light microscope. The whole process and its different stages, i.e. mitotic index such as prophase, metaphase, anaphase, telophase, have been described with much detail. Therefore, it is easy to determine the mitotic index at any moment, when a sample of cells is available. Cells are exposed to an antitubular agent, e.g. colchicine, that arrests the cell cycle in the metaphase stage of mitosis by disturbing the formation of the metaphase spindle. When samples of the cell population are taken at different times, then an increase of the mitotic index is observed so long as exposure is continued. However, the limited life span of arrested cells must be taken into account. Whatever blocking agent is used, degeneration of blocked metaphases will occur if exposure is longer than 240 min, as Aherne has observed (1977). The dose has to be carefully chosen, not to exceed the karyotoxic concentration. The biochemical effects of those spindle toxins on cell

metabolism have been explored extensively within the last 100 years. It seems likely that most of the biological activities of these spindle toxins can be explained by their ability to bind specifically with the cytoplasmic tubulin protein. The pharmacologically interesting part of colchicum autumnale is colchicine, a substance that is present in the plant in various optically active stereoisomers. One of these optically active substances, which has pharmacologically promising properties, has been synthesised by Schreiber in 1958 in a pioneering work. Nevertheless, the natural total extract is still used in pharmacological experiments. The total extract of colchicum autumnale and the periwinkle plants has numerous substances with complex chemical structures that have still not been fully elucidated. All periwinkle extracts have in common that they are only soluble in organic solvents and plant oils. For pharmacological application they have to be derivatised, on which there is a plethora of patents. Over the last 100 years, countless diligent scientists have studied the influence of these plant extracts on cell growth, especially on meiosis, mitosis and the migration behaviour of different cell types in vivo and in explants in vitro. The plant extracts, which do not affect the growth of the plants in which they are formed, cause the formation of multinucleated cells rich in chromatin, with deposition of intracellular complexes of ribonucleic acid, nucleoproteins and vinca extract. The targets of the plant extracts are cytoplasmic tubulin molecules, whose function in cellular migration is impaired.

This leads to the result that the cells are arrested in the metaphasis of mitotic division, which are impaired in cell division. In the absence of intact cytoplasmic mitotic spindle system, the chromosomes my disperse throughout the cytoplasm, which is regarded as exploded mitosis, or may occur in unusual groupings such as balls and stars. Since tubulins are also responsible in cancer cells for metabolic function, movement and metastasis, respectively, their influence on the migration behaviour of cancer cells is of great scientific and medical interest in prevention and treating of cancer. Plant extracts containing colchicine have been used for treatment of gout since the 18th century and is still taken at the first sign of symp-

toms. Early findings from the treatment of Familial Mediterranean Fever with colchicine have shown that the fragile leukocytes responsible for the fever episodes can be stabilised, with a significant reduction in fever episodes. Therefore, it is obvious to investigate the influence of these antitubulins on the migration behaviour of other body cells, especially tumour cells from the area of mesenchymal tumours. This will provide the basis for understanding how tumour cells metastasise to specific organs of the body. A number of clinical and research scientists have compiled their research in this publication, which we present for the benefit of public health. By using novel methods, our scientists were able to show that the migration behaviour of tumour cells, especially isolated fibrosarcoma cells from patients, is significantly inhibited in human therapeutic concentrations of spindle toxins, but not completely arrested.

2. Chapter: Joshua Lederberg on Colchicine 1942

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Lederberg (1942) reviewed the past works on the cytosolic effects of colchicine and compiled a set of data which led to his conclusion that colchicine introduces C-mitosis in the cell where the chromosomes never organize in a metaphase as they do not appear on the equatorial plate. Instead, they remain scattered about the cell, apparently up to seven to eight hours. The disorganization of the chromosomes due to colchicine results in polymorphic, pycnotic nucleus. Also, colchicine effects on spindle indicating that one physical correlative of the spindle inhibition is a modification of the viscosity rhythm of mitosis. The effect of colchicine is the reduction in that viscosity. However, the causal relation of these phenomenon is not clear yet. It is not revealed yet whether the colchicine effects directly on the cytoplasm viscosity or is the culmination of the latter an incidental consequence to a more direct inhibition of the spindle. Meanwhile, Lits, Kirshbaum & Strong (1938) worked on the effects of Colchicine on transplanted malignant lymphoid neoplasm in mice of C3H stain. This study grew tumor in healthy mice and grafted the tumor tissue into another group of mice. At various stages of its growth, the mice were observed. Then, the grafted mice were treated with colchicine and the effects were observed. The study found colchicine to be effective in suppressing the tumor to some extent. It was observed that if $\frac{1}{4}$o mg of colchicine was injected subcutaneously at a site far removed from a local fourteen-day growth of the transplanted tumor, the tumor mass did not continue to increase in size and lost its firm consistency.

FIGURE 1: Colchicum autumnale, root

FIGURE 2: Colchicum autumnale, flowers

In some instances, the tumor disappeared within two days after the second injection, whereas in other animals the growth remained palpable, although there was a marked reduction in tumor size. It was also observed that the recurrence of the tumor may not even be evident for two or three weeks. In one case no tumor recurred for five weeks. Most importantly, in no treated animal was there absolute suppression of tumor growth. When growth was suppressed, it was usually for two to three weeks. The tumor then resumed its former rate of growth and caused the death of the animal.

Colchicine is an alkaloid present in corns and roots of various Liliaceae, particularly Colchicum autumnale, the meadow saffron. Studies show that in its structural formula there is a nitrogen on a side chain and there is an acetanilide nucleus present (Lederberg, 1942).

Saffron extracts have been used since Byzantine times as an empirical specific for gout. It stimulates water and uric acid (Lederberg, 1942). However, it also causes intestinal inflammation and respiratory depression which is why this drug is

not widely used in modern practice. Temperature plays an important part in determining the toxicity of this drug. The warm-blooded animals or frogs living in a high temperature have 400 times the sensitivity as cold-blooded (Lederberg, 1942). Studies report specific paralysis or cardio parasympathetic terminations.

It has also been found that a modification of the Arneth leucocyte count, a lowered renal threshold for glucose, and a decrease of liver glycogen, all indicate this drug's interference with a renal mechanism. Colchicine poisoning, iodoacetate poisoning, and adrenal-cortical deficiency are all relieved by the administration of salt water or of cortical extract (Lederberg, 1942). There are also scattered reports in the French literature of stimulatory effects on parathyroid tissue. However, the general pharmacological effects of this drug have not yet been reconciled in any comprehensive theory on the mechanism of its action.

It is not based on the above-listed properties that Colchicine merits the attention that it receives from biologists today. Several other effects of this drug have been recorded which are traceable to its effects on cell division (Lederberg, 1942). The modifications which Colchicine introduces into cell division have been called the C-Mitosis.

Previously done works describe the C-Mitosis as follows. In the root-meristem of Allium cepa, concentrations of the drug varying from 0.0075g to 1g were applied by merely growing the roots in the test solution. Prophases proceeded normally and the polar caps show the possibility of appearing in the earlier stages. The spindle however does not become organized (Lederberg, 1942). As a result, the chromosomes never organize in a metaphase as they do not appear on the equatorial plate. Instead, they remain scattered about the cell, apparently up to seven to eight hours. The kinetochore is also somewhat influenced for it does not divide as quickly as it would have in a normal rhythm. The chromosome does however soon split along its length, and spread out so that x shaped chromosomes appear, the point of junction being the

still unsplit kinetochore (Lederberg, 1942). Gradually, this too divided yielding chromosomes in a tetraploid structure. The disorganization of the chromosomes due to colchicine results in polymorphic, pycnotic nucleus. These late C-telophases give the false appearance of a mitosis. These reconstituted nuclei are now tetraploid with respect to the parent (haploid) nuclei (Lederberg, 1942). Similarly, haploids can be made to yield homozygous diploids. This process of chromosome doubling can be repeated almost indefinitely.

In the reduction-division of the sporocytes, a fundamentally similar pattern applies. In somatic mitosis the presence of diplo-chromosomes and the absence of the spindle are diagnostic features in colchicine activity in the cell cycle. This mechanism of polyploidy depends on the inhibition of the spindle and is not to be confused with that induced in the resting phase by the hetero-auxin nor by that resulting from the absence of new wall-formation by purine alkaloids (Lederberg, 1942).

Cytological investigations on the effects of colchicine, particularly interesting to geneticists for obvious reasons, have been made on a great number of organisms, plant, and animal. Teratological effects resulting from distortion of cleavage were noted. The results give sufficient evidence to believe that the 'arrested metaphase' of the zoologists is equivalent to the C-mitosis of the botanists. The inhibition of spindle function is the prime cause.

While colchicine demonstrates C-mitosis with the clearest and greatest efficacy, it is by no means the only reagent that will induce polyploidy in the manner described. French workers have described a great many compounds including acenaphthene, p-dichlorobenzene, naphthalene and chloro-naphthalene, phenyl-urethane, ether, and others (Lederberg, 1942). There is no resolution of this list of chemical structure that slight modifications (demethylation) of the colchicine molecule destroy its C-activity.

Anesthetics have been represented in this list, however, and it has been suggested that colchicine acts as an anesthetic. The

following paragraphs deal with this view in detail.
In concordance with the colchicine's effects on spindle, a great number of consistent observations have been made indicating that one physical correlative of the spindle inhibition is a modification of the viscosity rhythm of mitosis (Lederberg, 1942). The effect of colchicine is the reduction in that viscosity. However, the causal relation of these phenomenon is not clear yet. It is not revealed yet whether the colchicine effects directly on the cytoplasm viscosity or is the culmination of the latter an incidental consequence to a more direct inhibition of the spindle. The supposed common behavior of anesthetics is decreasing viscosity which is interesting in this connection (Lederberg, 1942). Further progress in this direction is probably waiting for independent progress of research on the nature of the spindle.

Furthermore, some researchers have observed changes in water-permeability and electrical polarity of the cell membrane during sea urchin egg division. However, no reports appear in the literature with respect to colchicine effects.

Another research found the changes in the pH and osmotic pressure of colchicinized Tritichum seedlings. The increase in osmotic pressure reflects the very swollen appearance that meristematic cells have after effective treatment with colchicine (Lederberg, 1942). The pH change is discussed further below.

There is yet no rationale for colchicine effects here. The problem may however be tackled from a chemical viewpoint. If only in a negative way, the work there has been more fruitful. General considerations on the chemistry of cell-division are discussed further. If the relationships between activity and metabolism were there indicated to be complex and obscure, the colchicine situation can only emphasize this.

A study on anesthetic hypothesis tested the effects of colchicine on the activities of dehydrogenases, and checked the results against sodium cacodylate, which has similar cytological effects. The cacodylate had no effect on any of the substrates. Colchicine had no effect on succinic, glucose or glyco-

gen dehydrogenases, but partially inhibited, lactic and citric dehydrogenases. The source unfortunately was beef muscle and liver, in neither of which C-activity can be demonstrated (Lederberg, 1942). However, an interesting hypothesis to test would be that the lowering of pH in Triticum is a consequence of the accumulation of lactic and citric acid.

At any rate, colchicine does not work by blocking oxygen supply at any early stage. The effects of oxygen block are very much different. A group of researchers found out that colchicine concentrations 500x those required for complete division block in Strongylocentrotus have no effect on the oxygen consumption of these eggs. Researchers' further find out that drug does not affect respiration at all at 0.0002M and halves it at 0.0004M (Lederberg, 1942). Both of these concentrations probably are considerably in excess of that required for C-mitosis in their material, excised Zea roots, but no figures are given. The answer to this mechanism, like that for the anesthetic inhibition of other cell activities is not to be sought in the total respiration of metabolism but to some specific moiety thereof. In spite of these negative conclusions, an enzymatic hypothesis for C-activity remains a promising one, if not the only one that is susceptible to any form of attack.

Other observations on the enzymatic activity of colchicine are mostly negative. However, some researchers found a depression of proteolysis at a concentration of 0.0001 M (Lederberg, 1942) which may be at or not too much above the threshold for C-activity in their Zea roots.

A scholar working on rats found no inhibition of the acid-phosphatase activity of young rat testis by colchicine. Another study found an acceleration of malt diastase, and no direct inhibition of invertase by colchicine (Lederberg, 1942). Once again, no final conclusion is possible. The investigation is by no means complete: indeed, it has barely started.

In the course of some routine cytological studies on c-mitosis in the Allium-cepa root-tip, the author in Maron 1941 came across some indications of a gradient-of-susceptibility to col-

chicine in the onion root tip (Lederberg, 1942). This was followed up by some work in the Spring of 1942 which confirmed this conception as follows:

Onion bulbs are grown in the dark over tap water for 3-4 days until roots 1-2 cm long appeared. Then a stated colchicine solution was substituted for the water, replaced every few hours. Then root-tips were then cut off at stated times, fixed and stained for cytological study (Lederberg, 1942).
There are at least two possibilities in the interpretation of the gradient, requiring experimental test. Because of differential absorption in the region of differentiation or elongation there may exist an artificial concentration gradient down the root tip, or the gradient may be intrinsic in the cells. If the first interpretation survived test by cutting and culturing experiments, it will not be possible to use this approach. If the gradient is intrinsic, the results of attempts to extinguish or accentuate it would be more interesting. Such preliminary experiments with Pb, cyanide, methylene blue indicate only that the balance of figure-types is upset by the accumulation of prophases or of vegetable cells, in accordance with the independent properties of these reagents (Lederberg, 1942). At this point one can say that experiments are planned and will shortly proceed.

In 1932, the first empirical observations concerning the action of colchicine on tumors came out (Kirshbaum, 1938). The study noted an improvement in the health of cancer patients treated with Colchicum autumnale.

A study conducted later focused on the action of colchicine on a grafted sarcoma and presented the first scientifically controlled observations, indicating the possibilities of the use of the drug in the treatment of neoplastic growths (Kirshbaum, 1938). In a second series of experiments, the action of the drug was tested on Murray's guineapig liposarcoma and on tar cancer of the mouse. Biopsies showed a threefold increase in mitotic activity in human carcinomas of the cervix when 2 mg. of colchicine were injected subcutaneously every day for three days (Kirshbaum, 1938).

Another group of researchers observed a 700 per cent increase in the rate of mitosis in an adenocarcinoma of the sigmoid colon twelve hours after an intramuscular or subcutaneous injection of 1 to 4 mg. of colchicine (Kirshbaum, 1938). A similar action on the normal mucosa surrounding the tumor area was seen.

One researcher studied the effect of single and repeated administrations of colchicine upon mammary carcinoma C63. He showed that after one injection of colchicine the highest mitogenetic index in this carcinoma was 27.25 at twenty-four hours; the highest mitogenetic index in the Crocker sarcoma after colchicine administration was demonstrated in another study as 15.7 at nine hours (Kirshbaum, 1938).

One study noted the irradiated tumors of patients suffering from gout and after being treated with colchicine, they decreased quickly and noticeably in size. Small amounts of colchicine were injected every two days for two weeks into mice bearing grafted tumors (Kirshbaum, 1938). In two-thirds of the mice the tumors disappeared completely during this period, and in the remaining mice only small nodules remained. The histologic type of the tumor and the doses of colchicine were not recorded. A spontaneous epithelioma of the peritonsillar region in a dog regressed completely after forty days of treatment (Kirshbaum, 1938).

One study found it difficult to inhibit the growth of well-established grafts of transplantable tumors with colchicine without the appearance of severe toxic symptoms (Kirshbaum, 1938). It was concluded that colchicine in the doses used had no inhibitory effect on the growth of mouse sarcoma S37 *in vivo*. Growth *in vitro* of tumor obtained from an animal treated with colchicine was inhibited to a marked extent. Sarcoma S37 is less affected by colchicine than many normal tissues (Kirshbaum, 1938).

Another scholar detected a considerable inhibiting influence of colchicine upon the development of experimental tumors (crown-gall) in tomato plants (Kirshbaum, 1938). It has also

been found in previous studies that colchicine in amounts approaching the toxic dose produced hemorrhage in grafted tumors accompanied by a reduction in their ascorbic acid content and metabolism.

Kirshbaum (1938) continued in the similar direction by choosing mice of strong C3H stain and inducing lymphoid tumor in them. A spontaneous case of leukemia from which transfers were originally made arose in a female which had received 3.9 mg. of equilin benzoate in 303 days. This mouse showed a bilobed mediastinal mass, whitish in color, adherent to the sternum. The spleen was enlarged. The long bones were solid, and the pubis was osteoporotic because of prolonged estrogenic treatment (Kirshbaum, 1938). Both thymic and splenic transplants grew in the first transfer generation but, since the grafted splenic tissue produced a better growth, the local tumor resulting at this implantation site was used for regrafting. From then on local tumor was used as donor tissue. Grafting was accomplished by inserting a small piece of tumor tissue subcutaneously into the right axilla, using a trocar for the purpose. The tumor at the implantation site usually grew to a considerable size. The most satisfactory results were obtained when transplantation was done before any part of the tumor had become necrotic (Kirshbaum, 1938). The longer survival time in the ninth transfer generation could be ascribed to the poor condition of the donor tissue.

Twenty-three days or more after implantation a general leukemic state was present in most animals. Spleen and lymph nodes were enlarged and there was leukemic infiltration in the kidneys, liver, bone marrow, etc. The white blood count was usually elevated, with a high percentage of lymphoid cells. In some instances, a leukemic blood picture was not present, although there was a polymorphonuclear leukocytosis because of tumor necrosis (Kirshbaum, 1938). Just before death the tumor would decrease in size and the animal would become very emaciated.

The growth was transplanted into 104 mice with no failures (13 transfer generations) and no spontaneous regressions.

Starting with the fourth transplant generation (Series I) experiments were started to determine the effect of colchicine on the tumor. The drug was administered subcutaneously (Kirshbaum, 1938).

With Series I, $\frac{1}{4}$o mg. in 0.5 c.c. distilled water was injected every second day for three times, no further treatment being given. Injections were begun after the tumor had been growing in the hosts for fourteen days. Additional injections were administered on the seventieth and seventy-sixth days after transplantation when a recurrence of the regressed tumor made its appearance at seventy days, in the animal of this group which survived 101 days after implantation of the tumor. The same dosage was used for the test animals of Series II, but only 2 injections were given (Kirshbaum, 1938).
In Series III and IV, where the best results on survival time were obtained, the same amount of colchicine ($\frac{1}{4}$o mg.) was dissolved in only 0.1 c.c. of distilled water and injected subcutaneously every third day until the animal died or was killed. Injections were always made at a site far removed from the tumor. The mice used were young animals weighing 22-28 grams (Kirshbaum, 1938).

After it had been observed that tumors regress under the influence of colchicine therapy, a fifth series of animals was used for study of the histology of tumor regression and regeneration. Fifteen test animals of the ninth transfer generation comprised this group, with 4 additional tumor-bearing animals serving as controls (Kirshbaum, 1938). After the tumor had been growing fourteen days the first colchicine administration was made into all 15 test animals. Animals were killed eight, sixteen, twenty-four, forty-eight and seventy-two hours after the injection. At this time the 10 remaining animals were given a second dose, following which 5 animals were killed at the same intervals as after the first dose. Seventy-two hours after the second injection the 5 remaining animals received a third dose of the drug and were killed at similar intervals. Controls were killed fourteen, seventeen, twenty and twenty-three days after tumor implantation (Kirshbaum, 1938).
All test animals had a firm tumor about the size of a pea when

colchicine treatment was begun. Since central necrosis of these tumors does not set in until about the twenty-third day of growth the results could not be attributed to spontaneous degenerative phenomena. Peripheral and central portions of the tumor and all lymphoid tissues were fixed in Dustin's fluid and stained with Masson's stain (modification with light green), which is especially effective for chromatin material following this fixation. Sections were cut at 5 micra, paraffin imbedding being used. Imprints of the tumor stained with Pappenheim's combination of May-Grünwald and Giemsa solutions were also studied (Kirshbaum, 1938).

It was observed that following implantation of a few milligrams of the lymphoid tumor used, growth was relatively slow, but in fourteen days a solid mass the size of a large pea or kidney bean was present at the implantation site. Thenceforth the tumor increased in size more rapidly and by twenty-five days extended from the axillary to the inguinal fossa. The time at which the mice begin to fail varied, the survival time differing accordingly (Kirshbaum, 1938). The central portion of the tumor became necrotic after twenty-three days, and before the animal died there was usually a general emaciation with a corresponding reduction in tumor size. After more than twenty days of tumor growth, leukemic infiltration of spleen, lymph nodes, bone marrow, kidneys and liver were observed. The histologic picture of these tissues was comparable to that in human lymphatic leukemia with loss of normal architecture in spleen and lymph nodes, periportal and sinusoidal infiltration in the liver, and intertubular lymphoid accumulations in the kidney (Kirshbaum, 1938). The white blood count was usually elevated (15-30,000 cells per cu. mm.), but not to the extreme degree observed in certain other lines of mouse leukemia, where counts of 300,000 or more had been observed by one of the authors.

Kirshbaum (1938) concluded that if 0,25 mg of colchicine was injected subcutaneously at a site far removed from a local fourteen-day growth of the transplanted tumor, the tumor mass did not continue to increase in size and lost its firm consistency. In some instances, the tumor disappeared within two

days after the second injection, whereas in other animals the growth always remained palpable, although there was marked reduction in tumor size. Recurrence of the tumor might not be evident for two or three weeks. In one case no tumor recurred for five weeks. In no treated animal was there absolute suppression of tumor growth. When growth was suppressed, it was usually for two to three weeks. The tumor then resumed its former rate of growth and caused the death of the animal. Histologically the fourteen-day growth of transplanted tumor was a compact mass of lymphoid cells with a supporting reticular cell framework.

3. Chapter: Colchicine, chemistry and history of colchicum autumnale

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Summary
The distribution of colchicine in *Colchicum autumnale* has been studied by several workers, and there is general agreement that it occurs most abundantly in the seeds. According to a study, the ripe seeds contain from 0.4 to 0.9% of colchicine, whereas the content of the bulbs, sap, and leaves is essentially lower. From 0.38 to 0.4% of colchicine is found in the corms, and 0.72 to 0.75% in the seeds. The alkaloid is found to be located chiefly in the endosperm and the third layer of the seed coat. The toxic principle of *Colchicum autumnale* was first isolated in 1820, then later in 1833, a crystalline alkaloid was extracted, and it was named colchicine. In 1857, an amorphous initial material in the extraction process was named colchicine and the crystalline product colchiceine. Crystalline colchicine was first obtained in two separate studies in 1883 and 1884.

Pure crystalline colchicine, free from solvent, was first described in 1915 by using ethyl acetate as the medium for recrystallization and pale-yellow needles were obtained. Completely pure colchicine is colorless. With water, it forms a sesquihydrate , yellow rhombic crystals, and with chloroform, it forms two crystalline compounds, containing respectively one and two molecules of colchicine combined with one molecule of chloroform of crystallization. The crystals of the chloroform complex emit a bluish-white light when rubbed in the dark.

This behavior is not shown by pure (amorphous) colchicine. The chloroform can be removed by evaporation with water, and the colchicine is recovered from the crystalline complex by treating it with water and blowing in steam, followed by evaporation of the clear solution to dryness. This gives scales of analytically pure material, after drying in a vacuum at 100 degrees centigrade and then 130 degrees centigrade. The complete synthesis process discussed in detail will be one that was carried out by Schreiber and colleagues in 1959. It starts from purpurogallin and takes twenty steps via desacetylamino-colchicein to the natural (-) S- colchicine.

The molecular formula of colchicine is $C_{22}H_{25}O_6N$. The previous studies established the presence in the molecule of one readily hydrolyzable and three more difficultly hydrolyzable methoxyl groups, and also an acetylated primary amino group. The essential structural problem was, therefore, to determine the nature of the carbon skeleton of 16 atoms, and the positions of the substituents within this framework. This problem has not been completely solved, although the main features have been elucidated, and the structure of a closely related degradation product, deaminocolchicine methyl ether, which contains all but one of the carbon atoms of the $C_{16}H_9$ residue, has been unequivocally established.

Colchicine solutions are colored intensely yellow by strong mineral acids. Similar behavior is shown by colchiceine, and here also molecular complex formation is probably involved; the implied latent basicity appears to reside in an oxygen-containing group rather than in nitrogen. Although a neutral substance, colchicine is a highly toxic nitrogenous plant product and gives characteristic alkaloidal reactions with many of the usual alkaloid precipitants. Its dissociation constant was measured in 1916.

Colchicine has high solubility in water despite the absence of any of the groups usually associated with a high degree of water solubility. The substance is miscible with water in all proportions in the cold but is less soluble in hot water. A saturated solution at 82 degrees centigrade contains about 12% of

colchicine. If colchicine is hydrolyzed to colchiceine, liberating a hydroxyl group, the water-solubility decreases substantially. The high solubility of colchicine in water has not been satisfactorily explained, although it has recently been suggested that it is due to ionic resonance.

When tested with Colchicine, bromine water gives a crystalline monobromo compound and an amorphous dibromo compound, whereas a tribromo derivative forms by the action of excess bromine in methanol. The tribromo compound contains one labile bromine atom which displaces from the molecule when the compound is treated with methyl alcoholic potash. Two hydrolysis products of colchicine, namely, colchiceine and trimethylcolchicinic acid, also give tribromo derivatives with bromine in acetic acid.

Colchicine has the specific characteristic, also shown to some degree by other substances, of bringing cell division to an abrupt halt at a particular stage. This behavior has led to the unraveling of many interesting aspects of cytology and has also resulted in the elaboration of valuable and sensitive methods of assay of natural hormones and some of their synthetic counterparts. Above all, the action of colchicine on cell division has led to the discovery of methods for the artificial production of polyploid varieties of many plants. These new varieties have many attributes which are superior to those of the forms from which they are derived, and the artificial production of polyploids has considerable economic importance as well as scientific interest. That this is widely recognized is testified by the very extensive literature published during the past decade, from many different countries, on the artificial production of polyploid forms of plants by treatment with colchicine and other compounds.

The investigations which have established the main structural features of Colchicine are outlined in this chapter. Some details still require elucidation. In recent years intense interest has been aroused by the remarkable biological properties of colchicine, which were first revealed by the previous works dating back to the 1930s. The main interest remains due to

the fact that from the standpoint of molecular structure, colchicine may represent a unique type of plant product. One of the significant findings is regarding the use of isocolchicine; whenever it is used instead of colchicine, no C-mitotic action is observed. The latter differs from the former only in minor details of structure. There is at least one methoxy group in ring A is necessary for colchicine action; a ring C must be 7-membered, and the hydroxyl group should preferably be replaced by an amino group; the esterification of amino group in ring B increases the activity; and isocolchicine and its derivatives are less active. A proper distance should be maintained between esterified side chains of rings B and C. In isocolchicine, where the position of the methoxy group is reversed in ring C, the decrease in efficacy has been considered to be due to the presence of hydrogen bonds between the side chains of rings B and C. In the case of 'colchicine', the weak action is the result of the iso-form of this molecule. Though these data indicate the reactive groups responsible for colchicine action, the exact reaction involved in spindle inactivation is not yet fully clear.

Main Body:

Colchicum autumnale is a perennial plant common in England and is also found in Central and Southern Europe. Its name is stated to be derived from Colchis, the former name of a district of Transcaucasia, the Asia Minor, where several species of the plant grew. This district on the eastern shore of the Black Sea was the legendary home of the Golden Fleece, symbolical of wealth and fertility, and to the Greek mind, a region associated with sorcery (Cook and Loudon, 1959). Colchicine is an alkaloid found in the bulb and seeds of Colchicum autumnale (also called autumn crocus or meadow saffron). It is a bulbous plant that blooms in autumn in the wet meadows of central and southern Europe.

***FIGURE 1:** Colchicum autumnale, flowers*

Colcicum autumnale, commonly called "false saffron," "bastard saffron," "dog-killer," and "floating-light," is a bulbous liliaceous plant which blooms during the fall in the wet meadows of Central and Southern Europe, the leaves and seeds appearing the following spring. Its use as a quasi-specific remedy against the acute crises of gout dates back to ancient times, perhaps to the Byzantines. It was called "hermodact" by these people, the Arabs, Salernitians, and others. This name, however, includes three botanical species: quintofolium, or long hermodact, Egyptian colchicum, or small hermodact, and Colchicum autumnale, which was imported from the East. The first formula for this drug we owe to Jacques Psychriste, physician, and counsellor of Emperor Leon the Great (457–475 A.D.). The physicians of that time prescribed it mixed with large doses of scammony or alternated with Persian pills of aloe, for they attributed evacuant and cathartic properties to the drug. The irritating effect on the digestive tract was to be soothed by aromatics and sweetmeats. Colchicum was declared to be deadly by Dioscorides and its use was abandoned for centuries (Lits, 1933, Lits *et al.* 1938).

The toxic principle of colchicum was first isolated in a relatively pure state in 1820. Crystalline preparations were described in 1884, and important contributions to the chemistry of colchicine were made by in 1924 when a structural formula for the alkaloid was proposed (Cook and Loudon, 1959).
In recent years intense interest has been aroused by the remarkable biological properties of colchicine, which were first revealed by the work of Dustin in 1934 and Lits in 1938. Dustin observed that Colchicine is the methyl ether of an enolone containing three additional methoxy groups, an acetylated primary amino group and three non-benzenoid double bonds. A later study noted that the threshold regions of colchicine-mitotic activity are identical for both crystalline and amorphous forms; chloroform exerts no appreciable effect. One of the significant findings in Allium cepa is that whenever isocolchicine is used instead of colchicine, no C-mitotic action is observed. The latter differs from the former only in minor details of structure. The data collected by Dustin (1957) implies that: (a) at least one methoxy group in ring A is necessary for colchicine action; (b) ring C must be 7-membered, and the hydroxyl group should preferably be replaced by an amino group; (c) esterification of amino group in ring B increases the activity; and (d) isocolchicine and its derivatives are less active. A proper distance should be maintained between esterified side chains of rings B and C. This last statement is based on the fact that in isocolchicine, where the position of the methoxy group is reversed in ring C, the decrease in efficacy has been considered to be due to the presence of hydrogen bonds between the side chains of rings B and C (Sharma and Sharma, 1980). In the case of 'colchicine', the weak action is the result of the iso-form of this molecule. Though these data indicate the reactive groups responsible for colchicine action, the exact reaction involved in spindle inactivation is not yet fully clear. Pertinent suggestions have been made on the basis of results obtained with spindle poisons of different chemical structure (Sharma and Sharma, 1980).

Yet another facet of the cytological action of colchicine is reflected in the numerous studies of its effect on tumor growth. Although the promise of a therapeutic agent against cancer indicated by some of the earlier reports has not been

sustained by more extensive investigation, probably, the last word on this subject has not yet been written. In a recent review, it was suggested that more extensive studies should be made of the action of colchicine, combined with X-rays, on animal tumors. The very high toxicity of the alkaloid prevents its use for this purpose in any but the smallest doses, but when its structure is completely elucidated it may be hoped that the way will open for the synthesis of analogous compounds less toxic than colchicine, but with similar action on the cell nucleus.

Occurrence

Although colchicine is usually extracted from the meadow saffron this is by no means the only natural source of the alkaloid. A dozen species listed of *Colchicum* in which colchicine was detected and found it also in *lerendera caucasia* Bieb, and *Jerendera sobolifera* with a localization almost identical to that in *Colchicum.* A researcher extracted the colchicine from the whole plant of *Colchicum Cupani* Guss, and found the amount to be 0.161%, which is of the same order as the content of *Colchicum autumnale.* From histochemical studies of its distribution, it was concluded that colchicine is present mainly in cells in full activity and plays an essential part in the nutrition and growth of the plant (Cook and Loudon, 1959). According to a study, colchicine also occurs in large quantities in the following Liliaceae*: Bulbocodium, Tofieldia, veratrum anthenicum, Hemerocallis, Ornithogalum, and Tulipa,* and in traces in *Asphodelus, Fritillaria, Lloydia, and Muscular.* It is reported that *Androcymbium grandeur* MacBr., a plant from the central Sahara, contains colchicine distributed throughout its parts, and in a quantity comparable with *Colchicum autumnale. A team of scientists* extracted from the dried tubers of *Gloriosa superba* L. a mixture of alkaloids consisting chiefly of colchicine (assay showed the presence of 0.3% of colchicine in the dried tubers) together with small amounts of two other crystalline nitrogenous materials (Cook and Loudon, 1959). One of these crystallized from ethyl acetate in pale yellow leaflets, m.p. 177-178 degrees centigrade; the composition corresponded with $C_{33}H_{38}O_9$ or $C_{15}H_{17}O_4N$. The other also crystallized from ethyl acetate and formed

colorless needles, m.p. 267 degrees centigrade; its carbon and hydrogen content corresponded with the formula $C_{23}H_{27}O_6N$, which is that of a methylcolchicine (Cook and Loudon, 1959). The chemistry of these minor products does not appear to have been studied. Incidentally, by using ethyl acetate as the solvent, a group of scientists seem to have been the first workers to obtain colchicine in a pure crystalline form, free from the solvent of crystallization. By this means they obtained crystalline colchicine, not only from *Gloriosa superba but also from Colchicum autumnale* (Cook and Loudon, 1959). This was achieved in 1915, nearly a century after the earliest report of the isolation of colchicine.

The distribution of colchicine in *Colchicum autumnale* has been studied by several workers, and there is general agreement that it occurs most abundantly in the seeds. According to a study, the ripe seeds contain from 0.4 to 0.9% of colchicine, whereas the content of the bulbs, sap, and leaves is essentially lower (Cook and Loudon, 1959). Another study found from 0.38 to 0.4% of colchicine in the corms, and 0.72 to 0.75% in the seeds. The alkaloid is found to be located chiefly in the endosperm and the third layer of the seed coat (Cook and Loudon, 1959).

For the study of the distribution of colchicine in plants, use has been made of microchemical methods of detection and estimation. A study utilized the yellow color that colchicine gives with dilute mineral acids; color reactions also formed the basis of the work of another study carried out later, whereas some scientists used a microchemical reaction with platinum thiocyanate, which is stated to be sensitive to 2×10^{-7} g. of colchicine (Cook and Loudon, 1959). The methods for the estimation of this alkaloid revealed that the most reliable values were given by precipitation with phosphotungstic acid. Other methods of estimation in pharmaceutical preparations and in *Colchicum* seeds have been described. By mild acid hydrolysis colchicine is converted into colchicine, which gives an intense green color with ferric chloride. This also has been employed as the basis for methods of detection and estimation of colchicine (Cook and Loudon, 1959). Another study recommended the use of

dilute hydrochloric acid, instead of the concentrated acid used by earlier workers, for the preliminary hydrolysis to colchicine, and the method was adapted to the colorimetric estimation of colchicine for a study later on. Colchicine withstands putrefaction for 3 to 6 months, and the alkaloid, or an analogous substance, was detected in a corpse as long as 22 months after death. The subject appeared to have taken "coffee" prepared from autumn crocus (Cook and Loudon, 1959).

Extraction and Isolation

The toxic principle of *Colchicum autumnale* was first isolated in 1820 by a team of researchers who believed it to be veratrine (Seris, 1947). Later in 1833, another study extracted a crystalline alkaloid which was recognized as a distinct substance, and it was named colchicine (Cook and Loudon, 1959). In 1857, using the same method of extraction, another scientist was unable to obtain a crystalline product, but showed that his preparation was converted by boiling dilute hydrochloric or sulfuric acid into a crystalline material with the properties described in the study in 1833. He designated the amorphous initial material as colchicine and named the crystalline product colchiceine (Cook and Loudon, 1959). Colchicine is easily soluble in water, whereas colchiceine is sparingly so. Other workers have obtained alkaloidal preparations from autumn crocus by extraction with alcohol-containing sulfuric acid; these must be regarded as mixtures. The presence of colchicine in the plant cannot be regarded as established the balance of evidence is against its occurrence in the free state. The first extraction of the alkaloid without the use of a mineral acid was done in 1857 by a scientist who precipitated the colchicine with tannic acid and decomposed the salt (or complex) with lead oxide.

Crystalline colchicine was first obtained in two separate studies in 1883 and 1884. This, however, was not solvent-free, but contained firmly bound chloroform of crystallization which was not lost after standing in the air for a month and was only incompletely expelled by heating for *several* hours at 100 degrees centigrade (Cook and Loudon, 1959). Pure crystalline colchicine, free from solvent, was first described in 1915 by

scientists who used ethyl acetate as the medium for recrystallization and obtained pale yellow needles, m.p. 155-157 degrees centigrade. Completely pure colchicine is colorless. With water, it *forms* a sesquihydrate, yellow rhombic crystals, and with chloroform, it forms two crystalline compounds, containing respectively one and two molecules of colchicine combined with one molecule of chloroform of crystallization (Cook and Loudon, 1959).

Both studies of 1883 and 1884 obtained their colchicine preparations from alcoholic extracts of *Colchicum* seeds. The study in 1915 distilled the alcohol from the extract, dissolved the residue in water, and separated the aqueous colchicine solution from undissolved resin and fat. By repeated fractional extraction with chloroform, the researcher obtained a chloroform solution of colchicine that was concentrated to a viscous oil. Rosettes *of* crystals of the chloroform complex were obtained from the oil by standing below 0 degrees centigrade (Loudon, 1955). In 1884, the scientist, after the distillation of the alcoholic extract of *Colchicum* seeds, treated the residue with aqueous tartaric acid. The colchicine passed into the acid solution and this, freed from fat and resin, was extracted with chloroform, from which crystals were obtained after concentration by spontaneous evaporation of a solution in chloroform, alcohol, and petroleum ether. The study of 1915 noted that the crystals of the chloroform complex emitted a bluish-white light when rubbed in the dark. This behavior was not shown by pure (amorphous) colchicine. The chloroform is removed by evaporation with water, and the colchicine was recovered from the crystalline complex by treating it with water and blowing in steam, followed by evaporation of the clear solution to dryness. This gave scales of analytically pure material, after drying in a vacuum at 100 degrees centigrade and then 130 degrees centigrade (Loudon, 1955).

One of the studies carried out the isolation experiment as follows. The dark brown gum (330 g.) from an alcoholic extract of *Colchicum* seeds is diluted with 450 cc. of water and the solution, which contains undissolved solid and resinous material, is heated with 75 g of paraffin wax until the wax is molten.

The mixture is stirred vigorously and then allowed to cool. The solid wax, which dissolves the resin, is lifted from the surface, and the process is twice reported with fresh wax. The combined wax layers are thrice extracted with 100 degrees centigrade. of boiling water, and the aqueous extracts added to the solution of the alkaloid (Cook and Loudon, 1959).

A paste of filter paper pulp (50 g.) is then added to the aqueous colchicine solution. This is prepared by boiling filter paper with concentrated hydrochloric acid to effect complete disintegration, the mass is then washed with water until neutral. The mixture is filtered on a filter bed, to which some paper pulp has already been added, and yields a clear brown solution. The filter bed is boiled with a little water and then re-filtered. The combined filtrates are extracted with 12 portions of 200 cc. of chloroform, care being taken to ensure that the chloroform is free from hydrochloric acid. The addition of potassium carbonate to the yellow extract causes precipitation of some brown flocculent material, which is filtered from the dried solution. The latter is evaporated, leaving a golden-brown syrup (Cook and Loudon, 1959).

The sirup is redissolved in chloroform (150 cc.) and the solution passed through & column of alumina, 25 min. long and 3.5 cm. in diameter, which has been saturated previously with benzene (Cook and Loudon, 1959). Three bands are formed, an upper reddish-brown band, a larger bright yellow band, and a lower almost colorless band that contains the colchicine. The column is washed with chloroform until the yellowish color becomes colorless and yields no residue on evaporation. Distillation of the chloroform from the total elute gives a golden-yellow sirup which is distilled thrice with an equal volume of absolute alcohol to remove the residual chloroform. The residue is finally crystallized from ethyl acetate and yields 10 g. of colchicine as fine colorless needles, m.p. 148-150 degrees centigrade. A further 1.75 g. of slightly less pure material (m.p. 147-150 degrees centigrade) is obtained from the liquors. A second chromatographic purification, followed by crystallization from ethyl acetate, raises the m.p. to 155 degrees centigrade (Cook and Loudon, 1959).

Chemical Characteristics

The most extensive description of the chemical characterization of colchicine is that of the study in 1886. Although the researcher did not succeed in obtaining crystalline colchicine free from solvent, there is little reason to doubt that he was dealing with substantially pure material, which he obtained by evaporating the crystalline chloroform complex with water. The substance so formed had m.p. 143-147 degrees centigrade, after being dried at 110 degrees centigrade. Analysis corresponded with the formula $C_{22}H_{25}O_6N$, which has been confirmed by all subsequent work with colchicine derivatives (Loudon, 1955). In one important respect, the description alkaloid is a misnomer, as colchicine is not a base but a neutral substance. It does not contain a free amino group, nor does it form & well-defined series of salts as other alkaloids do. One study noted the formation of a saltlike compound with tannic acid, but it now seems more probable that this is a molecular complex. Colchicine solutions are colored intensely yellow by strong mineral acids. Similar behavior is shown by colchiceine, and here also molecular complex formation is probably involved; the implied latent basicity appears to reside in an oxygen-containing group rather than in nitrogen. Although a neutral substance, colchicine is a highly toxic nitrogenous plant product and gives characteristic alkaloidal reactions with many of the usual alkaloid precipitants. Its dissociation constant has been measured in 1916. The investigators give the value, $K = 4.5 \times 10^{-13}$ ($pK = 12.35$), which is in keeping with the lack of pronounced basicity. According to a study that made potentiometric measurements at the antimony electrode, colchicine has three dissociation constants, namely, pK, 1.8, 7.2, and 10.3 (Loudon, 1955).

A remarkable physical attribute of colchicine is its high solubility in water, despite the absence of any of the groups usually associated with a high degree of water solubility. A study states that the substance is miscible with water in all proportions in the cold but is less soluble in hot water. A saturated solution at 82 degrees centigrade contains about 12% of colchicine (Cook and Loudon, 1959). Furthermore, if colchicine is hydrolyzed to colchiceine, liberating a hydroxyl group, the

water-solubility decreases substantially. This high solubility of colchicine in water has not been satisfactorily explained, although it has recently been suggested that it is due to ionic resonance. The increase in solubility which attends to the esterification of acetic acid, $CH_3CONH \cdot CH_2 \cdot CO_2H$ (Schreiber *et al.* 1959), is a possible analogy to the behavior of colchicine and colchiceine.

A study describes several color tests and precipitation reactions with colchicine. Thus, it dissolves in concentrated nitric acid to a violet solution, which gradually becomes yellow; excess sodium hydroxide then transforms the solution to reddish yellow. Colchicine dissolves in concentrated sulfuric acid containing a trace of nitric acid to give first a yellow-green color, which becomes green, then blue and violet, and finally red and yellow. Dilution, followed by the addition of excess alkali, then gives a fine red color. Bromine water and iodine in potassium iodide (the latter only in acid solution) both give colored precipitates. Ferric chloride gives no color in neutral or acid solution, but if the acidified solution is boiled for a few minutes after the addition of ferric chloride an intense green color is formed. This is due to hydrolysis to colchicine. Mercuric chloride gives a citron-yellow precipitate with solutions of colchicine in dilute hydrochloric acid. Cadmium iodide also gives a precipitate - white in neutral solution, citron yellow in acid solution. Colchicine gives two complexes with auric chloride. The composition of one of *these* corresponds with that of the chloroaurate, $C_{22}H_{25}O_6N \cdot HCl \cdot AuCl_3$. This compound, m.p. 209 degrees centigrade, was also described in multiple other studies. The other, obtained by using excess colchicine, appears to have the formula $(C_{22}H_{25}O_6X \cdot HCl)_2AuCl_3$ (Cook and Loudon, 1959). Precipitates are obtained with salts of many complex acids. The use of phosphotungstic acid in the estimation of colchicine has been mentioned.

The action of bromine on colchicine was further investigated by another group of scientists who isolated mono-, di- and tribromo derivatives. Bromine water gave a crystalline monobromo compound and an amorphous dibromo compound, whereas a tribromo derivative was formed by the action of

excess bromine in methanol. The tribromo compound contained one labile bromine atom which was displaced from the molecule when the compound was treated with methyl alcoholic potash. Two hydrolysis products of colchicine, namely, colchiceine and trimethylcolchicinic acid, also gave tribromo derivatives with bromine in acetic acid. Colchicine and its two hydrolysis products all contain three hydrogen atoms readily replaceable by bromine (Cook and Loudon, 1959).

Colchicine solutions undergo autoxidation in the presence of light. By fractional precipitation of a chloroform solution of the oxidized material by petroleum ether, a researcher separated a brown resinous oxidation product, less soluble than colchicine, which he denoted as "oxydicolchicine." Analysis corresponded with the formula $(C_{22}H_{25}O_6N)_2O$, indicating the uptake of one atom of oxygen by two molecules of colchicine (Cook and Loudon, 1959). Because of the intractible nature of the oxidation product, this conclusion must be accepted with reserve, and further investigation is desirable. It is of interest, however, that in its toxicity and pharmacology "oxydicolchicine" showed significant differences from colchicine. It has been stated that "oxydicolchicine" is formed from colchicine by electrolytic oxidation, and also in the body. The pharmacological effects of colchicine in warm-blooded animals were believed to involve preliminary oxidation to "oxydicolchicine."

In this connection, interest attaches to the oxycolchicine described by previous studies. A researcher prepared this in 1890 by oxidation of colchicine with chromic acid in aqueous solution; the purified oxycolchicine was isolated as yellowish microscopic prisms, m.p. 266-268 degrees centigrade Analyses indicated the formula $C_{22}H_{23}O_7N$, and the formation of this compound appears to involve the oxidation of a reactive methylene group to a carbonyl (Cook and Loudon, 1959). There was evidence that oxycolchicine reacted with hydroxylamine, although an oxime was not isolated. According to a study in 1924, however, the compound gives a semi-carbazone, m.p. 220-223 degrees centigrade. This oxidation is of importance in indicating the presence of a reactive methylene group in the

colchicine molecule and because of the work in 1890 on "oxydicolchicine" the effect of oxycolchicine on animal organisms is of interest.

Structural Chemistry

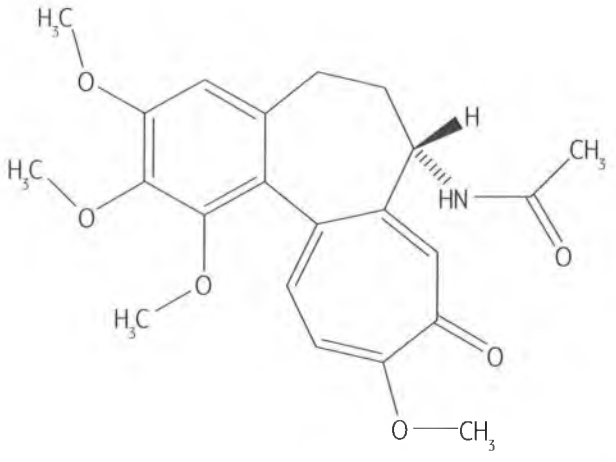
The molecular formula of colchicine is $C_{22}H_{25}O_6N$. The previous studies established the presence in the molecule of one readily hydrolyzable and three more difficultly hydrolyzable methoxyl groups, and also an acetylated primary amino group. These substituents account for the nitrogen atom and five of the six oxygen atoms. The remaining oxygen atom is present in a non-reactive carbonyl group. This has been established indirectly as colchicine and colchiceine are inert towards the usual carbonyl reagents (Cook and Loudon, 1959).

The essential structural problem was, therefore, to determine the nature of the carbon skeleton of 16 atoms, and the positions of the substituents within this framework. This problem has not been completely solved, although the main features have been elucidated, and the structure of a closely related degradation product, deaminocolchicine methyl ether, which contains all but one of the carbon atoms of the $C_{16}H_9$ residue, has been unequivocally established (Cook and Loudon, 1959).

The most important evidence bearing on the structure of colchicine has been derived from an elegant series of investigations. A structural formula was advanced by one of the studies that was recognized to be in doubt in certain minor respects, but these points of doubt have been clarified by later workers. Recent interest in the biological properties of colchicine has stimulated renewed chemical investigation, with the result that it has been necessary to revise the old structure in some important respects.

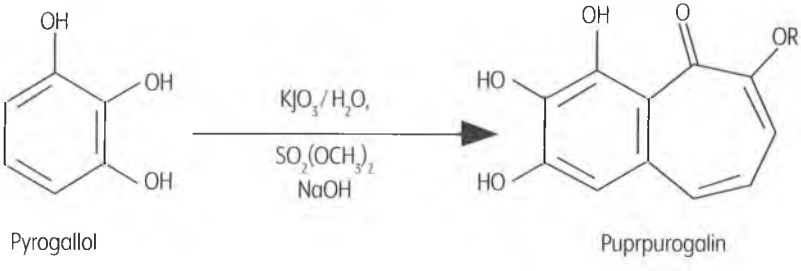
Synthesis of Colchicine by Schreiber Synthesis

For over a century, the known main alkaloid of *Colchicum autumnale* L., Colchicine, takes in a structural-chemical relationship and also has a special position in terms of its biological effects among the alkaloids.



The elucidation of its synthesis into the substance of formula below, the result of some classic investigations by various research groups, has been completed in the current decade. Here, a total synthesis of this natural substance is reported in context of the work done by Schreiber *et al.* in 1959.

Pyrogallol is oxidized to purpurogallin in the presence of KJO_3, H_2O, $SO_2(OCH_3)_2$, NaOH.

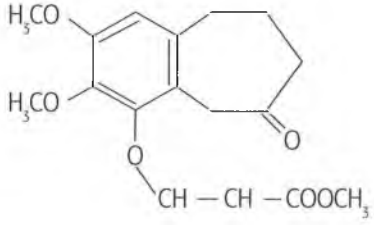


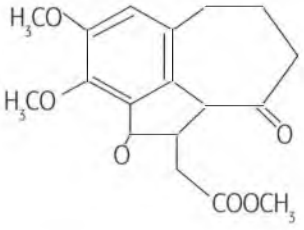
Pyrogallol Puprpurogalin

The OH side groups on the benzene ring are replaced with OCH_3. Whereas the OR group on the other ring is replaced with O double bonded to the carbon. The bonded oxygen atom on this ring is released during the reaction. This was carried out in the presence of H_2-Raney-Ni, Tetrahydrofuran

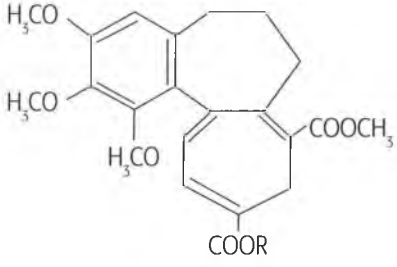
at 45 degrees centigrade, followed by the subsequent addition of $LiAlH_4$. After that, H_3PO_4 and H_2O were added in 1:1 ratio and kept at 70 degrees centigrade (Schreiber *et al.* 1959).

In the case of the triethylamine induced in tert-amyl alcohol and the subsequent addition of potassium tert. Amylate, the product of the previous reaction underwent condensation with methyl propiolate and the tricyclic hydroxy-α-pyrone is formed in an easy and uniform reaction (Schreiber *et al.* 1959). This annulation takes place via the intermediate products of the structures below. When appropriately modified reaction conditions are used, crystalized isomers of this type can be isolated in high yield.

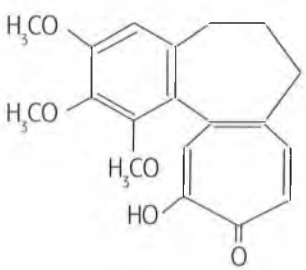




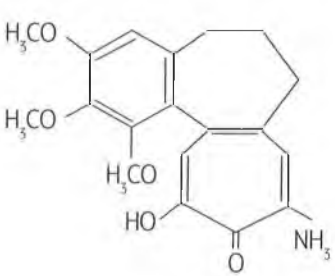
The trimethoxy-a-pyrone derivative goes off when heated with chloromethyl maleic anhydride under simultaneous elimination of carbon dioxide, a diene reaction occurs, with over 70 percent yield tetracyclic anhydride forms. The corresponding run using methanolysis and Dicarboxylic acid available after treatment with diazomethane diester is subject to treatment with potassium tert-amylate in benzene is extremely smooth with internal alkyl discharge resulting in a ring expansion and resulting in the product below (Schreiber *et al.* 1959).



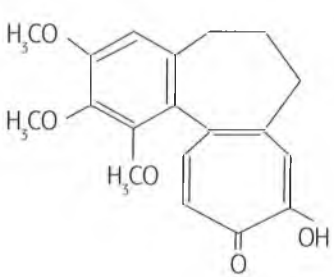
The tropolonization by partial alkaline Hydrolysis of this gives a monocarboxylic acid which can be obtained through two methods. The direct one and the more productive oxidative one with decarboxylation of the mono-adduct of the product and Osmium tetroxide by heating it in a weakly alkaline solution and in the presence of oxygen; the Carbomethoxy-tropolone that is formed has so far been isolated in yields of 20-30% (Schreiber *et al.* 1959). Decarboxylation of the corresponding carboxylic acid by brief heating to 260 degrees centigrade in the presence of quartz powder leads to the structure below with the isomeric arrangement of the α-Tropolone function (yield 64%) (Schreiber *et al.* 1959).



This arrangement can be reversed by evaluating an observation by another team of scientists on substituted, monocyclic α-chloro-tropones, followed by their implementation with ammonia under certain conditions corresponding α'-amino-tropones leads. In the present case, the tosylate which is required for this process, unfortunately, formed at the same time and the isomers were not separated.

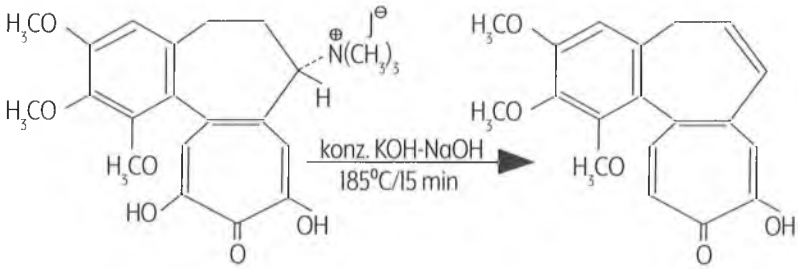


Amino - Tropone

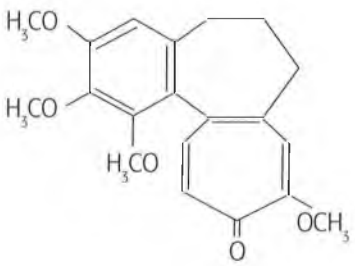


Free - Tropone

The ammonolysis of the non-crystallized tosylate mixture, however, gave a reaction product compared to 30 % Sulfuric acid basic content. Direct crystallization allowed the amino-tropone to be obtained (15% based on the above structure); in a likewise crystallized obtained component of the neutral portion (Fp 186 degrees centigrade) it is the rearranged acid amide. The amino-tropone and the free tropolone obtained therefrom by alkaline hydrolysis were found in the direct comparison according to their melting point, mixed sample, UV, and IR spectrum (38 or 45 bands) with deacetylamino-iso-colchicine-amide or desacetylamino-colchicein, as identical. These conversion products of colchicine are obtained by Hofmann degradation (Schreiber *et al.* 1959).



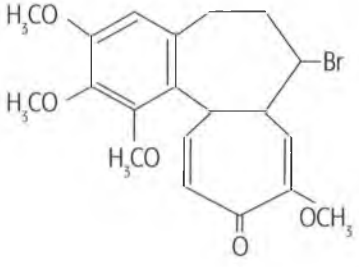
The reaction of free tropolone with diazomethane gave methyl ether mixture which could be determined by chromatography.



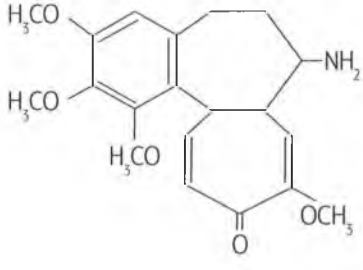
Their constitutional assignment is based on the fact that the isomer, which melts at 141 degrees centigrade, is uniformly that of the two possible amino tropones which when it reacts with ammonia, is identical to the Aminotropone that was totally synthetically obtained. As expected, the two methyl

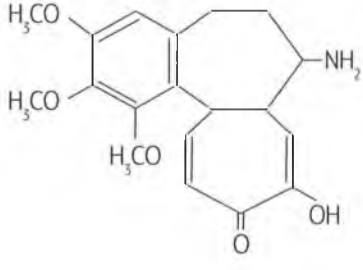
ethers behave significantly differently when reacted with Bromo succinimide (Schreiber *et al.* 1959).

Bromination of the above shown methyl ether underwent a rapid reaction which gave a mixture. From this mixture, in addition to the non-crystallized reaction products, an allylic Monobromide (shown below, m.p. 165 degrees centigrade; current reaction with $AgNO_3$) and a nuclear brominated isomer (m.p. 212 degrees centigrade; no reaction with $AgNO_3$) in an approximate ratio of 2:3 was obtained chromatographically (Schreiber *et al.* 1959).



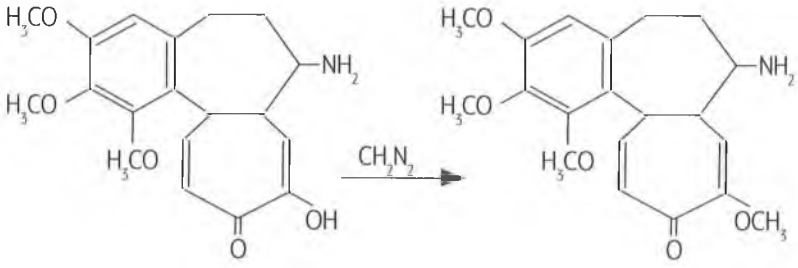
In contrast, the bromination of the isomeric methyl ether slows down significantly and, moreover, seems exclusively, according to the previous indications, to lead to nuclear brominated products. From the allylic bromide (m.p. 165 degrees centigrade) arose among those previously tried Ammonolysis conditions (NH_3 in C_2H_5OH at 100 degrees centigrade) predominantly a compound $C_{19}H_{19}O_4N_2$ (m.p. 235 degrees centigrade), in which it is an elimination product, besides a base $C_{19}H_{22}O_4N_2$ melting at 260 degrees centigrade (Schreiber *et al.* 1959).





The latter proved to be identical to the racemic Desacetyl-isocolchicin-amide, the one derived from (+/-)-deacetylcolchicein.

The melting points, the UV spectra, the IR spectra were recorded in KBr (41 bands), as well as the paper chromatographic behavior in the chloroform-formamide and n-butanol-acetic acid 9:1 ratio (Schreiber *et al.* 1959).



Effect of colchicine on cell

In recent years intense interest has been aroused by the remarkable biological properties of colchicine, which were first revealed by the work of Dustin in 1934 and Lits in 1938. Eigsti and Dustin (1957) observed that Colchicine is the methyl ether of an enolone containing three additional methoxy groups, an acetylated primary amino group and three non-benzenoid double bonds. A later study by Levan (1949) (full reference not available) noted that the threshold regions of colchicine-mitotic activity are identical for both crystalline and amorphous forms; chloroform exerts no appreciable effect. One of the significant findings in Allium cepa is that whenever isocolchicine is used instead of colchicine, no C-mitotic action is observed. The latter differs from the former only in minor details of structure. The data so far implies that: (a) at least one methoxy group in ring A is necessary for colchicine action; (b) ring C must be 7-membered, and the hydroxyl group should preferably be replaced by an amino group; (c) esterification of amino group in ring B increases the activity; and (d) isocolchicine and its derivatives are less active. A proper distance should be maintained between esterified side chains of rings

B and C (Sharma and Sharma, 1980). This last statement is based on the fact that in isocolchicine, where the position of the methoxy group is reversed in ring C, the decrease in efficacy has been considered to be due to the presence of hydrogen bonds between the side chains of rings B and C (Sharma and Sharma, 1980). In the case of 'colchicine', the weak action is the result of the iso-form of this molecule. Though these data indicate the reactive groups responsible for colchicine action, the exact reaction involved in spindle inactivation is not yet fully clear. Pertinent suggestions have been made on the basis of results obtained with spindle poisons of different chemical structure (Sharma and Sharma, 1980).

Effect of colchicine on tumor

Yet another facet of the cytological action of colchicine is reflected in the numerous studies of its effect on tumor growth. Although the promise of a therapeutic agent against cancer indicated by some of the earlier reports has not been sustained by more extensive investigation, probably, the last word on this subject has not yet been written. In a review by (Levine, 1945, 1946), it was suggested that more extensive studies should be made of the action of colchicine, combined with X-rays, on animal tumors. The very high toxicity of the alkaloid prevents its use for this purpose in any but the smallest doses, but when its structure is completely elucidated it may be hoped that the way will open for the synthesis of analogous compounds less toxic than colchicine, but with similar action on the cell nucleus.

4. Chapter: Vinblastine sulphate, chemistry and history

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Summary:
Vinblastine and Vincristine are alkaloids isolated from the plant *Catharanthus roseus* (Madagascar periwinkle) with antineoplastic properties. *Vinblastine* disrupts microtubule formation and function during mitosis and interferes with *glutamic acid* metabolism.

Effect of Vinca alkaloids on nucleic acids
It has been shown (Richards et al., 1966) that certain of the *Vinca* alkaloids, e.g., vinblastine, vincristine, and vinleurosine, inhibit the utilization of formate-^{14}C in the biosynthesis of nucleic acids by rat thymus cell suspensions. The DNA was affected much more than the RNA. Although the correlation was not exact, in general it was found that those alkaloids which arrested the growth of experimental tumors also inhibited the synthesis of DNA in thymus cell suspensions. In this regard, Jones et al., (1966) found that Vinblastine (VLBS) markedly inhibited the incorporation of formate-^{14}C and glycine-2-^{14}C into the DNA of rat thymus cell suspensions. In contrast, VLBS either did not affect or only slightly stimulated the labeling of the hypoxanthine and thymine in the "hydro-

lyzed acid-soluble" fraction. The labeling of the adenine and guanine both in this fraction and in the RNA was slightly decreased by the alkaloid. Incorporation studies using formate-, glycine-, or hypoxanthine-^{14}C indicated that VLBS interfered with reactions more particularly related to DNA synthesis. Time studies showed that the labeling of the DNA purines was affected before and to a greater extent than the DNA thymine. The acid-soluble fraction of thymus cells incubated with formate-^{14}C in the presence of VLBS contained increased amounts of radioactive serine and glycine. This increase over the control values did not appear until the incubation had been in progress for about 2 hours, whereas the effects of the alkaloid on DNA synthesis were detectable within 0.5 hour.

Effect of Vinca alkaloids on microtubules

Bensch and Malawista (1969) found that the periwinkle alkaloids in very low concentrations cause an intracytoplasmic sequestration of microtubule protein in the form of symmetrical, microtubular bodies. These crystals, which may measure up to 8 μ in length, appear within 30 minutes in L-strain fibroblasts *in vitro*, but they increase in incidence and size with time of exposure to the alkaloids. Similarly, if exposed to these compounds, human leukocytes *in vitro* contain identical crystalline structures. Neither colchicine nor puromycin prevents the formation of these bodies, the latter compound, however, retards crystal growth. The results of the experiments in which only the periwinkle alkaloids were used were essentially identical, with little difference in effect between the two periwinkle compounds or the different cell types (Bensch and Malawista, 1969). The same alkaloids, when used in a study of microtubules of polymorphonuclear leukocytes, again made the microtubules disappear but produced in these cells the intracellular bodies of singular regularity which fulfilled the criteria for definition of a crystal. This observation led to a systematic examination of the effects of the *Vinca* alkaloids vinblastine and vincristine sulfate on microtubules in mammalian cells. In another study, human leukemic lymphoblasts exposed to Velban showed prominent proteinaceous crystals and large complexes of ribosomes (which are arranged in clusters and helices) in association with fine granular, electron dense

material. This granular material showed relatively greater electron density than the crystals and lacked the prominent linearity of the crystals. In some cross sections tubular profiles were seen interspersed between the granular material and the ribosomes. It was suggested that the ribosomes are involved in the synthesis of the electron dense material which is subsequently organized into the prominent crystals. Treatment of cells or tissues that contain cytoplasmic microtubules with the vinca alkaloids result in disappearance of the microtubules, and the formation of highly regular crystals (Bensch and Malawista, 1969; Nagayama and Dales, 1970). Vinblastine crystals isolated from unfertilized sea urchin eggs have been found to be composed of tubulin complexed to vinblastine (Bryan, 1972; Bryan, 1971; Bryan, 1972). The finding that vinblastine caused crystal formation *in vivo* was quickly followed by the demonstration that it could cause the precipitation of tubulin *in vitro* (Bensch et al., 1969; Olmsted et al., 1970; Wilson et al., 1970), along with the assumption that the two effects were mechanistically related. However, precipitation *in vitro* and crystal formation *in vivo* seem to be produced by different mechanisms. Vinblastine precipitates tubulin in a concentration-dependent manner; usually high concentrations of vinblastine are required to bring about this effect. This appears, however, to be a non-specific effect of vinblastine (Wilson et al., 1970). Several early observations suggested that there must be other binding sites on tubulin with higher affinity than these. These and other data suggested that there might be many low-affinity binding sites for vinblastine on tubulin. It has been found that there are between 20 and 30 low-affinity vinblastine-binding sites per mole of chick embryo brain tubulin. These binding experiments were carried out by equilibrium dialysis with vinblastine in the millimolar concentration range; the protein ending up in the form of a vinblastine precipitate. The concentrations required to produce a mitotic index of 50 percent for all three analogs were approximately the same, 8×10^{-8} moles/l (Wilson, 1974). Second, low concentrations of the *Vinca* alkaloids slow the rate of decay of colchicine-binding activity (Wilson, 1970). Active *Vinca* alkaloids such as vinblastine, vincristine, and desacetylvinblastine possess this property. Low concentrations of vinblastine can

cause an aggregation of tubulin, which is seen as an increase in the sedimentation coefficient from 6 S to approximately 14 S. Low concentrations of vincristine produce the same effect. Wilson (1974) found that the tubulin-vinblastine complex would have an extrapolated sedimentation coefficient of about 9.5 S at zero protein concentration, which is consistent with a dimer of the 6 S tubulin. Thus, the binding of the *Vinca* alkaloids to a postulated class of high-affinity binding sites, which presumably prevents the tubulin from polymerizing, may induce the formation of tubulin dimers. This may be the initial event in the formation of crystals *in vivo*.

Another study by Bhattacharyya and Wolff (1976) further pursued the multiple binding sites of vinblastine and found that the rat brain tubulin possesses two distinct binding sites for vinblastine per molecule: a high-affinity site with an affinity constant of 6.2×10^6 M^{-1} and a low-affinity site with that of 8×10^4 M^{-1}. This study showed that vinblastine binding to the high- and low-affinity sites, respectively, accounts for the depolymerization and aggregation behavior of tubulin.

Effect of colchicine and vinblastine on animal cells.
Aside the interference with the assembly of microtubules, the alkaloids colchicine and vinblastine also affect several properties of the surface membrane of animal cells. They disorganize an apparent topographical separation between surface areas involved in membrane transport and in phagocytosis in rabbit polymorphonuclear leukocytes, inhibit the agglutination of these cells by concanavalin A and inhibit the concanavalin A-dependent adherence of red blood cells to transformed 3T3 fibroblasts (Waddell *et al.* 1974). It has also been found that these alkaloids also inhibit the spontaneous aggregation of hamster fibroblasts (BHK 21, clone 13) which occurs when these cells are shaken in suspension, after dispersal from monolayers by exposure to trypsin in the presence of EDTA (Waddell *et al.* 1974). The inhibition by alkaloids is less complete than can be obtained by treating the cells with high levels of trypsin, and the inhibited cells aggregate more than do polyoma-transformed cells.

In a different study it was found that the human monocytes treated with the microtubule-disrupting agents vinblastine and colchicine show enhanced migration into micropore filters (Crispe, 1976). This study revealed that when human blood monocytes are treated with the microtubular disrupting agents - colchicine and vinblastine - they show enhanced migration into micropore filters. The mechanism of this effect is likely due to the disruption of microtubules because the two agents bind to different sites on tubulin molecules, colchicine producing complete dissociation of microtubular protein and vinblastine precipitating it as crystals (Margulis, 1973), and there is no reason to suppose that they have any effects in common other than the disruption of the microtubular system. (2) The concentrations of the agents which produce maximal enhancement of migration (10^{-6} – 10^{-5} M) are sufficient to disrupt microtubules (Bhisey and freed, 1973) but were not sufficient in these experiments to cause loss of viability. Also, the known side effects of colchicine and vinblastine are the inhibition of various metabolic (including respiratory) pathways, and it is difficult to attribute an enhancement of motility to these effects. When treated with hexylene glycol, which appears to stabilize microtubules, their migration is inhibited (Crispe, 1976). These results suggest that disruption of the microtubules may facilitate deformation of the cell necessary for migration through small holes and that stabilization of the microtubules may increase cell rigidity and inhibit such migration.

Several *in vitro* methods are available for testing the biological activity of "antitubulins" (drugs that interfere with the structure or function of microtubules through the inhibition of tubulin polymerization into normal microtubules). Most involve the use of plant tissues or protozoa (Margulis, 1973; Deysson, 1968). Mammalian cells can hardly be expected to maintain identical reaction patterns. On the contrary, their reactions are diverse, so sophisticated, time-consuming techniques are needed to establish unambiguously the phenomenon of spindle disruption or to conduct quantitative dose-response studies with known antitubulins. Indeed, the most widely used parameter is the rise in mitotic index, which is the result of a temporary arrest in mitosis (De Brabander

et al., 1976). However, the mitotic peak is highly dependent on the culture conditions and the relative degree of synchrony of the cells. Alternatively, most toxic stimuli induce cultured cells to become rounded like mitotic cells (De Brabander *et al.*, 1976). Discerning disorganized mitotic cells is not possible without the time-consuming histologic techniques but even these procedures can produce ambiguous results due to the easy detachment of the rounded mitotic cells from the substrate by normal washing procedures. While examining the effects of colchicine (Eigsti and Dustin, 1955) and related drugs on cultured mammalian cells of different origin, De Brabander *et al.* (1976) found that some cell lines responded in a uniform and reproducible manner by producing multi-micronucleated cells that can easily be identified with phase-contrast microscopy. They found that this effect was restricted to disruption of mitotic microtubules, and so they developed it into a simple technique. Thus, a new culture model, which facilitated both mass screening of potential anticancer drugs acting on microtubules and quantitative experiments with known "antitubulins," was found (De Brabander *et al.*, 1976).

Microtubules appear to be important for the restriction of lamellipodial activity to a limited portion of the cell's margin and for the characteristic elongated spindle shape adopted by fibroblasts on two-dimensional surfaces since exposure to antimicrotubular agents results in an expansion of lamellipodial activity around the entire perimeter and a loss of the elongated shape (De Brabander et al., 1976; Gail and Boone, 1971; Goldman, 1971; Ivanova, 1976; Miszurski, 1949; Van der Schueren et al., 1978; Vasiliev et al., 1970; Vasiliev et al., 1969). There is a marked decrease in directed cell movement at displayed during wound healing (Vasilieve and Gelfand, 1976; Vasiliev et al., 1969) and in the rate of emigration of cells from cellular aggregates adherent to a substrate (De Brabander et al., 1976).

The involvement of microtubules in mediating the placement of lamellipodia has been demonstrated only for cells migrating in monolayers on artificial surfaces (usually glass or plastic). As an attempt to duplicate more exactly the *in vivo* situation of mesenchymal tissue, Armstrong and Armstrong (1979)

assessed the effects of antimicrotubular agents on motility of fibroblasts within three-dimensional aggregates maintained in organ culture. The morphology and motility of tissue cells involves several elements of the cytoskeleton (the microfilaments, intermediate filaments, and microtubules) (Abercrombie, 1973; Goldman et al., 1976). The use of drugs that selectively depolymerize one or another of the cytoskeletal components has proven useful in unraveling their respective functions (Borhers and De Barbander, 1975; Soifer, 1975). Consistent with the observations on cells in monolayer culture, motility of fibroblasts in mesenchymal aggregates is significantly reduced by antimicrotubular agents, suggesting that microtubules do play a role in the control of cell movement in intact tissues.

Use of *Vinca* Alkaloids for cancer

The *Vinca* alkaloids are established agents in the treatment of lymphomas, especially Hodgkin's disease, childhood acute leukemias, choriocarcinoma, and embryonal or mesenchymal carcinomas such as Wilms' tumor, neuroblastoma, rhabdomyosarcoma, and carcinoma of the testis (Creasey, 1975). Vinblastine is somewhat more effective than vincristine in the treatment of advanced Hodgkin's disease, whereas the latter drug is the more active against lymphosarcoma and reticulum cell sarcoma (Creasey, 1975). More striking, however, is that vincristine is of value in the therapy of acute lymphoblastic leukemia, a disease for which vinblastine is ineffective. The considerations of Valeriote et al., (1966) suggest that optimal effects of vinblastine require significant lengths of exposure to critical drug concentrations. However, in the clinic, toxicity has soared with more frequent injections, and this has discouraged exploration of other dose regimens. Vinblastine's antineoplastic activity in cancer chemotherapy is exercised through the disruption of cell mitotic spindles and the inhibition of cell division. Cells treated with vinblastine display the characteristic morphology called 'Sc-mitosis," in which cellular microtubules are destroyed and the cells are arrested at their metaphase (Palmer et al., 1960; Cutts, 1961). However, unlike other c-mitotic agents, such as colchicine and podophyllotoxin, vinblastine can induce in cells the formation of

highly ordered paracrystalline aggregates (Bensch and Malawista, 1968; Bensch and Malawista, 1968; Malawista and Sato, 1969; Fujiwara and Tilney, 1975).

Main Body:
Vinblastine Sulphate ($C_{46}H_{60}N_4O_{13}S$), the sulfate salt of vinblastine, and Vincristine ($C_{46}H_{56}N_4O_{10} \cdot H_2SO_4$) are alkaloids derived from the plant *Catharanthus roseus* (Madagascar periwinkle) and possess antineoplastic properties (Merck Index, 1976). They are not soluble in aqueous solutions. For application and experiments they must be transformed into their corresponding salts, i.e., sulphate. When we talk about Vinblastine here, we mean the salt, i.e., Vinblastine Sulphate.

It has been shown (Richards et al., 1966) that certain of the *Vinca* alkaloids, e.g., vinblastine, vincristine, and vinleurosine, inhibit the utilization of formate-^{14}C in the biosynthesis of nucleic acids by rat thymus cell suspensions. The DNA was affected much more than the RNA. Although the correlation was not exact, in general it was found that those alkaloids which arrested the growth of experimental tumors also inhibited the synthesis of DNA in thymus cell suspensions. In addition, thymus cell suspensions prepared from the glands of rats which had been given small doses of vinblastine incorporated much less formate into DNA than cells from the glands of untreated animals (Richards and Beer, 1964). These observations suggested that the inhibition by vinblastine of the biosynthesis of the nucleic acids in isolated thymus cell suspensions was probably quite closely related to the biologic activity of the compound in the intact animal.

In the biosynthetic pathways leading from the simple precursors such as formate and glycine to the polymeric DNA there are many intermediate reactions which might be inhibited by the alkaloid. This communication describes the effect of vinblastine (VLBS) on the synthesis *de novo* by thymus cell suspensions of the purine and pyrimidine bases present in the nucleotide pool. This has been studied by measuring the incorporation of radioactive formate and glycine into the bases, since these simple precursors are utilized as well-established

points in the biosynthetic pathways leading to the nucleic acids (Jones *et al.*, 1966). The radioactive bases are present - mainly as nucleotides - in the acid-soluble fraction of the cell suspensions. For analytical purposes the bases have first been liberated from the nucleotide pool by hydrolysis with perchloric acid and then separated and purified by conventional chromatographic procedures. The results of the study by Jones *et al.* (1966) show that, although VLBS strongly inhibited the incorporation of formate and glycine into the DNA of thymus cell suspensions, it did not inhibit the synthesis *de novo* of either the thymine or the hypoxanthine ring. Rather, the alkaloid appeared to stimulate somewhat the incorporation of exogenous formate into the derivatives of these bases in the acid-soluble fraction of the cell suspension. This suggests that VLBS may slightly stimulate either the formation or the use of activated 1-carbon intermediates. These results do not support the suggestion of McGeer and McGeer (1963) that VLBS may cause an over-all depression of purine biosynthesis. On the other hand, since the utilization of radioactive hypoxanthine in DNA synthesis was also markedly decreased, it appears that the alkaloid inhibits one or more of the reactions which load from inosinic acid to DNA (Jones *et al.*, 1966). VLBS affected the incorporation of formate into the DNA purines more than into the thymine, particularly after the cells had been exposed to the alkaloid for some time, e.g., 1.5 hour. This suggests that the reactions in the metabolism of the adenine and guanine intermediates may be particularly sensitive to the alkaloid. It may be significant in this connection that the synthesis *de novo* of hypoxanthine-^{14}C was slightly stimulated by VLBS, whereas the labeling of the acid-soluble adenine and guanine derivatives was not (Jones *et al.*, 1966).

Furthermore, the alkaloid inhibited the incorporation of labeled precursors into the adenine and guanine of the DNA much more than of the RNA, suggesting interference with a reaction more directly involved in DNA synthesis, e.g., the formation of the deoxyribonucleotides and/or the polymerase reaction. It is unlikely, however, that VLBS interferes with the formation of deoxyribonucleotides as a class since the synthesis of the acid-soluble thymine intermediates was not

inhibited. This indicates that the incorporation of formate-^{14}C into the DNA thymine is markedly reduced because the alkaloid inhibits one or more of the reactions which lie between thymidylic acid and DNA (Jones *et al.*, 1966). These reactions include the kinase-catalyzed steps leading to the formation of thymidine triphosphate and the polymerase reaction. However, the depletion of the supply of any of the other concurrently required deoxyriboside triphosphates would also inhibit the incorporation of the labeled thymine precursor.

Jones *et al.* (1966) showed through their work that VLBS interferes with the biosynthesis of DNA in thymus cells at some point after the formation of the simpler pyrimidine and purine nucleotides. However, the reactions involved in the biosynthesis of DNA are common to most mammalian tissues. The differences in the sensitivity of tissues to VLBS would seem, therefore, to be related to aspects of the metabolism of the cells other than those directly concerned with DNA synthesis. VLBS causes metaphase arrest of dividing cells. Studies have shown that VLBS may interfere *in vivo* with the synthesis of soluble RNA in the Ehrlich ascites tumor (Creasey and Markiw, 1964). They suggested that this might lead to disturbances in amino acid metabolism and interfere with the synthesis of protein such as that needed in the mitotic apparatus. It was found that in the presence of VLBS there was an increase in the total radioactivity of certain amino acids (serine, glycine and probably methionine) in the acid-soluble fraction of thymus cells incubated with formate-^{14}C. This increase may be due either to an enhanced synthesis of these compounds or to their decreased utilization as might result from a blockage in protein synthesis. The effect of VLBS on the radioactivity of the serine appeared only after the incubation had been in progress for about 2 hours, whereas the radioactivity of the DNA was affected from the start. This suggests that the two effects are only indirectly related. There is insufficient information at present to explain the biologic activities of VLBS in satisfactory biochemical terms. There is evidence (Beer, 1961; Creasey and Markiw, 1964; Richards et al., 1966), however, that the alkaloid interferes with nucleic acid metabolism, but it is not certain whether this is a direct effect or the result of a biochemical disturbance in some other

part of the molecular organization of the cell. More detailed studies are in progress in attempts to answer this.

Effect of Vinca alkaloids on nucleic acids

Vinblastine lowers ^{14}C-leucine incorporation into ascites cells. Vinblastine and vincristine inhibit DNA, RNA and especially protein biosynthesis in leukemic bovine lymphocytes and lead to inhibition of protein biosynthesis in cell-free systems. In case of the nucleic acid metabolism, they primarily inactivate the DNA-dependent RNA polymerase and reduce the intracellular nucleoside content. The colchicine used for comparison has similar binding sites and hence was chosen for the study (Warneoke, 1968).

Effect of Vinca alkaloids on mitosis

Periwinkle *(Vinca)* alkaloids, helpful in the therapy of neoplastic diseases, are known to affect cell division through action similar to that of colchicine (Palmer et al., 1960). Overdosage of these drugs primarily damages organs known to be rich in microtubules, such as neurons (Vy et al., 1967). Previous studies showed a rapid (but, for vinblastine, reversible) decrease and disappearance of the birefringence of the mitotic spindle of living eggs of a marine annelid during perfusion with these compounds; electron microscopy confirmed the attrition and disappearance of microtubules by such treatment (Malawista, 1968). In a later study, Bensch and Malawista (1969) studied the L-strain fibroblasts and human leucocytes. The fibroblasts in the tissue culture (Eagle, 1955) were exposed for 30 minutes to 24 hours to the periwinkle alkaloids which were added directly to the medium, resulting in final alkaloid concentrations ranging from 4×10^{-4} to 1×10^{-5} M. These cells, as well as control cultures, were subsequently fixed by addition of isotonic 2 percent phosphate-buffered glutaraldehyde solution (pH 5.9, temperature 24 to 30 degrees centigrade) at a ratio of 1:1, followed within 15 minutes by centrifugal separation of the cells from incubation medium-fixative mixture. Fibroblasts, but not leukocytes, were fixed also at 4 degrees centigrade and pH 7.4. The pellet was then overlaid with the fixative described above. Osmification (Palade, 1952) was carried out 1 to 240 hours after a 12-hour rinse of the cell pellet in isotonic phos-

phate buffer. The tissue embedded in epoxy resin (Maraglas) (Freeman and Spurlock, 1962) was examined in an Elmiskop. Similarly, suspensions of human leukocytes were incubated at 37 degrees centigrade for 2 to 3 hours in a phosphate-buffered Krebs-Ringer medium with 12% autologous serum, with or without the alkaloids (vinblastine 2.5 x 10^{-5} M, vincristine 1 x 10^{-4} M) (Bensch and Malawista, 1969). In other series of experiments, colchicine (final concentration 1 to 4 x 10^{-5} M) was added to the human fibroblast cultures 2 to 24 hours before addition of the periwinkle alkaloids. Similarly, suspension of human polymorphonuclear leukocytes were treated with colchicine 5 x 10^{-5} M 1 hour before vinblastine 2.5 x 10^{-5} M or with colchicine 2 x 10^{-4} M 1 hour before vincristine 1 x 10^{-5} M. In addition, leukocytes were exposed to 2 x 10^{-5} M puromycin (10 μ/ml) for 20 minutes before addition of vinblastine (2 x 10^{-5} M) or vincristine (1 x 10^{-4} M) (Bensch and Malawista, 1969). The exposure of the leukocytes to the *Vinca* alkaloids in all the experiments was 3 hours.

Effect of Vinca alkaloids on animal cells

Bensch and Malawista (1969) found that the results of the experiments in which only the periwinkle alkaloids were used were essentially identical, with little difference in effect between the two periwinkle compounds or the different cell types. Abnormal elongated intracellular crystalline bodies measuring up to 8 μ were identifiable in the 1 μ thick sections of the plastic-embedded material. Crystals, although encountered in all parts of a cell, were more abundant in the concave recess of the nucleus of the fibroblasts, normally an area of pronounced cytoplasmic activity, usually containing the Golgi apparatus, lysosomes, and the centrioles. The latter were sometimes partly surrounded by crystals, a feature which could also be found in the leukocytes. After 30 minutes to 1 hour exposure to the alkaloids, most of the crystals were observed in this juxtacentriolar location. Prolonged exposure was associated with dispersal of the cell organelles throughout a cell's cytoplasm.

The larger crystals predominated in dividing cells treated with the lower concentrations of vinblastine for 4-8 hr. These structures occupied, as confirmed by electron microscopy, a

considerable portion of a cell (Bensch and Malawista, 1969). Sections of these crystalline bodies parallel to their long axis revealed their fine structure to be reflected in highly regular arrays of electron-opaque lines which ran parallel to the long axis of these crystals. The predominant periodicity of these lines was 280 angstroms and was less frequently about 240 angstroms. However, a 200 angstroms periodicity was also observed repeatedly, particularly on examination of thinner sections; in this instance, the parallel lines consisted of rows of electron-opaque isodiametric (80 angstroms) dots which were spaced regularly every 240 angstroms. Dots forming a line were interconnected with one another by thin strands of electron-opaque material. The counterpart of a dot on a neighboring row was not present at the smallest possible distance, i.e., 200 angstroms, but at a certain greater distance (230-240 angstroms), thus giving rise to a second type of periodicity caused by rows of dots which formed a 22-degree angle with the short axis of a crystal (Bensch and Malawista, 1969). Sections perpendicular to the long axis of these crystalline bodies showed what appeared to be (at lower magnification) a regular pattern of circles conveying the impression of cross-sectioned stacks of microtubules. Closer examination at high magnification revealed each of these crystalline structures to consist of a hexagonal electron-lucent center surrounded by an electron-opaque rim of even width. This rim, which was shared with six surrounding tubules, measured approximately 80 angstroms in width. The outer diameter of each tubule was that of the predominant periodicity of the longitudinally sectioned crystals, namely 270-280 angstroms, the inner diameter thus being about 200 angstroms (Bensch and Malawista, 1969).

Relatively infrequently, collections of separate microtubules among a relatively dense population of ribosomes were observed in fibroblasts and mononuclear leukocytes but not in polymorphonuclear leukocytes. The diameter of each of these tubules was 270-280 angstroms, while the diameter of the tubules in control cells averaged 240 angstroms (Bensch and Malawista, 1969). Longitudinal sections through these collections of microtubules confirmed the increased numbers of ribosome in these areas if compared with the adjacent cytoplasm. Pre-

incubation of fibroblasts in media containing as much as 4 X 10^{-4} M colchicine for 24 hours followed by addition of vinblastine to the incubation fluid did not prevent the formation of microtubular crystals. The latter were identical with those found in cells exposed to vinblastine alone. Crystals were also present in leukocytes exposed to colchicine and vinblastine or vincristine; their incidence, intracellular distribution, and size were identical with that found in leukocytes exposed to the periwinkle alkaloids alone (Bensch and Malawista, 1969). Puromycin did not prevent the formation of crystals which, however, appeared smaller and fewer in their numbers.

There can be little doubt that the crystals formed after exposure of mammalian cells to periwinkle alkaloids consist mainly of microtubules; the structural resemblance between the tubular subunits of the crystals and normal microtubules is obvious. In addition, there is a complete absence of normal microtubules in the treated cells, except for occasional collections of tubules with a diameter identical with that found in the crystals. Moreover, colchicine, which is thought to occupy binding sites on its cellular target protein that are different from those held by periwinkle alkaloids (vinblastine) (Wilson and Friedkin, 1967), does not prevent formation of these crystalline bodies. Periwinkle alkaloids, however, block sites essential for the formation of normal tubular structures. Thus, vinblastine and vincristine bring about not only the formation of larger than normal microtubules and microtubule crystals but also a stabilization of these structures; the rigid requirements for the preservation of the microtubular structure by fixation at high temperature (37 degrees centigrade) and low pH also are not necessary after alkaloid treatment. Yet, in the experiments with *Pectinaria* eggs (Malawista, 1968), it was found that the process of spindle dissolution is fully reversible by diluting the concentration of vinblastine (but not vincristine) in the suspension medium (Bensch and Malawista, 1969).

The normal precarious equilibrium between the microtubular monomer and polymer leading to formation of tubules is also emphasized by the short period of time necessary for crystal formation. These crystalline bodies may occupy a relatively

huge volume estimated conservatively as 10 percent or more of the total cytoplasm of a cell; the short time interval between exposure and appearance of crystals in fibroblasts as well as the presence of voluminous crystals in the polymorphonuclear leukocytes, a cell type usually considered to carry out very little protein synthesis after its release into the bloodstream, indicates that normally most of the microtubular protein must be present in a disoriented state and must not be newly formed after exposure to the alkaloids (Bensch and Malawista, 1969). The presence of crystals in the experiments in which the leukocytes were exposed to puromycin supports this notion. However, prolonged exposure to the drug, with the concomitant sequestration of the microtubules in the form of crystals, may accelerate microtubular protein synthesis, as is borne out by the observed increase in number and size of the crystalline structures with time as well as the appearance of stacks of the single (abnormal in respect to their diameter) tubules between rows of ribosomes. Another striking feature is the proximity of crystals to centrioles in interphase cells, a finding which supports the assumption that these organelles may very well be a point of origin in the assembly of microtubules (Robbins et al., 1968).

The observed change in the normal cytoplasmic distribution of cell organelles, i.e., their dispersal after prolonged alkaloid exposure (12-24 hours), was in contrast particularly with the confinement of lysosomes to a small part of an untreated cell; this finding may reflect on one of the functions of microtubules, namely direction of the intracellular saltatory movements of cell particles (Freed, 1965). This function may be one of the requirements for the efficient merger of lysosomes with phagosomes (Malawaista, 1968). Indeed, there is evidence that the periwinkle alkaloids do not affect phagocytosis but retard intracellular digestion (Malawaista, 1968; Bensch, K. Unpublished).

Examination of the crystals per se suggests that the periwinkle alkaloids, like colchicine, permit the polymerization of the tubule protein to protofibrils. However, in the case of colchicine, the presence of ubiquitous fibrillary aggregates was

found; in cells treated with *Vinca* alkaloids, such aggregates were relatively rare. Instead, the assembly of protofibrils under the influence of vincristine and vinblastine apparently may proceed to formation of single cylindrical microtubules with a diameter 40 angstrom greater than that seen in untreated human leukocytes or L-strain fibroblasts (Bensch and Malawista, 1969). These single microtubules, however, are relatively rare when compared with the crystalline aggregates in these cells. The most surprising aspect of the latter is the fact that the wall of a tubule is shared with the six surrounding units. (Noteworthy of mention here is the partial common wall of the outer doublets of microtubules [fibers] of cilia [and spermatozoa] of species ranging from protozoa to mammals (Fawcett, 1966; Granick and Gibor, 1967; Behnke and Forer, 1967). A partial common wall is also normally present in the nine sets of triplets of a centriole.

Closer analysis of cross-sections of these crystals, as well as theoretical considerations, led to the conclusion that these tubules are hexagonal on cross-section and have a greater diameter of approximately 280 angstroms and a lesser diameter of 240 angstroms (Bensch and Malawista, 1969). Theoretically, electron microscopic study of sections of infinite thinness parallel to the long axis of a crystal should thus give line distances ranging front 140 to 280 angstroms; however, the greatest and by far most frequently encountered periodicity was 280 angstroms, although smaller line distances were found, predominantly 240 and 200 angstroms (Bensch and Malawista, 1969). Obviously, the sections studied showed only an occasional crystal cut perfectly parallel to its long axis, and more importantly, the sections were usually at least 500 angstroms thick; these factors should give rise to a variety of patterns. Thus, for instance, the 200-angstrom periodicity may be produced by a superimposition of the zigzag of tubular walls if cut and looked at from a certain angle. But a grazing cut through the surface of a crystal would probably produce an identical pattern. Remarkable about the 200-angstrom periodicity is also the fact that the lines consist of regularly spaced dots which in turn, in conjunction with dots on neighboring lines, give rise to an oblique periodicity of 240 angstroms. The lines

of the latter form an angle of 22 degrees with the transverse diameter of a crystal (Bensch and Malawista, 1969). This poses a question of whether this is indicative of a helix with a pitch angle of 68 degrees. Of course, this coiling of the microtubule wall would be due to a spiral arrangement of its subunits which are assumed to consist of protofibrils (Andre and Thiery, 1963; Pease, 1963; Ledbetter and Porter, 1964; Moor, 1968). According to the literature, 10-13 filamentous subunits (protofibrils) are thought to be bundled together symmetrically to form a microtubule cylinder with a 50-60 angstrom spacing of the subunits (Ledbetter and Porter, 1964; Grimstone and Klug, 1966). The hexagonal pattern observed in their experiments suggests that the walls of the microtubule crystals may consist of protofibrils arranged in multiples of six. Thus 12 longitudinal filaments per microtubule would be spaced at 70 angstroms center-to-center, 18 (four per side of the hexagon) spaced at approximately 45 angstroms (Bensch and Malawista, 1969). Neither of these calculated spacing distances agrees with those found in normal plant or animal microtubules. The hexagonal form of the cross-sectioned tubules is also of interest in light of recent studies which found occasional hexagonal mitotic spindle fibers in yeast cells recovering from exposure to an anaerobic medium; the diameter of these microtubules was different from that found randomly in the cytoplasm or nuclei (Bensch and Malawista, 1969).

Krishan (1970) used human leukemic lymphoblasts (CCRF-CEM) (Foley et al., 1965) from suspension cultures and treated them with 10 μg/ml of vinblastine sulfate for 3 hours. Cell buttons retrieved after centrifugation were fixed in 2 percent glutaraldehyde at room temperature for 15 minutes, washed in 6 percent buffered sucrose, and postfixed in 2 percent osmium tetroxide. Dehydration in graded acetone series was followed by embedding in an Epon-Araldite mixture. Thin sections were stained with lead citrate solution and examined in a Philips 300 electron microscope.

Krishan (1970) observed that the crystals induced by Velban (VLBS) or vincristine sulfate (VCR) in human leukemic lymphoblasts (CCRF-CEM) were morphologically similar to

those described earlier in L-929 fibroblasts, platelets, human neurons and rabbit neurons, and oligodendroglia (Bensch and Malawista, 1969; Krishan, unpublished observations; Schochet et al., 1968; Schochet et al., 1969; White, 1968). The ribosome-associated, electron dense material showed a faint linear arrangement and greater electron density than the crystals. In the photomicrographs obtained during the study, ribosomes were interspersed in small clumps between the rows of the electron dense granular material. Although fine linearity of the electron dense material was evident, no apparent resemblance to the "abnormal large microtubules" reported earlier (Bensch and Malawista, 1969) or to the prominent linear arrangement of the crystals could be recognized at this stage.

Earlier evidence from histochemical studies and enzyme extractions (Krishan and Hsu, 1969) has clearly suggested that the VLBS- and VCR-induced crystals are proteinaceous and do not contain appreciable amounts of either RNA or DNA. Incubation of cultures with puromycin, actinomycin D, *p*-fluorophenylalanine (Bensch and Malawista, 1969; Krishan and Hsu, 1969), or cycloheximide before and/or during the exposure to VCR or VLBS has failed to prevent the crystal formation. Preliminary evidence from leucine-^{3}H labeling experiments (Krishan, unpublished observations) also suggested that a major part of the crystals is assembled from preformed proteins, possibly the precursors of the microtubular-filamentous elements (Krishan, 1970). Ribosome-associated complexes described variously as granule-lamellae or polysome-lamellae complexes have been reported recently in plant cells (Bartels and Weier, 1967), in cells from the proximal renal tubules of a monkey, and in adenoma cells from a human adrenal cortex (Hoshino, 1969). While the ribosome-lamellae complexes in the monkey renal tubular cells cannot be related to drug administration, it is not known whether the material from the human adrenal cortex was obtained from a patient who was being treated with drugs (Krishan, 1970). The presence of large amounts of ribosomes and helical polyribosomes in association with crystals and with the masses of electron dense material suggests that in addition to arresting cells in mitosis and causing the formation of crystals from preformed

cellular proteins, VLBS and VCR may stimulate the further production of protein subunits, the probable precursors of the crystals (Bensch and Malawista, 1969). In many sections the electron dense material associated with the ribosomes shows a faint linearity while in some other sections it shows reduced electron density and the presence of tubular profiles identical in diameter to these seen in the cross sections of the crystals. Based on these observations, it is reasonable to speculate that the following events occur in cells exposed to *Vinca* alkaloids VLBS and VCR in concentrations used in the study done by Krishan (1970). (1) Initial breakdown of cellular microtubules and reformation of their constituent and/or precursor proteins into crystals showing a linear arrangement of filaments in longitudinal sections and tubular profiles in cross sections. (2) Stimulation of further synthesis of these precursor proteins through the activity of the ribosomes and helical polyribosomes. The electron dense material seen in association with the ribosomes which shows a faint linearity but is much more electron dense than the constituent material of the crystals, could be this newly synthesized precursor protein. (3) Reorganization of this electron dense material into tubular profiles followed by elimination of the ribosomes and the formation of the crystalline structures.

In a study by (Wilson et al., 1970), the precipitation of structural proteins other than the colchicine-binding microtubular proteins by vinblastine was demonstrated. This study raised the consideration that whether the protein forming the intracellular cytoplasmic crystals are similar to colchicine-binding microtubular proteins (Borisy and Taylor) and the proteins precipitated by vinblastine and vincristine from the supernates of the cell homogenate (Marantz et al., 1969; Bensch et al., 1969). Krishan and Hsu (1971) worked with colchicine-^{3}H labeling of vinblastine- and vincristine-induced crystals in tissue culture cells and found the presence of colchicine-binding microtubular proteins in these structures.

Treatment of cells or tissues that contain cytoplasmic microtubules with the vinca alkaloids result in disappearance of the microtubules, and the formation of highly regular crystals

(Bensch and Malawista, 1969; Nagayama and Dales, 1970). Vinblastine crystals isolated from unfertilized sea urchin eggs have been found to be composed of tubulin complexed to vinblastine (Bryan, 1972; Bryan, 1971; Bryan, 1972). The finding that vinblastine caused crystal formation *in vivo* was quickly followed by the demonstration that it could cause the precipitation of tubulin *in vitro* (Bensch et al., 1969; Olmsted et al., 1970; Wilson et al., 1970), along with the assumption that the two effects were mechanistically related. However, precipitation *in vitro* and crystal formation *in vivo* seem to be produced by different mechanisms. Vinblastine precipitates tubulin in a concentration-dependent manner; usually high concentrations of vinblastine are required to bring about this effect. This appears, however, to be a non-specific effect of vinblastine (Wilson et al., 1970). For example, vinblastine could precipitate many acidic proteins, including muscle actin, and also nucleic acids (for example, double-stranded DNA) (Wilson, 1974). All the proteins that were vinblastine-precipitable could also be precipitated by calcium ions, and Wilson (1974) argued that vinblastine was acting as an "organic cation."

Effect of Vinca alkaloids on microtubules

Several early observations suggested that there must be other binding sites on tubulin with higher affinity than these. First, the concentration range in which the *Vinca* alkaloids exert their disruptive effects on microtubules is quite low, lower in fact than the concentration range commonly used for colchicine. The concentrations required to produce a mitotic index of 50 percent for all three analogs were approximately the same, 8×10^{-8} moles/l (Wilson, 1974). Second, low concentrations of the *Vinca* alkaloids slow the rate of decay of colchicine-binding activity (Wilson, 1970). Active *Vinca* alkaloids such as vinblastine, vincristine, and desacetylvinblastine possess this property, whereas weaker or inactive derivatives (for example, leurosidine and catharathine) are less able to produce this effect, or do not possess this ability at all (Creswell, 1972). Another important observation, made by Weisenberg and Timasheff (1970) was that low concentrations of vinblastine can cause an aggregation of tubulin, which is seen as an increase in the sedimentation coefficient from 6 S to approximately 14 S. Wilson

(1974) found that low concentrations of vincristine produce the same effect. Another study found that the tubulin-vinblastine complex would have an extrapolated sedimentation coefficient of about 9.5 S at zero protein concentration, which is consistent with a dimer of the 6 S tubulin. Thus, the binding of the *Vinca* alkaloids to a postulated class of high-affinity binding sites, which presumably prevents the tubulin from polymerizing, may induce the formation of tubulin dimers. This may be the initial event in the formation of crystals *in vivo*.

Bhattacharyya and Wolff (1976) revealed that the rat brain tubulin possesses two distinct binding sites for vinblastine per molecule: a high-affinity site with an affinity constant of 6.2 x 10^6 M^{-1} and a low-affinity site with that of 8 x 10^4 M^{-1}. The dual action of vinblastine on the state of aggregation of tubulin can be ascribed to the presence on that protein of two distinct and different binding sites for the alkaloid. The high affinity site (K_A about 6 x 10^6 M^{-1}) binds vinblastine at concentrations where antimitotic effects and the disappearance of cytoplasmic microtubules are observed *in vivo*. A lower affinity site (K_A about 8 x 10^4 M^{-1}) is associated with various aggregation phenomena both *in vitro* and *in vivo* (Ventilla et al., 1975; Weisenberg and Timasheff, 1970; Bryan, 1972). A comparison of the properties of these two sites showed that both sites are specific; vinblastine effects at these concentrations were not observed with the other acidic proteins examined. On the other hand, the precipitation occurring at $>10^{-3}$ M vinblastine was observed with a variety of proteins (Bhattacharyya and Wolff, 1976).

Occupancy of the lower affinity site appeared to overcome the blocking of polymerization caused by occupancy of the high affinity site, without apparently interfering with binding to this site. This showed that the high-affinity site is labile, with a $t_{1/2}^{37°}$ of 3.5 hours, is protected by colchicine, and is unaffected by salt, whereas the low-affinity site is stable but is inhibited by salt. Binding to both sites is rapid. The high-affinity binding constant of vinblastine to tubulin (6.2 x 10^6 M^{-1}) corresponds to the half-maximal concentration of vinblastine need to prevent polymerization of tubulin *in vitro*, whereas the low-affinity binding constant (8 X 10^4 M^{-1}) corresponds to the half-maxi-

mal concentration of vinblastine required to aggregate tubulin (Bhattacharyya and Wolff, 1976). This showed that vinblastine binding to the high- and low-affinity sites, respectively, accounts for the depolymerization and aggregation behavior of tubulin. Although colchicine and vinblastine do not compete with each other for their respective binding sites, some sort of "long-range" or allosteric interaction between the colchicine and vinblastine sites must occur since occupancy of one site protects the other. Whether the conformational changes in tubulin upon colchicine binding (Ventilla et al., 1972) have effects on vinblastine binding, and whether vinblastine will have similar conformational effects remains to be seen.

Evidence suggests that vinblastine enhances colchicine binding in both 3T3 and SV3T3 cells when added to the growing population prior to the addition of colchicine. Enhanced binding is temperature dependent, of high affinity, i.e., bound colchicine is not easily dissociated, slowly reversible and inhibited by podophyllotoxin (Kralovic and Voelz, 1977). These binding properties coincide with those set as criteria for colchicine specific binding to extracted tubulin (Wilson and Bryan, 1974). It is also in agreement with the specific colchicine binding to intact cells reported by others (Wilson and Friedkin, 1967; Taylor, 1965; Gillespie, 1971). Vinblastine, proportional to its concentration, enhances colchicine binding in 3T3 and SV3T3 cells without changing cell permeability to colchicine (Kralovic and Voelz, 1977). Enhanced binding could not be attributed to *de novo* synthesis of colchicine binding proteins possibly triggered by an imbalance of the ratio of assembled to free microtubule subunits. Binding increased slowly, was temperature dependent, of high affinity, slowly reversible, and inhibited by podophyllotoxin. Vinblastine could possibly enhance colchicine binding by depolymerizing microtubules into subunits, thereby increasing colchicine binding sites.

Aside the interference with the assembly of microtubules, the alkaloids colchicine and vinblastine also affect several properties of the surface membrane of animal cells. They disorganize an apparent topographical separation between surface areas involved in membrane transport and in phagocytosis in rabbit

polymorphonuclear leukocytes, inhibit the agglutination of these cells by concanavalin A and inhibit the concanavalin A-dependent adherence of red blood cells to transformed 3T3 fibroblasts (Waddell *et al.* 1974). It has also been found that these alkaloids also inhibit the spontaneous aggregation of hamster fibroblasts (BHK 21, clone 13) which occurs when these cells are shaken in suspension, after dispersal from monolayers by exposure to trypsin in the presence of EDTA (Waddell *et al.* 1974). The inhibition by alkaloids is less complete than can be obtained by treating the cells with high levels of trypsin, and the inhibited cells aggregate more than do polyoma-transformed cells.

Vinblastine is effective at much lower concentrations than colchicine, colcemide is an intermediate. Waddell *et al.* (1974) argue that these concentrations are similar to those required to alter the shape of BHK cells in culture from fibroblast-like to epithelial-like. Lumi-colchicine shows slight inhibition, but only at concentrations 100 times higher than the colchicine from which it was prepared by UV irradiation. The onset of inhibition is fast, occurring within the few minutes resolvable by the assay, and some disaggregation is commonly observed when vinblastine, for example, is added to a partly aggregated cell suspension. In contrast to these effects on spontaneous aggregation, the agglutination of trypsinised BHK21 cells by concanavalin A seems to be wholly insensitive to the same inhibitors. This is true both for C13 cells (in which case lectin-induced and spontaneous aggregation are superimposed in the assay) and for trypsinised polyoma-transformed BHK21 cells.

Vinblastine has a very different structure from colchicine and inhibits aggregation at much lower concentrations. Lumi-colchicine, which shares at least some of the non-microtubule effects of colchicine, is essentially inactive. It therefore seems more likely that the effects observed by Waddell and colleagues (1974) resulted from interference with the assembly of microtubules than from some secondary, non-specific effect of the alkaloids on the cell surface. A remaining problem is that the effect of vinblastine is at least partially reversible, whereas effects of vinblastine on microtubules in some systems are not.

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Vinblastine has a very different structure from colchicine and inhibits aggregation at much lower concentrations. Lumi-colchicine, which shares at least some of the non-microtubule effects of colchicine, is essentially inactive. It therefore seems more likely that the effects observed by Waddell and colleagues (1974) resulted from interference with the assembly of microtubules than from some secondary, non-specific effect of the alkaloids on the cell surface. A remaining problem is that the effect of vinblastine is at least partially reversible, whereas effects of vinblastine on microtubules in some systems are not.

Microtubules are probably present in trypsinised BHK cells, although another study found them rather sparse and randomly scattered throughout the cytoplasm. It is an intriguing problem how the integrity of microtubules could modify the probability that suspended cells will adhere to one another.

In a different study by Crispe in 1976, it was found that the human monocytes treated with the microtubule-disrupting agents vinblastine and colchicine show enhanced migration into micropore filters. When treated with hexylene glycol, which appears to stabilize microtubules, their migration is inhibited. These results suggest that disruption of the microtubules may facilitate deformation of the cell necessary for migration through small holes and that stabilization of the microtubules may increase cell rigidity and inhibit such migration. Their study revealed that when human blood monocytes are treated with the microtubular disrupting agents - colchicine and vinblastine - they show enhanced migration into micropore filters. The mechanism of this effect is likely due to the disruption of microtubules because the two agents bind to different sites on tubulin molecules, colchicine producing complete dissociation of microtubular protein and vinblastine precipitating it as crystals (Margulis, 1973). Also, the concentrations of the agents which produce maximal enhancement of migration (10^{-6} – 10^{-5} M) are sufficient to disrupt microtubules (Bhisey and freed, 1973) but not sufficient to cause loss of viability.

Hexylene glycol inhibits migration markedly at 0.1 M. Similar concentrations have been found to affect the mitotic apparatus (MA) of sea urchin embryos, stabilizing the isolated MA (Kane, 1965) and causing hypertrophy of the MA in living cells (Rebhun et al., 1975), where the effect may be antagonized by colchicine. This suggests that the agent can penetrate the cell membrane. Its effect is not restricted to MA microtubules, and it has been used in the isolation of intact microtubules from rat brain homogenates (Wuerker and Kirkpatrick, 1972).

In the absence of any other known effect on the cell locomotor system, the most likely action of hexylene glycol in Crispe's (1976) experiment is on cytoplasmic microtubules. If this is

so, both sets of results are consistent with the hypothesis that disruption of the microtubular system decreases cell rigidity and thereby facilitates deformation necessary for the migration of cells through small pores, whereas stabilization of microtubules prevents such deformation and therefore impedes migration. It may be that the inverse relationship between microtubular integrity and migration rate demonstrated by Crispe (1976) is relevant to activation by cytotaxins. Monocytes showing accelerated migration in a chemotactic gradient must deform during their passage into micropore filters, and it is possible that a labeling of the microtubular cytoskeleton is a prerequisite for such acceleration.

The depression of motility by vinblastine at high concentration has also been observed in neutrophils (Ward, 1971), where motility enhanced by a chemotactic gradient was inhibited 50% by 6×10^{-5} M vinblastine, 5×10^{-4} M colchicine or 8×10^{-4} M colcemid. It seems unlikely that this depression of motility is because of these agents on microtubular integrity; respiratory depression is a more convincing explanation. Therapeutic doses of colchicine as used in gout would, if evenly distributed *in vivo*, give concentrations well below those which enhanced monocyte migration. However, in cancer patients, vinblastine sulphate is given in doses of up to 0.5 mg/kg which, if evenly distributed through total body water, would produce a concentration around 7×10^{-7} M (Crispe, 1976). The experiments reported by Crispe (1976) show that *in vitro* migration is significantly enhanced at this concentration, so it is possible that monocyte migration or deformability is affected in these patients. Cells of the mononuclear phagocyte system appear to be important in resistance to cancer (although most of the evidence relates to experimental animals) (Remington et al., 1975), and it is possible that an effect on monocyte migration is superimposed on the antimitotic and other effects of vinblastine when it is used as an oncolytic drug.

Several *in vitro* methods are available for testing the biological activity of "anti-tubulins" (drugs that interfere with the structure or function of microtubules through the inhibition of tubulin polymerization into normal microtubules). Most

involve the use of plant tissues or protozoa (Margulis, 1973; Deysson, 1968).

Mammalian cells can hardly be expected to maintain identical reaction patterns. On the contrary, their reactions are diverse, so sophisticated, time-consuming techniques are needed to establish unambiguously the phenomenon of spindle disruption or to conduct quantitative dose-response studies with known anti-tubulins. Indeed, the most widely used parameter is the rise in mitotic index, which is the result of a temporary arrest in mitosis (De Brabander *et al.*, 1976). However, the mitotic peak is highly dependent on the culture conditions and the relative degree of synchrony of the cells. Alternatively, most toxic stimuli induce cultured cells to become rounded like mitotic cells (De Brabander *et al.*, 1976). Since it is virtually impossible to discern disorganized mitotic cells from prenecrotic cells with phase-contrast microscopy, time-consuming histologic techniques are necessary, but even these procedures may produce ambiguous results because the rounded mitotic cells are easily detached from the substrate by normal washing procedures. While examining the effects of colchicine (Eigsti and Dustin, 1955) and related drugs on cultured mammalian cells of different origin, De Brabander *et al.* (1976) found that some cell lines responded in a uniform and reproducible manner by producing multi-micronucleated cells that can easily be identified with phase-contrast microscopy. Because this effect was restricted to disruption of mitotic microtubules, they developed it into a simple technique. Thus, a new culture model, which facilitated both mass screening of potential anticancer drugs acting on microtubules and quantitative experiments with known "antitubulins," was found.

De Brabander *et al.* (1976) found this culture model to have the many advantages. The use of mammalian cells (either transformed or not), simplicity of the techniques (phase-contrast microscopy or simple microscopy after Giemsa staining, or 3 percent Toluidine blue 5 min staining), and ease with which it lent itself to quantification were the most prominent gains from it. The model was based on the uniform multi-micronucleation response induced by antitubulins in MO cells,

the epithelioid-type C3H mouse embryo cell line. The specificity (towards antitubulins) of this response was ascertained using many substances, including most of the known antitubulins and several nonrelated cytostatic or cytotoxic compounds. The uniformity of the response was established with the use of time-lapse observation of large numbers of cells and quantitative approaches. The results obtained in this model with the standard antitubulins (colchicine, vinblastine, vincristine) showed similar effects. The major difference between colchicine and the *Vinca* alkaloids was that colchicine was less reversible, which might be an indication of stronger intracellular binding of the (De Brabander *et al.*, 1976).

The *Vinca* alkaloids acted synergistically with colchicine when threshold subactive doses were combined, although it is known that they bind at a different site on tubulins. Several substances that have been claimed or were suspected to interfere with microtubules were tested. The results showed that the substances which were indeed active with MO cells included colchicine, vinblastine, vincristine, podophyllotoxin, rotenone, griseofulvin, mercaptoethanol, benomyl, methyl benzimidazol-2-yl carbamate, and R 17934. Compounds that were inactive on these mammalian cells in culture included isopropyl carbanilate and melatonin, both of which are known to be active in other systems.

In culture, fibroblast motility involves the protrusion of flattened cell processes or lamellipodia which characteristically are restricted to a limited portion of the cell's perimeter (Abercrombie, 1961; Ambrose, 1961). In the absence of collision with other cells, the position of the leading lamella persists for considerable periods, ensuring that the cell moves in a relatively straight line (Gail and Boone, 1970; Vasilieve and Gelfand, 1976). During locomotion in culture, fibroblasts tend to become elongated in shape as the leading lamella advances over the substratum (Abercrombie, 1961; Trinkaus et al., 1971; Vasilieve and Gelfand, 1976). Microtubules appear to be important for the restriction of lamellipodial activity to a limited portion of the cell's margin and for the characteristic elongated spindle shape adopted by fibroblasts on two-dimensional

surfaces since exposure to antimicrotubular agents results in an expansion of lamellipodial activity around the entire perimeter and a loss of the elongated shape (De Brabander et al., 1976; Gail and Boone, 1971; Goldman, 1971; Ivanova, 1976; Miszurski, 1949; Van der Schueren et al., 1978; Vasiliev et al., 1970; Vasiliev et al., 1969). There is a marked decrease in directed cell movement at displayed during wound healing (Vasilieve and Gelfand, 1976; Vasiliev et al., 1969) and in the rate of emigration of cells from cellular aggregates adherent to a substrate (De Brabander et al., 1976).

The involvement of microtubules in mediating the placement of lamellipodia has been demonstrated only for cells migrating in monolayers on artificial surfaces (usually glass or plastic). As an attempt to duplicate more exactly the *in vivo* situation of mesenchymal tissue, Armstrong and Armstrong (1979) assessed the effects of antimicrotubular agents on motility of fibroblasts within three-dimensional aggregates maintained in organ culture. The morphology and motility of tissue cells involves several elements of the cytoskeleton (the microfilaments, intermediate filaments, and microtubules) (Abercrombie, 1973; Goldman et al., 1976). The use of drugs that selectively depolymerize one or another of the cytoskeletal components has proven useful in unraveling their respective functions (Borhers and De Barbander, 1975; Soifer, 1975). Vertebrate fibroblasts maintained in monolayer tissue culture show characteristic alterations in form and movement upon exposure to antimicrotubular agents (De Brabander et al., 1976; Gail and Boone, 1971; Goldman, 1971; Ivanova, 1976; Miszurski, 1949; Van der Schueren et al., 1978; Vasiliev et al., 1970; Vasiliev et al., 1969). Lamellipodial extension and retraction continues indicating lack of involvement from microtubules. The cells do, however, lose their elongated shape and leading lamella. In this condition, net cell translocation is markedly diminished (Gail and Boone, 1971). These findings suggest that microtubules function to maintain cell polarity by stabilizing the leading lamella, restricting lamellipodial activity to a limited portion of the cell's margin. Armstrong and Armstrong (1979) used three-dimensional aggregates which reflected more closely the organization of the mesenchymal tissue *in vivo*. They observed that the pharmacological concen-

trations of the antimicrotubular agents colcemid, vinblastine, colchicine, and R17934 (nocodazole) produced a marked decrease in the migration of mesenchymal cells in cell aggregates. Concomitant with the decrease in cell movement, the antimicrotubular agents produced a decrease in the frequency of the elongated shape. When the aggregates of dissimilar cells are paired, cells of one type usually migrate over the surface of the partner aggregate to envelop it (Armstrong, 1978; Armstrong and Niederman, 1972; Holtfreter, 1939; Steinberg, 1970; Townes and Holtfreter, 1955).

Use of *Vinca* Alkaloids for cancer

The *Vinca* alkaloids are established agents in the treatment of lymphomas, especially Hodgkin's disease, childhood acute leukemias, choriocarcinoma, and embryonal or mesenchymal carcinomas such as Wilms' tumor, neuroblastoma, rhabdomyosarcoma, and carcinoma of the testis (Creasey, 1975). Responses have been obtained with some frequency in such other diseases as mycosis fungoides, Letterer-Siwe disease, oat cell carcinoma of the lung, breast carcinoma, the blastic crisis stage of chronic myelocytic leukemia, and some central nervous system neoplasms (Creasey, 1975, with extensive references). This of course does not represent an exhaustive list, but rather an indication of the areas of most useful application. It should also be stressed that there are significant differences in the spectra of activity of vinblastine and vincristine. Vinblastine is somewhat more effective than vincristine in the treatment of advanced Hodgkin's disease, whereas the latter drug is the more active against lymphosarcoma and reticulum cell sarcoma (Creasey, 1975). More striking, however, is that vincristine is of value in the therapy of acute lymphoblastic leukemia, a disease for which vinblastine is ineffective.

Although the use of weekly injections of vinblastine is standard with these agents, experiments carried out with transplantable mouse tumors show that daily injections produce antitumor effects clearly superior to those of single large doses (Johnson et al., 1963; Creasey, 1966). Furthermore, the theoretical considerations of Valeriote et al., (1966) suggest that optimal effects of vinblastine require significant lengths

of exposure to critical drug concentrations. However, in the clinic, toxicity has soared with more frequent injections, and this has discouraged exploration of other dose regimens. Creasey (1975) studied the major toxic manifestations of therapy with the *Vinca* alkaloids. The most striking feature found in this study was that leukopenia, the most prominent side-effect of vinblastine, is relatively uncommon with vincristine, which produces a range of neurologic and neuromuscular symptoms. Many studies (Tobin and Sandler, 1966, 1968; Sandler et al., 1969; Gottschalk et al., 1968) have described the clinical changes encountered. The most consistent finding is depression and finally loss of the Achilles deep tendon reflex (Creasey, 1975). Most studies have not indicated changes in conduction velocity, but one report showed that this is slowed (McLeod and Penny, 1969). In general, it would appear that there is a selective effect on the muscle spindle (Sandler et al., 1969), together with axonal degeneration and demyelination comparable to that seen experimentally (Gottschalk et al., 1968; McLeod and Penny, 1969; Bradley et al., 1970). Many of these neurologic symptoms could result from the biochemical and microtubular interactions.

In the last few years, it has become evident that the future of the *Vinca* alkaloids, and possibly of most other types of anticancer drugs, lies in their integration into intensive schedules of combination therapy. Considerable progress is being made in devising and applying drug combinations to clinical disease; many of the most successful of these protocols involve *Vinca* alkaloids. The major principles behind the selection of components, apart from the activity against the disease, are avoidance of cumulative toxicity by use of agents with differing limiting side-effects, and employment of drugs of different mechanisms of action in the hope of inflicting a variety of metabolic lesions on the target cells (Creasey, 1975). Further improvements are to be expected when increased knowledge of the human pharmacology of these agents is obtained and applied to determining logical combinations. However, it is only when such multiagent schedules have evolved to encompass other modalities such as surgery, radiation, and immunotherapy in a concerted approach to cancer, that therapeutic

benefits such as are now obtained in only a few diseases will be achieved in all neoplasia.

Vinblastine's antineoplastic activity in cancer chemotherapy is exercised through the disruption of cell mitotic spindles and the inhibition of cell division. Cells treated with vinblastine display the characteristic morphology called 'Sc-mitosis," in which cellular microtubules are destroyed and the cells are arrested at their metaphase (Palmer et al., 1960; Cutts, 1961). However, unlike other c-mitotic agents, such as colchicine and podophyllotoxin, vinblastine can induce in cells the formation of highly ordered paracrystalline aggregates (Bensch and Malawista, 1968; Bensch and Malawista, 1968; Malawista and Sato, 1969; Fujiwara and Tilney, 1975). The *in vivo* vinblastine-induced tubulin paracrystals have a short rod-like structure with a hexagonal cross-section and contain equimolar quantities of vinblastine and tubulin (Fujiwara and Tilney, 1975; Bryan, 1972; Wilson et al., 1978). Electron microscopic studies have shown that these paracrystals contain a large number of "macrotubules" packed in parallel array along their long axis and that each "macrotubule" in turn, consists of two intertwined helical aggregates of tubulin, 180 degrees out of phase with each other (Bensch and Malawista, 1968; Bensch and Malawista, 1968; Fujiwara and Tilney, 1975). *In vitro*, each tubulin a-ß dimer contains two vinblastine binding sites. Upon binding vinblastine, the protein can self-associate to form rings, coils, and tubular aggregates. Nevertheless, there have been no reports so far of the *in vitro* formation of vinblastine-tubulin paracrystals.

In their ultracentrifugal study, Na and Timasheff (1982) demonstrated that in the presence of vinblastine, tubulin undergoes an isodesmic, indefinite self-association (Na and Timasheff, 1980) which is enhanced by Mg^{2+} ions (Na and Timasheff, 1980). The associated species observed under such conditions, although small in size, have a vinblastine stoichiometry identical with that found in the *in vivo* vinblastine-tubulin-paracrystals (Na and Timasheff, 1980). Following these self-association studies as a guideline, they succeeded in inducing the tubulin-vinblastine complex to form a paracrystalline aggregate similar to those observed *in vivo* and noted the results. Purified calf brain tubulin was induced to self-aggregate *in vitro* into paracrystalline structures by the anti-cancer drug vinblastine. The size, shape, optical birefringence,

FIGURE 1: *several cross-sectioned small crystals are present in the upper half of the figure, fibroblasts incubated with vinblastine sulphate 2 x 10-5 M (curtsey of Dr Namunescu).*

and drug stoichiometry of these tubulin-vinblastine paracrystals were similar to those of paracrystals formed *in vivo*. Similar structures can be formed with vincristine and desacetylvinblastine, but not with colchicine or podophyllotoxin (Na, 1982).

5. Chapter: Pharmacology, biochemistry and histology of anti tubulin agents

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Summary: Centrioles
Robbins and Gonatas (1964) found that the mammalian cells of the HeLa (S3) strain, when exposed to spindle inhibitors, undergo several morphological transformations during interphase as well as during mitosis. Centrioles are cytoplasmic proteins found in eukaryotic cells. They support the division of cells by combining with microtubules and chromatin. In addition to tubulin, they also might have receptors for anti-tubulin agents, which might have a modifying influence on mitoses. Centrioles are most often found associated in pairs (the diplosome). Usually during interphase these are next to the nuclear envelope, though in some cell types they are near the cell surface. Sometimes these pairs are arranged end-to-end, but more commonly they come together to form a right angle (Fulton, 1971). Centrioles undergo an extraordinarily precise ritual in relation to mitosis. The interphase cell has 2 centrioles (a diplosome); these separate to the poles at the beginning of division (Fulton, 1971). During the ensuing karyokinesis each centriole duplicates, so that at the end of mitosis each daughter cell receives 2 centrioles. Since microtubules were first observed they have been found in association with centrioles, though orderly arrays of microtubules can also form in the absence of centrioles.

VBLS & Biochemistry
Jones *et al.* (1966) showed through their work that VLBS interferes with the biosynthesis of DNA in thymus cells at some

point after the formation of the simpler pyrimidine and purine nucleotides. However, the reactions involved in the biosynthesis of DNA are common to most mammalian tissues. The differences in the sensitivity of tissues to VLBS would seem, therefore, to be related to aspects of the metabolism of the cells other than those directly concerned with DNA synthesis. VLBS causes metaphase arrest of dividing cells. Studies have shown that VLBS may interfere *in vivo* with the synthesis of soluble RNA in the Ehrlich ascites tumor (Jones *et al.*, 1966). They suggested that this might lead to disturbances in amino acid metabolism and interfere with the synthesis of protein such as that needed in the mitotic apparatus. Warneoke and Seeber (1968) found that Vinblastine lowers ^{14}C-leucine incorporation into ascites cells. Vinblastine and vincristine inhibit DNA, RNA and especially protein biosynthesis in leukemic bovine lymphocytes and lead to inhibition of protein biosynthesis in cell-free systems. In case of the nucleic acid metabolism, they primarily inactivate the DNA-dependent RNA polymerase and reduce the intracellular nucleoside content (Warneoke and Seeber, 1968). COL used for comparison has similar binding sites and hence was chosen for the study. Wilson *et al.*, (1970) found that vinblastine sulfate can precipitate several proteins in addition to microtubule protein. Those proteins which precipitated with vinblastine sulfate, all of which were derived from structure, also precipitated with Ca^{2+} ions. Their results suggest that vinblastine sulfate, presumably acting as a cation, precipitated proteins by combining with sites which could also combine with Ca^{2+} ions.

COL & mitoses

The effect of COL is particularly expressed when it affects cells that are in the pre-mitotic, mitotic, or post-mitotic stage of their life (Mairold, 1943). COL acts primarily on growing cells, regardless of whether this involves plasma growth or elongation growth. COL is not unique in this respect, the same is known about the phytohormone auxin, it is not only a hormone of elongation growth, but it can also influence division growth in a positive or negative sense. It is the same with COL. It not only interferes with division growth - as the name "mitotic poison" might suggest - but even more striking

is its morphogenetic effect, which it achieves by changing the direction of elongation growth (Mairold, 1943). The similarity to a hormonal effect has already been noticed by many researchers and COL has repeatedly been referred to directly as a "phytohormone". In his study, Mairold (1943) found that the cell division of algae exposed to COL (*Spirogyra, Zygnema, Cladophora*) did not proceed normally. Studies on plasmolysis on form and time were carried out on colchicinated *Helodea*, *Allium* and *Spirogyra*-cells. In all three subjects, colchicination resulted in a shortening of the plasmolysis time, suggesting a change of state of the cortical cytoplasm (Mairold, 1943). At higher temperatures (26-30 degrees centigrade), tissue ruptures occurred on the hypocotyl of *Lupinus albus* under the effect of COL, similar to those observed in laboratory air. But no effect of COL was observed on blue-green algae (*Nostoc*, *Tolypotrix*) as representatives of acaryotic organisms. The phytopharmacological studies (in the sense of power) on seedlings of *Lupinus albus* revealed that at a dilution of 1:90,000, the COL solution has an inhibitory effect on the growth of the root of *Lupinus* at room temperature (Mairold, 1943). The growth inhibition increased with increasing duration of exposure, until finally the colchicinated roots completely stopped growing in length. An increase in the COL concentration up to 0.005 percent increased the growth inhibition; a further increase in the concentration no longer resulted in an increase in the effect. Simultaneously with the inhibition of length growth, there was a promotion of primary thickness growth primarily above the root tip, which led to the formation of the well-known club roots (Mairold, 1943). In concentrations below 0.001 percent, there was no growth-inhibiting effect on the roots of Lupinus at room temperature, but a growth-promoting effect. This growth-promoting effect was most evident at COL concentrations of 0.0001 to 0.0002 percent (Mairold, 1943). The efficacy of COL on plant objects is considerably enhanced by an increase in temperature during the exposure period. In relation to the root growth of *Lupinus*, the temperature quotient Q10 is between 5 and 6 under certain conditions (Mairold, 1943). The lower the test temperature, the higher the concentration threshold of COL, at which promotion of length growth still occurred. At higher tempera-

tures (26-30 degrees centigrade), even dilutions below 0.001 percent had a growth-inhibiting effect (Mairold, 1943). The formation of clubs on the roots was also promoted and accelerated by an increase in temperature during COL exposure. In concentrations that induced clubbing at higher temperatures, it was absent at lower temperatures, therefore the clubs could grow through despite leaving the root in the same solution when transferred to lower temperatures. If the temperature was increased by 10 degrees centigrade, the COL effect increased 7 to 8 times with regard to clubbing (Mairold, 1943). Using clubroot formation as an easily detectable criterion of COL effect, a quantitative and qualitative biological detection method for COL was worked out. It made it possible to test the COL content of the drug, *Semen Colchici*, without difficulty (Mairold, 1943).

The quantitative COL detection method has shown that the alleged COL content of Veratrum in the drug Radix Veratri cannot exceed 0.02 percent. On root hairs of *Tradescantia fluminensis* and *Nasturtium officinale*, COL treatment caused the appearance of clubbed or spherical aphids (Mairold, 1943). After transfer to pure water, the club originally located at the tip of the root hair may grow through. COL amorphous and COL crystalline have essentially the same effect on plasmolysis; the chloroform content (approximately 13% w/w) of COL crystalline was therefore irrelevant in plant cells (Mairold, 1943), which might be in contrast to human cells. Root growth of *Lupinus* was more strongly inhibited in solutions of COL crystalline than in the corresponding solutions of COL amorphous. Roots of *Colchicum autumnale* were found to be largely immune to externally applied COL, even under conditions where COL was otherwise particularly intense (30 degrees centigrade). A number of *Liliaceae* supposedly containing COL (*Tofieldia, Asphodelus, Ornithogatum*) proved not to be immune to COL (Mairold, 1943).

COL & mitoses

The effect of COL on mitosis is characteristic; it produces a picture dubbed "COL-mitosis" or "c-mitosis" by a study in 1938 (Biesele, 1958). Since similar effects are produced

by many other agents, the use of the term is by no means restricted to cell division influenced by COL. Biesele (1958) found that COL attacks the secondary bonding of the gelated spindle protein into fibers, but not the initial formation of the gel. This led to his speculation that perhaps COL acts through the mitotic centers and kinetochores. He also found that actomyosin threads treated with COL no longer contracted when exposed to ATP. Sacroblast ribbons grown in tissue culture were disrupted by COL at 10^{-8} M or more, apparently because of disorientation of a system of extended protein micelles, which were probably protofibrils of actomyosin (Biesele, 1958).

Anti-tubulin agents & microtubules interaction

COL and several other compounds (pharmacological but not chemical analogs) are apparently fine tools for analyzing the assembly of microtubule protein into tubules. Microtubules underlie the development of asymmetric cell shapes. Slow morphogenetic movements that involve microtubule polymerization tend to be COL sensitive. Microtubules may be considered "cellular skeletons," especially with respect to the development of new form, but they also may be intimately involved in other complex functions of eukaryotic cells. Assembly of microtubule protein into mitotic spindle microtubules may account directly for the generation of force in mitosis (Margulis, 1973). If this concept applies, chromosomal movement in mitosis should be considered morphogenetic. Furthermore, conformational changes in microtubule protein may account for the chemosensitivity of the nervous system of higher animals. If this theory is correct, a precise understanding of the interactions of tubulin proteins with small molecules may eventually elucidate those wonderous processes of touch, hearing, olfaction, chemotaxis, and generation of action potentials. The fundamental motility of the primitive (9 + 2) flagellate (Margulis, 1973) may have been put to work in the elaboration of these far more complex sensory systems, yet they all may be based on ATP-mediated mechanochemical transductions involving tubulins.

COL & tumor, in humans, animals, plant

Amoroso (1935) reported experiments on the inhibition of tumor growth in mice and a dog by injection of COL, although

uniform results have not been obtained by subsequent workers. Ludford (1936) also carried out experiments on mice and dogs to study the influence of COL on tumors. He observed arrest of mitosis in tissue cultures of both normal and malignant cells. In addition, he also noted that some of the arrested cells that were exposed for a longer time died. Brues (1936) found that COL, produces arrest in metaphase of a maximum number of mitoses when injected subcutaneously in aqueous or oily solutions but these results are obtained only within certain limits of dosage. Larger doses prevent cells from entering mitosis, but it has only partial effects in a smaller dosage. COL, octahydro-COL, N-acetylcolchinol and four derivatives of the latter all produce similar effects to COL, but in a considerably higher dosage. Dimethyl- and trimethyl-colchicinic acids are ineffective in any sublethal or lethal dosage. In another study, Oughterson *et al.*, (1937) studied the effect of COL on tumors in a group of patients who received the drug either subcutaneously or intramuscularly. The subcutaneous and intramuscular injection of COL in the doses used was not accompanied by any general toxic symptoms. Many patients, however, showed evidence of local irritation manifested by local soreness and redness, and in some instances by paresthesia. While these symptoms all disappeared spontaneously, nevertheless the discomfort in some patients was of such degree as to make it desirable to use some other method for administering the drug. They concluded that the COL technique makes it possible in some instances to obtain a more accurate index of the rate of growth of the tumor than can be obtained by ordinary methods. In 1937, another study by Nebel and Ruttle showed the inhibition of spindle formation under the influence of COL. They found that in dividing cells of animals and higher plants, cells with the doubled chromosome number are formed due to the inhibition of spindle formation by COL. As growth progressed in plants, didiploid and mixochimeric shoots were formed. Tetraploids were obtained by this means in many genera of herbaceous plants. But various genera do not show the effect of polyploidy equally well.

COL & fibrocytes

In a study in 1939, Bucher studied two karyoclastic poisons - COL and trypaflavine (3,6-diamino-10-methyl-acridiniumchlo-

rid) - regarding their effects on the growth rhythm and on cell division in fibrocyte cultures. He found that COL inhibits the division rhythm - apart from an initial damage in the 2nd-3rd hour only insignificantly, whereas trypaflavin in suitable concentrations blocks the onset of new mitoses more or less completely (trypaflavin effect). He argued that the characteristic of COL is the blocking of mitoses in the first stage of metaphase (COL effect), whereas trypaflavin mitoses proceed without delay and the cytoplasmic damage by the two karyoclastic poisons recedes far behind the conspicuous nuclear damage. Also, COL causes chromatin pyknosis of the various stages of karyokinesis, especially metaphase; characteristic for trypaflavin action is the formation of chromatin bridges in the ana-, telo-, and reconstruction phases. He also found that the mitotic disorders of more severe degree caused by "strong" pharmaceutical concentrations are largely similar to each other. He then compared the mitotic disorders caused by these two substances with mitotic disorders of other etiologies, as well as with the pathological cell divisions frequently encountered in carcinomas and cancerized cultures.

Haas (1940) observed a change in the core content and the plasma structures when looking at mussel eggs in the dark field after the use of pharmaceuticals could be determined. The determination of the upper limit concentrations at which turbidity or coagulation processes occurred on the nucleus or plasma led to the finding that several pharmaceuticals are characterized by a specific effect on the cell nucleus, while the nucleus-damaging effect of other substances is bound to almost the same concentrations that also damage the cytoplasm. The division of the investigated pharmaceuticals into a group of cytoplasmic and a group of nuclear toxins is in agreement with the results obtained in sea urchin eggs in determining the change in respiratory metabolism and the influence on the nuclear division processes (Haas, 1940).

Anti-tubulin agents & chondrocytes

In a study by Ehrlich *et al.*, (1974) embryonic chick cranial bone was cultured in the presence of the antimicrotubular agents - COL and vinblastine - and with several other com-

pounds known to affect the cellular handling of collagen. Secretion of procollagen, quantitated by light microscope autoradiography, was correlated with the extent of conversion of procollagen to collagen and with rates of collagen and noncollagen-protein synthesis. COL inhibited procollagen secretion and conversion to collagen and specifically inhibited collagen synthesis. Cells exposed to COL revealed an increased number of dilated Golgi-associated vacuoles and vesicles, some of which contained parallel aggregates of filamentous structures (Ehrlich *et al.*, 1974). Their observations suggest that the pathway of at least a fraction of procollagen secretion by osteoblasts includes the Golgi complex. Disruption of microtubules may interfere with the movement of Golgi-derived vesicles, and the resulting accumulation of collagen precursors in the Golgi complex may lead secondarily to an inhibition of synthesis. Although vinblastine also inhibited both procollagen secretion and conversion to collagen, the observed reduction in general protein synthesis and striking changes in the ultrastructure of the rough endoplasmic reticulum complicated interpretation of the effects. Interpretation of the effects of cytochalasin B was limited because the cellular response in cranial bone was markedly heterogeneous and the drug caused an inhibition in the incorporation of radiolabeled amino acids into both collagen and noncollagen protein.

Vinca alkaloids and cancer

In 1960, Johnson *et al.*, described the experimental activity of a new clinically confirmed antitumor compound, vincaleukoblastine ($C_{46}H_{58}O_9N_4$), as the sulfate (VLBS). Its greatest activity was seen against the P-1534 acute lymphocytic leukemia in DBA/2 mice. Late as well as early stages of this leukemia were significantly affected by this compound. No synergistic or additive effects have been observed in combination therapy with other antitumor compounds. A second indole-indoline alkaloid, leurosine, isomeric with VLBS, has also been obtained from Vinca rosea Linn, with similar demonstrable experimental antitumor activity. Two other alkaloids, vindoline ($C_{25}H_{32}O_2$) and catharanthine ($C_{21}H_{24}O_2N_2$), also obtained from *Vinca rosea*, were devoid of antitumor activity singly or in equimolar concentrations but have been postulated as the

biogenetic precursors of VLBS and leurosine. Preliminary studies in vitro demonstrated that certain compounds could reverse the growth-inhibitory activity of VLBS against human monocytic leukemia cells. These compounds were coenzyme A, aspartic acid, tryptophan, α-ketoglutaric acid, ornithine, citrulline, arginine, and glutamic acid. VLBS and leurosine are representatives of a new class of clinically active antitumor compounds which may interfere with the cellular metabolic pathways leading from glutamic acid to urea, and from glutamic acid to the citric acid cycle.

Histology of cancer cells

During their histochemical and ultrastructural study, Robins and Gonatas (1964) found that the lysosomes, represented by the multivesicular bodies in HeLa cells, form clusters and become circumferentially disposed instead of occupying the polarized juxtanuclear position characteristic of these organelles. Under the electron microscope they showed to have acquired a dense osmiophilic core that is separated from the bounding unit membrane by an electron lucent halo. The Golgi apparatus fragments under the influence of spindle inhibitors and takes up a circumferential distribution in a pattern similar to that of the lysosomes (Robbins and Gonatas, 1964). On the ultrastructural level, no significant modifications in this organelle were observed by them. In addition, they also noted numerous 60-80 angstroms fibrils in the interphase cell, coursing through the cytoplasm as well as a paucity of spindle microtubules. Also, a striking similarity was seen between the behavior of the lysosomes in the drug-treated interphase cell and the untreated, normal mitotic cell (Robbins and Gonatas, 1964).

COL & poisoning in humans

Eigsti and Dustin (1955) published their findings on the pharmacological aspect of COL. They reported that vomiting, diarrhea, bloody stools, and a progressive paralysis of the central nervous system are the most evident signs of toxicity. Death occurs within several hours in warm-blooded animals, or several days in cold-blooded vertebrates, after injections of the largest closes. In 1906, COL was called "this most remarkable

slow poison." Progressive nervous paralysis leading to respiration arrest, appears to be the main cause of death, whatever the animal tested (Eigsti and Dustin, 1955). While describing the disturbances possibly related to mitotic poisoning, Eigsti and Dustin (1955) related the pharmacological effects to the histological changes resulting from spindle destruction. It was noticed that the leukocytosis-promoting effect of COL, which nearly led to the discovery of its action on mitosis, is probably only remotely linked to mitotic arrest. Its origin may be the action of the drug on the central nervous system. However, it is associated with some of the first descriptions of tissues altered by COL and has often been quoted as the origin of modern cytological work in this field (Eigsti and Dustin, 1955). A substance that arrests the mitoses taking place in the bone marrow for some hours and destroys many of them would be expected to depress blood formation.

COL & fever

Dinarello *et al.*, (1976) studied the patients with familial Mediterranean fever (FMF) who were part of a double-blind trial of daily COL as prophylaxis for their disease and observed their leukocyte functions while receiving COL or placebo. The patients on COL, compared to those on placebo, had significantly fewer neutrophils and monocytes accumulating at skin-window sites 24 hours after the initial abrasion (Dinarello *et al.*, 1976). Skin window technique is used in immunology where the top layer of skin is scraped off making it possible to identify the immune response that would occur with a diminished physical barrier in the host and observe mobilization of leukocytes. Because the early phase of the skin-window response was normal in these patients, the decreased late response may be related to a failure to amplify the initial inflammatory reaction. The reduced capacity to generate a normal inflammatory response may account for the failure of these patients to develop full attacks while taking COL.

COL & poisoning in animals

Extensive cellular destruction has been found in the bone marrow of mice (Eigsti and Dustin, 1955). Considerable congestion and a decrease in the number of nucleated cells are

the consequence of this destruction. The idea of COL having some direct hormonal action led to some curious experiments which are important to consider because COL has often been used for the detection of hormone-stimulated growth. During the breeding season, the fish *Rhodeus amarus* displays brilliant reel "nuptial colours," which are related to the expansion of chromatophores and to local hyperaemia. These colours appear in animals treated with male hormones. COL alone has the same effects. Nuptial colours are displayed by fish subjected for 10 minutes to a 1.5/1000 solution, or for 35 minutes to a concentration of 0.75 /1000 (Eigsti and Dustin, 1955). COL and hormones add their effects, and the full skin changes could be produced in 2 hours instead of 20 hours with hormone alone. COL had no action on the weight of ovaries of mice similarly injected, or on the seminal vesicles of rats injected with testosterone. Neither do results of experiments on silk-worms justify the conclusion that COL is "hormone-mimetic." The only possibility is that through nonspecific action, this toxic drug could stimulate the secretion of hormones by endocrine glands, in particular the pituitary (Eigsti and Dustin, 1955). Furthermore, the mitotic changes induced by COL in a Crocker sarcoma of the mouse were first described in 1934 (Eigsti and Dustin, 1955). It is not surprising that the discovery of a specific action upon mitosis, the metaphase arrest, attracted wide attention afterwards. This research made clear for the first time the possibility of arresting cell division with chemicals acting specifically. Such a relation had been demonstrated several years earlier but COL, being such a unique chemical, helped greatly in convincing researchers of the possibility of cancer chemotherapy. Eigsti and Dustin (1955) grasped immediately the potentiality of this new approach. Their publication and the demonstration marked a turning point and led many people to work on neoplastic growth

COL & plants and algae

Shyam and Sarma, (1974) found that the effects of COL have been extensively studied on several higher plants, and in recent years to some extent on algae. But no reports seem to exist concerning COL effects on any member of Volvocales. They focused their study on the effects of COL on the cell

division of *Conium pectorale*, a member of colonial Volvocales (Chlorophyceae). The effects of COL on a colonial algal flagellate *Gonium pectorale* (Volvocales, Chlorophyceae) were recorded for the first time. While lower concentrations up to 0.5 percent of COL did not produce any effects, 1 percent concentration of this alkaloid brought about contraction of chromosomes, accumulation of metaphase plates, polyploid nuclei, multinucleate cells, and cell enlargement (Shyam and Sarma, 1974). At this concentration, increase in chromosome number was the outstanding effect, such increase ranged from partial doubling, to diploidy and tetraploidy. There was no case where the treatment affected 100 percent of the cells of colony, since unaffected cells within the same colony showing normal chromosome number were observed (Shyam and Sarma, 1974). The affected cells generally did not to divide further instead, they died.

Main Body: Vindoline alkaloids

Alkaloids are substances of widely divergent structures whose chief similarity are their natural origin and basic nature. Biogenetically, the origin of COL remains obscure, and no relationship with any other groups of alkaloids has been detected (Bentley, 1957). The biologically active antitumor compounds described by Johnson *et al.*, (1960) represented a new and previously unknown class of indole-indoline alkaloids. Indole alkaloids are believed to be derived from tryptophan (Bentley, 1957). Previously described indole alkaloids include the ergot alkaloids, the harmala alkaloids of the yohimbine and physostigmine groups, *Strychnos* alkaloids which include strychnine, and the Rauwolfia alkaloids. In their study, COL did not have the selective effects seen with VLBS. Arrested metaphases of the C-mitotic type were also demonstrated by such diverse agents as other alkaloids, sulfhydryl reagents, antifolics, purines, certain amino acid analogs, quinones, phenols, and polyanions (Biesesele, 1958). The preliminary tissue culture studies indicated an antimetabolite action, perhaps connected with glutamic acid. An additional observation which suggested antimetabolite action was the clinical remissions obtained by Hertz and colleagues (Garcia, 1954) in women with Methotrexate-resistant choriocarcinoma. The clinical

results of indicated that VLBS not only affects a different clinical spectrum of malignancies than is affected by COL, but also has fewer toxic or side-effects (Hodes et al. 1959; Hodes et al., 1960). Thus, VLBS and related active compounds, regardless of mechanism of action, are examples of clinically active substances of new chemical compositions which provide a new and heretofore unknown lead in cancer chemotherapy.

COL chemistry and mode of action

Although the COL molecule contains a variety of molecular groupings (e.g., methoxyl, methoxymethyleneketone, acetylamino) it cannot be said from the results of the investigation carried out by Brues (1936) that any single group is essential for the mitotic inhibiting action of COL. The compounds examined in that study included COL derivatives in which the methoxymethylene group had been first hydrolyzed to hydroxymethylene, then replaced by iodine, and finally completely eliminated, the last two stages being accompanied also by modification of the ketonic group (conversion into a phenolic hydroxyl group). Also, some of the compounds examined had the acetylamino-group intact, others had this group hydrolyzed to the free amino-group, while in yet another case the amino-group was replaced by a hydroxyl. All of these derivatives showed activity (Brues, 1936). The inactivity of dimethyl- and trimethyl-colchicinic acids is of interest. In the former case the suppression of activity was attributed to demethylation of one of the three methoxyl groups in ring I, but trimethyl-colchicinic acid appeared to be anomalous, for the only modifications in the COL molecule were the hydrolysis of the methoxymethylene group to hydroxymethylene and the hydrolysis of the acetylamino-group to the free amino group, whereas neither of these changes is necessarily accompanied by loss of activity (Brues, 1936). Possibly the inactivation of trimethylcolchicinic acid is associated with the presence of the basic groups and also the strongly acidic hydroxymethylene group in the same molecule. The lack of specificity suggested by these results appeared to warrant the examination of synthetic compounds of analogous structure.

No substances are effective on mitosis in the small doses required in the case of COL itself, and the toxicity roughly

follows the same relative dosage with different compounds, except in the case of the colchicinic acids (which are ineffective on mitosis), and possibly in the case of the nitrogen-free carbinol, in which the lethal dose is yet undetermined (Brues, 1936).

However, the fact that the lethal effect of COL appears only after several hours, when the mitotic effect is beginning to wear off, suggests that the two effects may be dissociated, and this question requires further investigation. There is obviously a wide gap between the effective and lethal doses of COL (and its salicylate) and those of the other substances. Some of the other differences of dosage may, however, depend upon variations in solubility and absorbability.

VLBS & biochemistry

Jones *et al.* (1966) showed through their work that VLBS interferes with the biosynthesis of DNA in thymus cells at some point after the formation of the simpler pyrimidine and purine nucleotides. However, the reactions involved in the biosynthesis of DNA are common to most mammalian tissues. The differences in the sensitivity of tissues to VLBS would seem, therefore, to be related to aspects of the metabolism of the cells other than those directly concerned with DNA synthesis. VLBS causes metaphase arrest of dividing cells Cardinali et al., 1961; Cutts, 1961). Studies by Creasey and Markiw (1964) have shown that VLBS may interfere *in vivo* with the synthesis of soluble RNA in the Ehrlich ascites tumor (Jones *et al.*, 1966). They suggested that this might lead to disturbances in amino acid metabolism and interfere with the synthesis of protein such as that needed in the mitotic apparatus. It was found that in the presence of VLBS there was an increase in the total radioactivity of certain amino acids (serine, glycine and probably methionine) in the acid-soluble fraction of thymus cells incubated with formate-^{14}C. This increase may be due either to an enhanced synthesis of these compounds or to their decreased utilization as might result from a blockage in protein synthesis. The effect of VLBS on the radioactivity of the serine appeared only after the incubation had been in progress for about 2 hours, whereas the radioactivity of the DNA was affected from the start. This

suggests that the two effects are only indirectly related. There is insufficient information at present to explain the biologic activities of VLBS in satisfactory biochemical terms. There is evidence, however, that the alkaloid interferes with nucleic acid metabolism (Beer, 1961; Creasey and Markiw, 1964; Richards et al., 1966), but it is not certain whether this is a direct effect or the result of a biochemical disturbance in some other part of the molecular organization of the cell. More detailed studies are in progress in attempts to answer this.

Warneoke and Seeber (1968) found that Vinblastine lowers ^{14}C-leucine incorporation into ascites cells. Vinblastine and vincristine inhibit DNA, RNA and especially protein biosynthesis in leukemic bovine lymphocytes and lead to inhibition of protein biosynthesis in cell-free systems. In case of the nucleic acid metabolism, they primarily inactivate the DNA-dependent RNA polymerase and reduce the intracellular nucleoside content (Warneoke and Seeber, 1968). The COL used for comparison has similar binding sites and hence was chosen for the study. The vinca alkaloid vinblastine (VBLS) is considered to exert its antimitotic effects through an action on microtubules. Evidence that it interacts directly with microtubule proteins in physiological concentrations provides additional strong support for the hypothesis (Wilson et al., 1970). Furthermore, high concentrations of VBLS are capable of precipitating at least one kind of microtubule protein, COL-binding protein, from cell-free extracts, and VBLS has been employed as a tool for the purification of microtubule proteins (Wilson et al., 1970). However, several biochemical effects of the vinca alkaloids have been reported which may not be related to the ability of these agents to disrupt microtubule structures. One such effect is aggregation of polyribosomes in eucaryotic cells and in a procaryotic organism, *E. coli*, which is considered to contain no microtubules (Wilson *et al.*, 1970). Other effects include inhibition of DNA and RNA synthesis.

Wilson *et al.*, (1970) found that precipitation by VBL is not restricted to microtubule proteins, but is a property of several proteins, all of which are derived from cell structure. They also found that the same proteins are precipitated by

calcium ions. Their results suggest that VBLS is acting as a cation, combining with sites that also combine with Ca^{2+}. The interpretation is further supported in the case of one of the proteins by the evidence of the additivity of VBLS and Ca^{2+} (Wilson *et al.*, 1970). It is also supported by preliminary experiments with another alkaloid cation, strychnine; in experiments exactly parallel to those with VBLS, strychnine at 5×10^{-3} M precipitated 85 percent of the erythrocyte membrane protein from solution (Wilson *et al.*, 1970). Several alkaloid cations have higher affinities for the ionized groups of proteins than do Ca^{2+} ions (Bugenberg De Job, 1949). These results cast no doubt on the efficacy of VBLS in precipitating COL binding proteins (Olmsted et al., 1970; Krishan and Hsu, 1969); however, they do indicate that other proteins may be precipitated. It is possible that the conditions of precipitation by VBLS will influence the selectivity of the reaction. Wilson *et al.*, (1970) found that the protein of the hyaline layer of fertilized sea urchin eggs is precipitated by VBLS at 37 degrees centigrade but not at 0 degree centigrade, while temperature has little effect on the precipitation of actin or the proteins of erythrocyte membranes. The effects of other variables such as pH and ions other than Ca^{2+} were not investigated systematically. A further complication is that VBLS, presumably acting as a cation, can precipitate DNA and ribosomes (Wilson *et al.*, 1970). They found that VBLS at a concentration of 3.3×10^{-3} M gave complete precipitation of 0.4 mg of calf thymus DNA in 1.2 ml of 10 mM sodium phosphate buffer, pH 6.8, at either 0 or 37 degrees centigrade. Similarly, this drug precipitated at least 65 percent of isolated chick oviduct ribosomes under the same conditions (Palmiter et al., 1970). Thus, precipitation by VBLS can provide a valuable procedure in the fractionation of certain proteins, as has already been seen for COL-binding proteins but is not highly specific for the identification of particular proteins, as the COL- binding reaction has been shown to be. Their findings are consistent with the idea that several proteins of structure share important common properties. Those proteins which Wilson *et al.*, (1970) precipitated with VBLS are all derived from structures and similarities among some of them, especially similarities of amino acid composition and "actin-like" properties.

Centrioles

It is essential to grasp the concept of centrioles before diving into the anti-tubulin agents such as COL and vinblastine since they affect the spindle formation which is directly related to the centrioles. Centrioles are most often found associated in pairs (the diplosome). Usually during interphase these are next to the nuclear envelope, though in some cell types they are near the cell surface. Sometimes these pairs are arranged end-to-end, but more commonly they come together to form a right angle (Fulton, 1971). Centrioles undergo an extraordinarily precise ritual in relation to mitosis. The interphase cell has 2 centrioles (a diplosome); these separate to the poles at the beginning of division (Fulton, 1971). During the ensuing karyokinesis each centriole duplicates, so that at the end of mitosis each daughter cell receives 2 centrioles. It is yet unknown what controls the production of new centrioles, which go from 2 to 4 to 2 during each division cycle, but the cell exerts control - positive or negative - over the time of production of new centrioles, and over the quantity produced (Fulton, 1971). Whatever the control, it must be very precise, because centriole duplication ordinarily does not get out of phase with nuclear division- neither too few nor too many centrioles are produced. Since microtubules were first observed they have been found in association with centrioles, though orderly arrays of microtubules can also form in the absence of centrioles. In cells that are not dividing, centrioles are often surrounded by a small number of microtubules (Fulton, 1971). The spindle fibers, which are composed of microtubules, are regularly associated with centrioles, but also form in mitotic cells in organisms that do not have centrioles. The microtubules of the asters, the amphiaster, and the continuous and chromosomal fibers of the mitotic apparatus all come to focus on the centrioles (Fulton, 1971). These microtubules usually do not directly contact the centrioles.

Anti-tubular agents & centrioles

Some antimicrotubular agents like COL, vinblastine, and vincristine (Olmstead and Borisy, 1973; Wilson et al.,1974.), cause a disruption of the microtubules, whereas others such as ethanol, hexylene glycol (Kane, 1965; Kirkpatrick, 1969)

and deuterium oxide (Gross and Spindel, 1960: Tilney and Gibbins, 1969) stabilize these organelles. They impair the secretion of many cell products, e.g., hormones such as insulin (Lacy et al., 1968; Malasisse et al., 1971) and thyroid hormone (Neve et al., 1972; Williams and Wolff, 1972). More recently, microtubules have also been implicated in the secretion of the macromolecules of connective tissue matrices. It has also been found that COL and vinblastine, as well as other antimicrotubular agents, inhibit both secretion and synthesis of collagen in vitro (Dehm and Prockop,, 1972). COL and vinblastine decrease the rate of secretion of collagen by cells isolated from chick embryo tendons by about 70 percent, and the amount of intracellular collagen concomitantly increases about two-fold (Dehm and Prockop,1972). In a biochemical and ultrastructural study on bone cells, Ehrlich *et al.*, (1974) found that COL, in addition to the effect on secretion, also depressed collagen synthesis, whereas vinblastine inhibited synthesis of collagen as well as other proteins. These authors suggested that the reduction in collagen formation by COL is due to an accumulation of collagen in the Golgi vacuoles, with secondary inhibition of its synthesis. Likewise, COL and vinblastine depress both secretion and synthesis of glycosaminoglycans by isolated chick-embryo chondrocytes, but, contrarily, do not greatly reduce the synthesis of collagen by cartilaginous tibia anlagen (Jansen and Bornstein, 1974). An analgen is a crystalline compound used as an antipyretic and analgesic, employed chiefly in rheumatism and neuralgia. It is a complex derivative of quinoline. Based on these findings, it is usually assumed that microtubules are involved in the intracellular translocation of secretory vacuoles, and that the primary effect of antimicrotubular agents in connective tissue cells is on secretion.

Anti-tubulin agents & microtubules

Margulis (1973) argued that COL and several other compounds (pharmacological but not chemical analogs) are apparently fine tools for analyzing the assembly of microtubule protein into tubules. Since cellular structures composed of microtubules take on many forms depending on the type of cell, its physiological conditions and stage in division, and so forth, it may be difficult to even recognize COL-sensitive protein *in*

vivo. Bhisey and Freed (1971) induced an alteration from gliding to ameboid motility by subjecting macrophages to VBLS and COL. Although a 55-angstoem subcortical fibril was consistently present, a new 100-angstroem filament appeared to be COL-induced. These investigators concluded that a shift from gliding to amoeboid movement is associated with loss of microtubules and increased prominence of microfilamentous structures. Such induced microfilaments may be composed of tubulin. However, in general, "microfilaments" at best are still a miscellaneous collection of organelles.

Even sensitivity to cytochalasin as a criterion for the identification of cytoplasmic microfilaments is in doubt (Sanger and Holtzer, 1971). Yet at least one class of microfilament seems not at all to be composed of the COL-binding protein (Wessells et al., 1971). Neurofilaments too are unlikely to be tubulin (Shelanski and Freit, 1972). COL at high concentrations apparently interacts nonspecifically with microtubule protein (Wilson, 1970). At lower concentrations, 1 mole of COL binds per tubulin dimer; this apparently inhibits subunit protein from assembling into tubules (Borisy and Taylor, 1967a, b). Cilia and flagellar subunits lose their sensitivity to low COL after they have assembled, presumably because the COL-binding sites between the protein molecules are no longer exposed. This seems to be the best interpretation of a vast body of data (Margulis, 1973). Unlike mitotic chromosome movements, melanosome transport, cilia, and flagella movement, axostyle bending in *Saccinobaculus*, and cell extension in *Stentor* (Huang, 1971) are probably best explained by a model involving the sliding of microtubules past each other mediated by the microtubule-associated Mg^{2+} ATP-ATPase (dynein) system (Gibbons, 1965, 1971). "Microtubule sliding" movements are far faster than COL-sensitive movements and involve the functioning of completed morphological structures. Slow COL-sensitive movements seem to be accompanied by the formation of microtubule-based structures, as illustrated by the observation that cilia and flagella regeneration is sensitive to COL even though cilia and flagella movement is not (Margulis, 1973). It seems that in some biological situations such as the mitotic chromosome movements, outgrowth of

cilia and flagella, migration of the oral membranellar band in *Stentor* and outgrowth of axopods in *Echinosphaerium*, formation and function are the same process. This class of processes can be characterized by both their slow velocities and the pattern of their sensitivity to COL and its pharmacological analogs demecolcin (demecolcin and Colcemid are trivial and trade names, respectively, for N-desacetyl-N-methyl-COL), vinblastine, griseofulvin, podophyllotoxin, and so forth (Deysson, 1968).

Microtubules may be considered "cellular skeletons," especially with respect to the development of new form, but they also may be intimately involved in other complex functions of eukaryotic cells. Assembly of microtubule protein into mitotic spindle microtubules may account directly for the generation of force in mitosis (Margulis, 1973). If this concept applies, chromosomal movement in mitosis should be considered morphogenetic. Furthermore, conformational changes in microtubule protein may account for the chemosensitivity of the nervous system of higher animals (Atema, 1972). If this theory is correct, a precise understanding of the interactions of tubulin proteins with small molecules may eventually elucidate those wonderous processes of touch, hearing, olfaction, chemotaxis, and generation of action potentials. The fundamental motility of the primitive (9 + 2) flagellate (Margulis, 1970) may have been put to work in the elaboration of these far more complex sensory systems, yet they all may be based on ATP-mediated mechanochemical transductions involving tubulins.

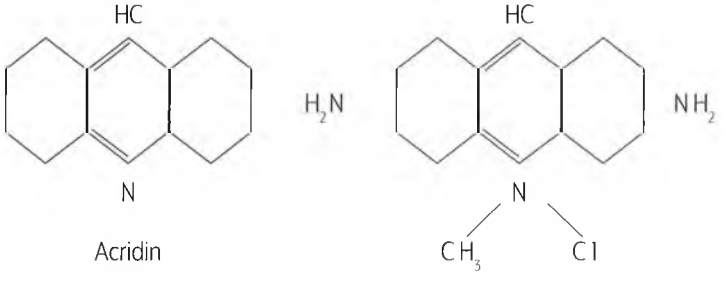
COL & mitoses

Mazia (1956a) found that COL attacks the secondary bonding of the gelated spindle protein into fibers, but not the initial formation of the gel. This led to his speculation that perhaps COL acts through the mitotic centers and kinetochores. Lettre (1952) showed that the effect of COL on mitosis was the inhibition of a reaction between ATP and a contractile system of actomyosin type. COL in concentrations to about 2.5×10^{-8} M (which is near or below the usual low threshold of COL mitotic activity) brought about a 25 to 30 percent decrease in the rate of the viscosity of heart actomyosin gels

induced by ATP (Barany and Palis, 1952). He also found that actomyosin threads treated with COL no longer contracted when exposed to ATP. Sacroblast ribbons grown in tissue culture were disrupted by COL at 10^{-8} M or more, apparently because of disorientation of a system of extended protein micelles, which were probably protofibrils of actomyosin (Godman, 1955). The effect of COL on mitosis is characteristic. It produces a picture dubbed "COL-mitosis" or "c-mitosis" by Levan (1938). Since similar effects are produced by many other agents, the use of the term is by no means restricted to cell division influenced by COL. In distributed c-mitosis occurring in onion roots exposed for 8 hours to COL, D' Amato (1948a) found chromosomes in groups of 10 and 6, 11 and 5, and 12 and 4, commonly; in groups of 13 and 3, 14 and 2, and 9 and 7 less often; and only occasionally in groups of 8 and 8. Tropolone at 10^{-4} M reversed the metaphase arrest cause by 2 x 10^{-6} M COL in a sudden effect that appeared after 16 hours of simultaneous exposure of the fibroblasts to the two agents (Benitez at al., 1953).

COL, mitoses & karyoclastic poisons

COL and trypaflavine (3,6-diamino-10-methyl-acridiniumchlorid) are the main representatives of the so-called karyoclastic poisons according to Dustin (1929). These substances are also primarily karyoclastic in their effect on tissue cultures, while a damaging influence on the cytoplasm, although not completely absent, is quantitatively and qualitatively far behind the nuclear damage. Based on his study on rabbit fibrocytes,



3,6-diamino-10-methyl-acridiniumchlorid

Bucher (1939) suggested that the two drugs may also be placed in two different groups of karyoclastic poisons. In tissue culture both poisons have an inhibitory effect on the division rhythm. However, while this inhibition remains quite slight in the COL films, trypaflavin can completely or partially prevent the new appearance of mitoses for several hours after a certain latency period (typical trypaflavine effect).

These results agree well with the findings obtained from earlier animal experiments with regard to the trypaflavin effect, but not with regard to the COL effect. According to a previous study in 1929 by Dustin, COL causes stimulation of the division activity in the first phase, then a blockade of the mitoses in the metaphase ("stathmokineses") in the second phase, which were in consensus with Bucher's (1939) findings. As for the difference concerning the first phase, it could be due to the different methodology, or because there is a difference in principle between the COL effect on the cells *in vivo* and *in vitro*. Also, the results from animal experiments do not agree with each other. In contrast to Dustin (1929), Brues (1936) did not find any stimulation of the division activity by COL in his investigations on regenerating rat livers. Furthermore, the influence of the cell division rhythm *in vitro* can be clarified perfectly only by means of the film analysis.

The previous investigations by Ludford (1936) on tissue cultures and *in vivo* show that the accumulation of the cell division figures is caused by the mitotic arrest in the metaphase without being able to exclude a certain stimulation of the mitotic event. Determination of the mitotic coefficient, i.e., the number of new mitoses starting every hour, is virtually impossible without observing film *in vivo* and *in vitro* (Bucher, 1939). The mitoses visible at certain intervals can be determined by counting, the number of which will deviate greatly from the former as soon as the mitoses can no longer finish or only vary more in their expiration time.

The successes shown in animal experiments by the studies of Amoroso (1935), Peyron *et al.*, (1936 and 1937), had met with no approval in the literature or, nevertheless, only very

limited approval when Bucher (1939) published his study. Chemotherapeutic experiments on humans done by Oughterson et al., (1937) seemed to not only be premature but not at all desirable because of the extraordinary toxicity of the autumn crocus alkaloid (still effect at dilutions of 1:100 million *in vitro*). Under certain circumstances, COL could be used to increase radiation sensitivity in X-ray therapy under cautious internal medical observation.

In contrast to COL, trypaflavin had not been given much hope for anticancer therapy before Bucher (1939) and its effect on tissue cultures had not yet been studied. In the case of this pharmacone, the findings of Bucher (1939) on tissues *in vitro* were in complete agreement with the results obtained in animal experiments in the previous studies by Dustin (1929), Mayer (1935) and others. The appearance of new mitoses is almost prevented in concentrations of 1:800000 after a certain latency period and even completely at a concentration of 1:600000 during several hours. He called this phenomenon the typical trypflavine effect. This characteristic effect of trypaflavine could be used to try to inhibit the forced cell division activity in malignant tumors. However, such studies by Dustin and Grégoire (1933) and Dustin (1934) on mice with crocker's sarcoma did not lead to a positive result (Bucher, 1939). Another study by Hüssy (1930) was reported to have seen an effect on mouse carcinoma with two other acridine derivatives, i.e., phosphine-acridine-nitrate and phosphine-acridine-imino, and yet another one by Lewin (year not available) obtained satisfactory results with trypaflavin in local cancer treatment in humans. Trypaflavin, which pharmacologically belongs to the general paralytic protoplasma toxins and can lead to paralysis of the respiratory center, is probably too dangerous for general therapy in sufficient doses to inhibit tumor growth. A local inhibitory influence on tumors is within the range of possibility and its bactericidal effect would be added as a desirable side effect of trypaflavin in infected exulcerated tumors (Bucher, 1939).

Like the division rhythm, the course of single mitosis is also influenced differently by COL and trypaflavin. The average duration of COL mitoses increases the longer the toxin effect

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The successes shown in animal experiments by the studies of Amoroso (1935), Peyron *et al.*, (1936 and 1937), had met with no approval in the literature or, nevertheless, only very

limited approval when Bucher (1939) published his study. Chemotherapeutic experiments on humans done by Oughterson et al., (1937) seemed to not only be premature but not at all desirable because of the extraordinary toxicity of the autumn crocus alkaloid (still effect at dilutions of 1:100 million *in vitro*). Under certain circumstances, COL could be used to increase radiation sensitivity in X-ray therapy under cautious internal medical observation.

In contrast to COL, trypaflavin had not been given much hope for anticancer therapy before Bucher (1939) and its effect on tissue cultures had not yet been studied. In the case of this pharmacone, the findings of Bucher (1939) on tissues *in vitro* were in complete agreement with the results obtained in animal experiments in the previous studies by Dustin (1929), Mayer (1935) and others. The appearance of new mitoses is almost prevented in concentrations of 1:800000 after a certain latency period and even completely at a concentration of 1:600000 during several hours. He called this phenomenon the typical trypflavine effect. This characteristic effect of trypaflavine could be used to try to inhibit the forced cell division activity in malignant tumors. However, such studies by Dustin and Grégoire (1933) and Dustin (1934) on mice with crocker's sarcoma did not lead to a positive result (Bucher, 1939). Another study by Hüssy (1930) was reported to have seen an effect on mouse carcinoma with two other acridine derivatives, i.e., phosphine-acridine-nitrate and phosphine-acridine-imino, and yet another one by Lewin (year not available) obtained satisfactory results with trypaflavin in local cancer treatment in humans. Trypaflavin, which pharmacologically belongs to the general paralytic protoplasma toxins and can lead to paralysis of the respiratory center, is probably too dangerous for general therapy in sufficient doses to inhibit tumor growth. A local inhibitory influence on tumors is within the range of possibility and its bactericidal effect would be added as a desirable side effect of trypaflavin in infected exulcerated tumors (Bucher, 1939).

Like the division rhythm, the course of single mitosis is also influenced differently by COL and trypaflavin. The average duration of COL mitoses increases the longer the toxin effect

lasts, as does the number of pathological karyokineses (already roughly visible), until finally the overwhelming majority of the cells undergoing division do not get beyond metaphase at all (stathmokineses) (Bucher, 1939). In contrast, under the effect of trypaflavin, all mitoses, as far as they still occur, proceed relatively quickly without any coarse disturbances visible in the film. The finer chromosomal defects are also quite different in the COL and trypaflavin mitoses, except for the most severe degrees of damage. The damage caused by COL mainly affects the metaphase while the damage caused by trypaflavin especially affects the ana-, telo- and reconstruction phases (Bucher, 1939). The fundamental studies on the effect of X-rays on cell division on the corneal epithelium of urodelar larvae by Alberti and Politzer (1924) show that a primary effect and - separated from it by a mitosis-free intermediate time - a secondary effect can be distinguished (Bucher, 1939). The primary effect is characterized by nuclear pyknosis in the different phases of the cell division process, by chromatin bridges between the daughter nuclei and by an angular position of the often-unequal daughter nuclei, as well as by the formation of so-called pseudoamitoses. In the secondary effect, the pyknoses recede; instead, there is a deflection of chromosomes and partial nucleation, often even a scattering of chromosome (fragments) around the whole cell (Bucher, 1939). Chromatin bridges, almost always combined with deviation of daughter nuclei, as well as necrosis and, therefore, remnants of necrotic nuclei also outside the cells in the form of strongly stainable structureless spheres occur in the secondary effect (Bucher, 1939).

COL causes nuclear pyknosis of the different mitotic stages, especially of metaphase and scattered chromosomes are often found, sometimes also a scattering of chromosomes in the whole cell, and chromosomes then often show a longitudinal cleavage (Bucher, 1939). The damage thus corresponds partly to the primary effect and partly to the secondary effect of irradiation; this applies to both COL and trypaflavin mitoses. In contrast to the COL, the trypaflavin (apart from the strong concentrations) causes much less frequently pyknomitoses, which can show the ring- or cup-shaped appearance

of the chromatin clump observed in the secondary effect or then quite strange forms (Bucher, 1939). Likewise, scattered chromosomes and cells with an irregular scattering of chromosome (fragments) recede quantitatively, although they may appear qualitatively the same as in the COL mitoses in more severe poisoning. The formation of chromatin bridges between the daughter stars, i.e., chromatin arrangements, is characteristic for the trypaflavin effect which are often located not quite symmetrically and very often can occur together with a deviation of the daughter stars or are likely to be responsible for this angular position (Bucher, 1939).

The chromatin formations in the form of longer rods, dumbbells, clubs, etc., are presumably derived from such bridge formations due to chromosome pyknosis. In the case of more severe poisoning, necroses are found more frequently in the trypaflavin experiments than in the case of COL necroses, i.e., spheres of chromatin, some of which may lie outside the cells (Bucher, 1939). Perhaps these formations do correspond to the "spherules" described by the Dustin (1929) and Mayer (1935), although according to these studies they should have nothing to do with pycnoses. Thus, various chemical (COL, trypaflavin, etc.) and physical effects (for example, X-rays) can produce morphologically quite similar mitotic disorders in quite different cells (fibrocytes, corneal epithelia, carcinoma cells, etc.) (Bucher, 1939). There are disorders which - considered only for themselves - could be addressed as specific for a certain trauma. And yet there are differences when the overall effect of the damaging agent in question are analyzed (Bucher, 1939). Two substances can influence the division rhythm in the same way, but the course of single mitosis is quite different. With the finer morphological disturbances - especially with the weaker poison concentrations - a far-reaching differentiation is possible since one substance first causes these differentiations (e.g., COL causes pycnoses) and another substance first predominantly cause those mitotic disturbances (e.g., trypaflavin causes chromatin bridges) (Bucher, 1939). At the strong toxic concentrations, the disturbances then become more or less unspecific. When a chemical compound is examined for its effect on the growth rhythm and single mitosis in tissue

culture, certain particularly conspicuous disturbances stand out strongly (COL or trypaflavin effect), but without these phenomena being specific for the substance in question (Bucher, 1939). More or less specific, however, is their effect taking into account all symptoms.

According to these findings, it is therefore also expedient after the investigations on tissue cultures to assign the two karyoclastic poisons COL and trypaflavin to two different groups. It would be interesting to examine how the other karyoclastically acting substances classified into the groups in question behave in this respect. More or less the same mitotic disorders are also frequently encountered in cancerized cultures and in carcinomas.

Haas (1940) observed a change in the core content and the plasma structures when looking at mussel eggs in the dark field after the use of pharmaceuticals could be determined. The determination of the upper limit concentrations at which turbidity or coagulation processes occurred on the nucleus or plasma led to the finding that several pharmaceuticals are characterized by a specific effect on the cell nucleus, while the nucleus-damaging effect of other substances is bound to almost the same concentrations that also damage the cytoplasm. The division of the investigated pharmaceuticals into a group of cytoplasmic and a group of nuclear toxins is in agreement with the results obtained in sea urchin eggs in determining the change in respiratory metabolism and the influence on the nuclear division processes (Haas, 1940).

Anti-tubulin agents & metaphase

The effects of the alkaloid COL as a mitotic poison were first described by Lits (1934) and Dustin (1934) and the nature of its action has been further studied by Ludford (1936). In common with a number of apparently unrelated substances, notably the cacodylates, it has been shown to arrest cell division during metaphase and to maintain cell thus affected in this phase of mitosis for several hours following administration; this occurs both in tissues *in vivo* and in explanted tissue (Brues, 1936). It is, of course, most readily seen in tissues in which the mitosis

rate is normally high, notably in the glands of the intestine, and it occurs as well in malignant tumors and in explants from them. Several derivatives of COL have been investigated (Brues, 1936) with regard to their effects as mitotic poisons. The test object was the regenerating rat liver (Brues, 1936), in which the arrested mitosis is conspicuous and is easily distinguished from the normal mitosis. In the case of the regenerating rat liver (in which the normal rate of cell division is known with reasonable accuracy), under suitable conditions, the number of mitoses seen in arrested metaphase after administration of COL over a given length of time is equal to the number of mitoses which would normally have occurred and gone on to completion during that time (Amoroso, 1935). Brues (1936) carried out a study to determine the effects on mitosis of various compounds derived from COL, to assess the importance of the various molecular groupings of COL and to determine whether similar mitotic affects may be shown with COL derivatives which are devoid of the high toxicity of the parent alkaloid.

It is essential to select as a test-object a tissue with a high normal rate of mitosis in which the abnormal arrested metaphase can be unequivocally distinguished from the normal metaphase. Experimental tumors fulfil the first of these criteria but owing to the frequency of abnormal mitotic figures it is often impossible to say whether a given mitosis shows the toxic effects of a mitotic poison or not (Brues, 1936). Moreover, in tumors many cells in apparently normal later stages of division are seen after effective doses of the drug have been given and, at least in the case of certain transplanted sarcomata (probably owing to inadequate blood supply), the effect may be confined to the borders of the tumor and in some sections missed altogether.

The most satisfactory tissue for the study by Brues (1936) was liver in the process of restoration following subtotal hepatectomy. The colchicine employed in these experiments was obtained from the Hoffman La Roche Laboratories. Since this substance contains about 25 percent chloroform of crystallization, a few rats were injected with amorphous colchicine, which contains no chloroform, with entirely similar results as far as the cytological picture was concerned (Brues, 1936).

Colchicine was dissolved in 0.9 percent saline solution before administration. When large quantities were used, it was first dissolved in a minimum amount of alcohol, to facilitate solution in water. In order to control the use of alcohol in administration of this and other substances, comparable amounts of alcohol alone have been injected into animals during hepatic regenerations, without any resulting abnormalities of mitosis being detectable (Brues, 1936). In the cane of the rat, the average mitosis rate during the period of rapid regeneration was nearly as great as that in most experimental tumors; and histological study was facilitated by the large size of the hepatic cells and by the highly characteristic distribution of the chromosomes under the influence of a mitotic poison.

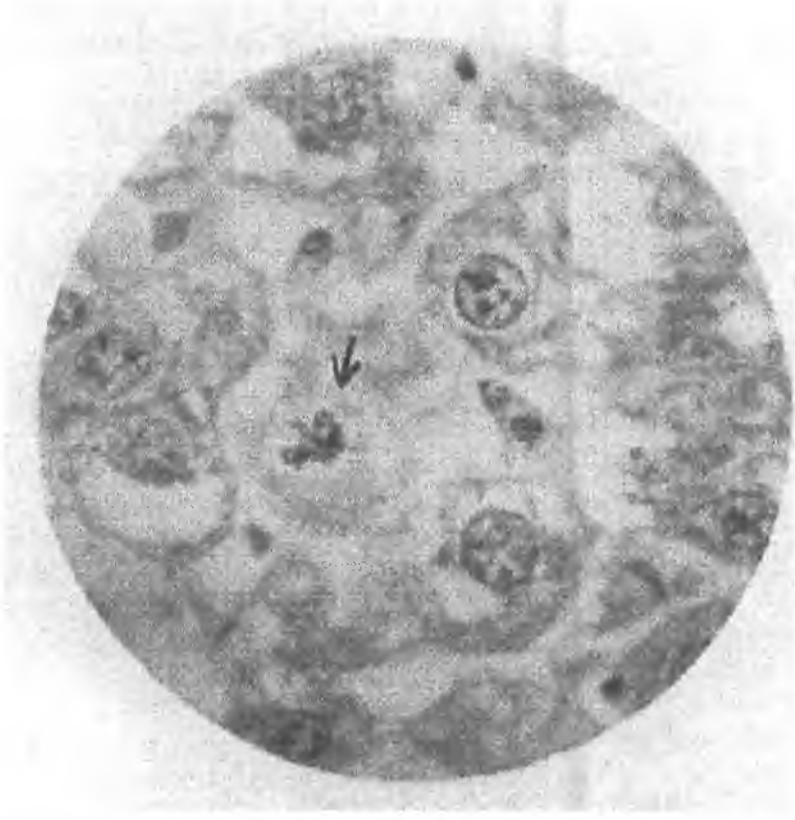

FIGURE 1: Normal metaphase as indicated by arrow in regenerating liver 30 hours after operation, x 600 (from BRUES).

In the normal hepatic cell mitosis, the chromosomes form a very compact group shortly after the disappearance of the nuclear membrane during prophase and remain so throughout the division - except in prophase it is impossible to distinguish and count the individual chromosomes (Brues, 1936). On the other hand, after parenteral administration of a suitable dose of COL, the chromosomes scatter widely throughout the cell shortly after disappearance of the nuclear membrane, as if they repelled one another, and it is often possible to count them. In addition, the cell in this arrested stage has the rounded border characteristic of a cell in mitosis and in most cases the cytoplasm stains much more lightly with eosin (Brues, 1936). Except under the influence of a very small dosage, these figured were abnormal and in the same section with normal metaphases and later stages of mitosis, making it possible to distinguish the abnormal from a normal one. An appropriate dose of sodium cacodylate gives essentially the same picture in this organ.

In vivo, the COL action is most likely a direct one. COL leads to the damage of a division-inhibiting factor in the cell, while

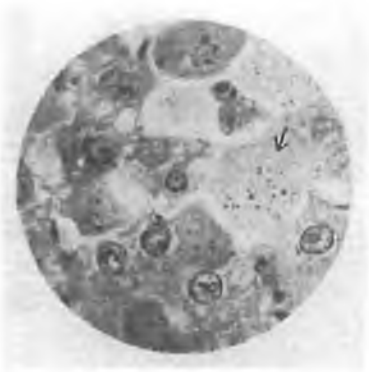

***FIGURE 2**: Abnormal mitoses in regenerative liver 2 days after operation and 8,5 hours after COL Treatment (100 mcg s.c) One abnormal FIG In indicated by arrow, x 600, from BRUES.*

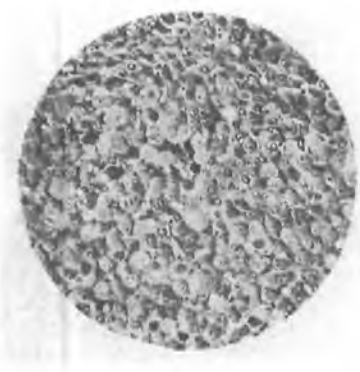

***FIGURE** 3 Low power view of the liver, showing large numbers of arrested mitoses, x 95, from BRUES*

the fibrocytes of the tissue culture already show a maximum growth rhythm uninfluenced (Bucher, 1939). Therefore, it is difficult to induce a growth-promoting effect in the culture by any chemical substance at all - perhaps apart from certain hormones. In animal experiments, the latency period is naturally longer (usually 6-8 hours) than in the experiments *in vitro* (1-2 hours in the films).

In addition to the general minor depressive influence on the mitotic rhythm, COL in tissue culture results in a stronger transient inhibition, which reaches its maximum within 120 min to 180 min after toxin addition and is then compensated for in the following 60 min to 120 min. A similar mitosis-poor or mitosis-free interval was also observed after ultraviolet irradiation. Apparently, this is not a reaction characteristic of any action, but a nonspecific, reversible damage to the division regulator, which is overcome in the course of a certain time. Similar observations have already been made before by Alberti and Politzer (1924) after the X-ray irradiation of the corneal epithelium of urodelar larvae. In general, the mitotic disorders caused by COL had some similarities with the cell divisions damaged by X-rays. In contrast to these conditions, the mitotic inhibitory effect of trypaflavine (diaminometlyacridinium in stronger concentrations) could not be overcome so quickly.

The most striking effect of COL on the course of cell divisions was the blockage in the first stage of metaphase - the typical COL effect. This phenomenon occurred both *in vitro* and *in vivo* therefore, and Dustin (1938) proposed to call these arrested mitoses stathmokineses (stathmocinbses, divisions stathmocinétiques); the name expresses the main action of this alkaloid. This stathmokinetic effect of COL has for some years been attributed by a practical significance for the therapy of neoplasms. Studies in 1927 by Amoroso and Hayes (full reference not available in brues, 1936) saw that tumors in patients who received COL for the treatment of acute gout attacks responded better to X-ray irradiation. As cell divisions could not be completed and new cells continued to divide - possibly even to an increased extent - and marched into metaphase, there was a strong accumulation of karyokineses. This accu-

mulation was particularly high in neoplasms, which in and of themselves already had a higher mitotic coefficient, so that the already greater radiation sensitivity of malignant tumors was increased even further.

COL, metaphase & tumours

The action of COL upon division of normal and malignant cells has been studied by several investigators. Dustin (1934) described a remarkable increase in the number of mitoses in malignant growths in animals following the injection of COL. COL applied to cultures of normal and malignant cells of animals causes an arrest of mitosis for several hours, and that prolonged application destroys the cells held up in division while the resting cells still survive (Ludford, 1936). A single injection in animals produces an arrest of mitosis for several hours. The mitoses arrest in the metaphase, which is due to the failure of the mitotic spindle to form and function in the normal manner (Oughterson *et al.*, 1937). The largest accumulation of arrested mitoses occurs in tissue in which cell division is normally of frequent occurrence. This phenomenon has been utilized by many studies (Brues, 1936; Allen *et al.* (year not available)) as an index of the rate of cell growth. It, therefore, seemed that this method might prove of value in studying the growth characteristics of human tumors. In their experiment, the effect of COL on tumors was studied in a group of 21 patients who received the drug either subcutaneously or intramuscularly. 15 of the 21 patients had control biopsies before receiving the COL. 11 patients showed definite arrest of mitosis in the metaphase (Oughterson *et al.*, 1937). A biopsy was performed, when possible, to establish the diagnosis, and to serve as a control. COL was then administered, and in most instances a second biopsy was performed, or the entire tumor was removed 9 hours later. In certain inaccessible tumors it was impossible to perform a biopsy before the removal of the tumor. However, the COL effect could be observed and compared with similar tumors. In some cases, biopsies were taken at intervals varying from 4 to 18 hours after injection (Oughterson *et al.*, 1937). The drug was diluted in saline solution and administered either intramuscularly or subcutaneously. Doses varying from 1 mg to 4 mg were used,

and as little as mg has produced a typical COL effect in some tumors (Oughterson *et al.*, 1937).

The number of mitotic figures was still increasing up to 12 hours, at which time there was an increase of over 700%. In some fields, in the 12-hour specimen, as many as 50% of the cells were in mitosis (Oughterson *et al.*, 1937). The accumulation of mitotic figures after COL could not be attributed to delivery of the tumor on the abdominal wall, since control biopsies taken before and 10 days after the procedure showed approximately the same number of cells in mitosis per high power field. The repeated biopsies were not responsible for the changes noted, as similar accumulations of mitoses after COL had been observed in other intestinal tumors that were not subjected to multiple biopsies (Oughterson *et al.*, 1937). The 12-hour specimen represents the entire tumor and a large portion of adjacent bowel. The sections of this tumor far removed from the sites of the small biopsy taken at the earlier period, as well as the normal mucosa beyond the tumor mass, showed the same striking accumulation of mitoses.

Anti-tubulin agents & histology

Both short- and long-term effects and the ability of the cells to recover after prolonged exposure to these drugs were studied by Moskalewski *et al.*, (1975). During the experiments, they found that the antimicrotubular agents caused marked changes in the Golgi complex. As an attempt to determine whether these alterations were directly due to the absence of microtubules or were secondary to inhibition of the synthesis of matrix components, the effects of puromycin, a potent protein synthesis inhibitor, on cellular fine structure was also examined (Moskalewski *et al.*, 1975). They used chondrocytes isolated from cartilaginous epiphyses of guinea pig fetuses as experimental system, aggregated them, and maintained them in organ culture. Because of the potential usefulness of this system for other studies on the function and metabolism of cartilage, the morphology of the chondrocyte aggregates was recorded for up to 25 days, i.e., beyond what was necessary for the experimental purposes proper (Moskalewski *et al.*, 1975).

It is generally accepted that the Golgi complex is involved in the synthesis and secretion of matrix components in connective tissue cells. There is considerable morphological and biochemical evidence indicating that the formation of both collagen and proteoglycans is initiated in the granular endoplasmic reticulum, and then completed during transport through the cisternae of the reticulum to the Golgi complex, as well as within this latter system, where the molecules finally accumulate in vacuoles before being released to the extracellular space by exocytosis (Moskalewski *et al.*, 1975).

During recent years antimicrotubular agents have been found to interfere with both secretion and synthesis of collagen in cultured bones and fibroblasts (Dehm and Prockop, 1972; Ehrlich et al., 1974), and of glycosaminoglycans in isolated chondrocytes (Jansen and Bornstein, 1974). Based on these findings, it has been suggested that disruption of microtubules interferes with the movement of Golgi vacuoles to the cell surface, and this results in accumulation of secretory products in the Golgi complex, which then secondarily leads to an inhibition of synthesis. In their study, aggregates formed and cultured in the presence of COL and vinblastine showed a reduced content of intercellular matrix (Moskalewski *et al.*, 1975). This reduction comprised both collagen fibrils and matrix granules, the latter demonstrated to contain proteoglycans by ruthenium red staining (Luft, 1971; Thyberg et al., 1973). Considerable amounts of intercellular matrix were laid down in the treated aggregates. The chondrocytes retained part of their capacity for synthesizing and secreting extracellular macromolecules despite the disappearance of the microtubules and the structural alterations of the Golgi complex (Moskalewski *et al.*, 1975). It is evident from their results that the reduction of matrix deposition in aggregates treated with COL and vinblastine could be due to a primary disfunction of the Golgi complex as well as to a disturbance in the transport of secretory vacuoles.

Anti-tubulin agents & tumours

There have been numerous reports showing considerable numbers of mitotic figures in the hemopoietic organs of normal

healthy animals and in the neoplastic tissues of tumor-bearing animals after COL administration (Amoroso, 1935). This suggests an interrelationship between mitosis and COL. But the effect of COL in slowing down the rate of growth of neoplastic tissue was yet to be reported. To test this supposition, Amoroso (1935) carried out a series of experiments and tested the extent to which COL might affect new growths. He took two groups of mice. One group had twelve tumor-bearing mice which were injected subcutaneously on alternate days with small doses of COL. The treatment lasted for two weeks. Another group of twelve tumor-bearing mice was used as control. The tumors at the end of the first week showed much less growth in the first group compared to the control group, while at the end of the second week there was no macroscopically recognizable tumor tissue present in the 66.6 percent of the injected mice and only minute nodules were detected in the remaining 33.3 percent (Amoroso, 1935). These nodules finally showed complete regression and no tumor tissue could be recognized eight weeks later. In the control group, a marked development of the tumor was noted. In another experiment of this series, the percentage of mice with no recognizable tumor tissue at the end of two weeks was 100 percent (Amoroso, 1935).

Based on these results, Amoroso (1935) then carried out similar experiments on dogs and found that of several dogs treated or under-treatment with COL, the changed observed in a spontaneous tumor in the peritonsillar region in of the dogs was sufficiently striking to warrant recording. There was a progressive diminution in the size of the growth and within six weeks, only a small scar remained at the site of the original growth (Amoroso, 1935). In 1936, Ludford also carried out experiments on mice and dogs to study the influence of COL on tumors. He observed arrest of mitosis in tissue cultures of both normal and malignant cells. In addition, he also noted that some of the arrested cells that were exposed for a longer time died.

Vinca alkaloids & cancer

While investigating *Vinca rosea* Linn (periwinkle), certain crude fractions of the plant were submitted for evaluation by

Johnson *et al.*, (1960). The fractions were discovered to give interesting and, in some cases, profound activity against the P-1534 leukemia, an acute lymphocytic leukemia transplanted in DBA/2 mice. These findings led to a prompt phytochemical investigation which resulted in the obtaining of three new alkaloids: leurosine, virosine, and perivine (Svoboda, 1958). Leurosine was found to have some activity against this experimental neoplasm. Shortly after these compounds were obtained, another study by Noble, Beer and Cutts (1958) reported obtaining another new alkaloid as a sulfate from *Vinca rosea*. They suggested the name Vincaleukoblastine (VLBS) for this compound. The most striking biological action of VLBS was its leukopenic action in normal rats, which was used for its bioassay (Cutts, 1958; Noble et al., 1958). It was subsequently demonstrated that VLBS also markedly inhibited the P-1534 leukemia (Noble et al., 1958; Johnson et al., 1959). Following these results, Johnson and his colleagues (1960) carried out some experiments with these compounds to suggest a possible hypothesis for the mechanism of action of this example of a new class of antineoplastic agents.

The plant *Vinca rosea* Linn (periwinkle) is an apocynaceous, ever-blooming, pubescent herb or sub-shrub which has been shown to be a source of many alkaloids. It has enjoyed a popular reputation in indigenous medicine in various parts of the world. The detection of its activity against the P-1534 leukemia is particularly significant because this tumor system has detected other clinically useful antitumor agents (Johnson et al., year not available) and has been sensitive enough to study structure-activity relationships of active compounds which correlated with the clinical activity (Grezon et al., 1959; Krakoff et al., (year not available); Miller et al., 1959; Miller et al., 1960). Of the alkaloids studied, one isomeric with VLBS – "leurosine" – has also shown a demonstrable retardation of the P-1534 leukemia (Johnson *et. al.*, 1960). It has generally been of a lower order of activity than VLBS, and less consistent. The indole alkaloid, catharanthine, and the dihydroindole alkaloid, vindoline were of theoretical interest (Gorman et al., 1959). Vindoline-like and catharanthine-like molecules each approximate one-half of the leurosine and VLBS mole-

cules (Johnson *et al.*, 1960). A solution containing equimolar proportions of these two alkaloids has an infrared absorption spectrum which approximates those of VLBS and leurosine (Garcia, 1958). These compounds either singly or in an equimolar solution have been devoid of any anti-P-1534 activity, as have all other pure alkaloids from this plant which have been tested (Johnson *et al.*, 1960). It would seem possible on biogenetic, chemical, and physical grounds to speculate that vindoline and catharanthine may represent precursors of the biologically active VLBS and leurosine molecules. The mechanism of action of VLBS is of consider able practical importance. Metaphase arrest of the type referred to as C-mitosis has been observed *in vitro* in human cells of malignant origin, but not all cells examined were equally susceptible (Johnson *et al.*, 1960). The classical example of an agent with C-mitotic activity is the alkaloid COL. This may be only coincidental insofar as usefulness of VLBS is concerned according to Johnson and his colleagues (1960).

COL & cancer

While looking into the role of COL in cancer research, Eigsti and Dustin, (1955) found that the combined action of COL and X-irradiation on animal and plant materials has been studied in several laboratories but no decisive results appear to have been obtained. All the studies on neoplastic cells point towards the same inescapable fact: COL was considered a treatment for gout prior to 1934 and was also believed to have some favorable action against cancer. Malignant cells, especially in animal tumors, often display "spontaneous" mitotic abnormalities. Few studies have compared them to those induced by COL and suggested that the cells were under the influence of some mitotic poison acting like COL (Gutman and Yu, 1952). They argued that this may be lactic acid (Vibert, 1907). However, these spindle disturbances often appear to be the consequence of more deep-seated nuclear changes, closely related to the cause of malignancy itself, and leading to chromosome breakages and rearrangements. However, it was noted in early human carcinomas that the spindle changes appeared first (Mugler and Hauswald, 1952). The behavior of such cells when brought under the influence of COL is of

great importance to determine whether a specific destruction of malignant cells by a spindle poison is possible. The effect of COL on cancerous growths was studied by Eigsti and Dustin (1955) either by injecting the animals with the drug, or by explanting the abnormal cells *in vitro* and using the methods of tissue culture. This last procedure has been followed with a mammary carcinoma (Van Heerswynghels, 1935) and a sarcoma of the mouse, and with Ehrlich mouse carcinoma growing as an "ascites tumor" in the abdominal cavity (Lambers, 1951; (Sanno, 1911). Concentrations of 100 x 10^{-6} M to 1.25 x 10^{-6} M inhibit outgrowth from the explants and arrest cell divisions. This effect is still evident on carcinoma cells at a concentration of 0.5 x 10^{-6} M (Eigsti and Dustin, 1955). In culture containing explants of both tumor and embryonic kidney, the latter showed the greatest cellular destruction following the mitotic arrest. Differences of sensitivity between various strains of carcinomas were found, while the Crocker sarcoma showed fewer arrested metaphases (Lits, 1934, 1936).

COL cancer & histology

A study in 1938 by Deckner carried out cytological examination of 30 human carcinomas and found an unusually large number of nuclear divisions and among them an unusually large proportion of pathological forms, but no division figure specific for the cancer cell. These were morphologically the same disturbances which Alberti and Politzer (1924) observed experimentally after X-ray irradiation or Bucher (1939) observed after exposure to COL and also to trypaflavin in non-cancerous cells. Similar findings were also obtained in another study by Mauer (1938) with cancerized fibroblast cultures. It has been noted by various authors that multipolar mitoses and deviations from normal chromosome number (especially often extreme hyperchromasia) are found relatively frequently in malignant tumors and in cancerized cultures. Both disorders are also suggested in the COL experiments, but not in the trypaflavin experiments. Thus, COL is closer to carcinogenic substances than trypaflavin. It should be remembered that COL is also a phenanthrene derivative (Bucher, 1939). Biologically, however, there is still a big difference between carcinoma cells and cells forced to undergo

morphologically similar pathological mitoses under experimental conditions. The latter show the mitotic disturbances only transiently; then they perish or, if the damaging agent is removed in time, can return to normal division (still after 20-hour exposure to COL 1:25 million). Cancer cells, on the other hand, retain the impaired division mechanism in subsequent generations. In the case of tumor cells, in addition to their vital expressions (cell division, metabolism, etc.), the hereditary structure has also been affected (Bucher, 1939).

Anti-tubular agents& histology

Both microtubule inhibitors - VBLS and COL - decrease collagen synthesis and secretion but in so doing lead to different ultrastructural changes in cells. The effects of VBLS are manifest in both the RER and the Golgi complex, resulting in a vesiculation of the RER cisternae and a diminution of the Golgi complex (Ehrlich *et al.*, 1974). COL appears to affect only the Golgi complex, leading to enlarged Golgi-associated vacuoles. The changes seen with VBLS are consistent with the generalized depression of protein synthesis produced by the compound. The more limited changes in the Golgi complex seen with COL were associated with a 30 percent reduction in collagen synthesis and with the appearance of abnormal filamentous structures within Golgi-associated vacuoles (Ehrlich *et al.*, 1974). The latter findings suggest that collagen is secreted at least in part via the Golgi pathway and that this secretion is dependent on a normally functioning microtubular system.

COL & fever (FMF)

Dinarello *et al.*, (1976) studied the patients with familial Mediterranean fever (FMF) who were part of a double-blind trial of daily COL as prophylaxis for their disease and observed their leukocyte functions while receiving COL or placebo. Leukocytes taken from these patients while on prophylactic doses of COL produced normal quantities of leukocytic pyrogen, ingested bacteria normally, and migrated normally in chemotactic chambers (Dinarello *et al.*, 1976). In addition, these patients had normal numbers of circulating T and B lymphocytes as well as normal blastogenic responses of their peripheral lymphocytes to mitogenic stimuli. The patients on

COL, however, had significantly fewer neutrophils and monocytes accumulating at skin-window sites 24 hours after the initial abrasion (Dinarello *et al.*, 1976). Skin window technique is used in immunology where the top layer of skin is scraped off making it possible to identify the immune response that would occur with a diminished physical barrier in the host and observe mobilization of leukocytes. Because the early phase of the skin-window response was normal in these patients, the decreased late response may be related to a failure to amplify the initial inflammatory reaction. The reduced capacity to generate a normal inflammatory response may account for the failure of these patients to develop full attacks while taking COL.

COL & poisoning

Eigsti and Dustin (1955) published their findings on the pharmacological aspect of COL. The nineteenth century medical literature contains many references to *Colchicum* preparations (Delioux and Savignac, 1876). In 1941, a woman of 42, attempting suicide, swallowed 60 1-mg pills of colchicine "Houdé." She lived eight days after this very high dose; delayed lethality is nearly always found in colchicine poisoning. The principal findings were the persistence of mitotic changes long after the ingestion of colchicine, indicating that this substance is only slowly metabolized, the evidence of a general toxic reaction, and considerable changes in the liver, where the proliferation of hepatic cells was made evident by the mitotic "stasis" produced by spindle destruction. These changes were once considered at the time as evidence of mitotic stimulation by colchicine, but they are probably only an indirect effect, since the alkaloid destroyed hepatic cells and later arrested the mitoses needed for regeneration. One other similar pathological description has recent been published. This was a case of acute poisoning. A five-year-old girl swallowed an unknown number of seeds. These were later identified as belonging to the genus Colchicine. Repeated vomiting and abdominal pain were the first signs of toxicity. The central temperature rose, and the pulse became fast. Death followed in 38 hours. Cerebral oedema was conspicuous. Small haemorrhagic clots were seen on the pericardium and the peritoneal serosa. The duodenal mu-

cosa was swollen and dotted with many haemorrhagic zones. Evidence of mitotic poisoning was visible in the liver, where some cells were in a condition of arrested metaphase. Others showed evidence of degenerative alterations. Arrested metaphases were conspicuous in the bone marrow; a small number could be found in the duodenal mucosa. Pycnotic destruction of lymphocytes in lymph glands, Peyer's patches, and the thymic cortex was probably the result of the combined action of the mitotic poison and of the general alarm reaction (Selye, 1950). Colchicine was detected by a biological method, while chemical reactions remained negative. Large quantities were found in several organs, in particular the liver, the kidney, and the brain. Extracts from these tissues displayed a typical spindle-poisoning effect when brought into contact with chick fibroblast cultures. In the complex changes which take place when a large dose of colchicine is absorbed in man, it is evident that some are related to the poisoning of cell division, for instance bone-marrow inhibition (Brown, 1940; Seed et al., 1940), while others, such as the destruction and regeneration of liver cells, and the evidence of stress, are of a more complex nature. Vomiting, which may appear shortly after the drug is taken, is one major sign of a series of disturbances which dearly have nothing to do with the cytological effects which have been studied so far (Eigsti and Dustin, 1955)

Death occurs within several hours in warm-blooded animals, or several days in cold-blooded vertebrates, after injections of the largest closes. In 1906, COL was called "this most remarkable slow poison." (Dixon, 1906). Progressive nervous paralysis leading to respiration arrest, appears to be the main cause of death, whatever the animal tested (Eigsti and Dustin, 1955). To test this nervous action of COL an experiment was performed nearly 50 years ago which gives a remarkable demonstration of the sensitivity of the nervous system towards COL. While the injection of even the largest doses killed a cat only after several hours, the intracerebral injection of the drug had a spectacular and rapid action. Very soon the blood pressure was found to increase, and the respiration became rapid and deeper. After 35 minutes, a sharp fall in the blood pressure indicated vasomotor paralysis (Dixon, 1906). One hour after the injection, the animal

died of respiratory paralysis. An important series of findings in rats and cats pointed to the nervous system as one of the principal causes of the various effects of COL poisoning. It was observed that vomiting cannot be, as was sometimes thought, the consequence of pathological modifications of the gastrointestinal tract brought about by mitotic arrest. The same is true for diarrhea, a frequent symptom, which would appear to be a consequence of intestinal congestion and ulcerations (Fuhner and Rehbein, 1915). No diarrhea and almost no vomiting is found in animals injected with barbiturates, even when the dose of COL is lethal. The central temperature falls sharply after COL (Eigsti and Dustin, 1955). This may be partly a result of stress and nonspecific toxicity (Clark and Barnes, 1940, Selye, 1950), but the curves indicate that the decrease taking place in the first ten hours has another cause (Eigsti and Dustin, 1955). This is now believed to be a central nervous effect (Ferguson, 1952, 1953). Another fact points in the same direction: Animals treated with COL display an increased sensitivity (Eigsti and Dustin, 1955). While unanesthetized cats die only after eight to ten hours, the same dose of COL brought death in less than two hours when the animals had received barbiturates previously (Ferguson, 1952, 1953). Barbiturate or ether anesthesia also proved to be abnormally dangerous in animals which had received the alkaloid first. Arterial constriction leading to high blood pressure was also noted. Experiments of brain transection in the cat demonstrated that this also was a consequence of a central nervous stimulation (Ferguson, 1952, 1953). However, other territories of the nervous system are affected by COL. The neuromuscular apparatus appears to be the most sensitive, though only after repeated administration of the alkaloid can the modifications be detected. An atrophy of the hind quarters of cats injected daily with 0.05 mg per kg of body weight was observed after two weeks (Eigsti and Dustin, 1955). The leg muscles were converted into thin strands. There was no evidence of muscular damage. Abnormal responses to acetylcholine were observed. There was no true neuromuscular block. Anesthetic properties have also been noted; these are probably of central origin (Eigsti and Dustin, 1955). Death often follows a period resembling narcosis. In the dog, this appears before the muscle paralysis. In cold-blooded animals, the nervous

changes may be very slow to appear. In frog, kept at low temperature, reflexes disappear progressively, the corneal being the last, and this not until several weeks after an injection of colchicine (Fuhner and Rehbein, 1920).

While describing the disturbances possibly related to mitotic poisoning, Eigsti and Dustin (1955) related the pharmacological effects to the histological changes resulting from spindle destruction. It was noticed that the leukocytosis-promoting effect of COL, which nearly led to the discovery of its action on mitosis (Dixon, 1906, Dixon and Malden, 1908), is probably only remotely linked to mitotic arrest. Its origin may be the action of the drug on the central nervous system. However, it is associated with some of the first descriptions of tissues altered by COL and has often been quoted as the origin of modern cytological work in this field (Eigsti and Dustin, 1955). A substance that arrests the mitoses taking place in the bone marrow for some hours and destroys many of them would be expected to depress blood formation. Extensive cellular destruction has been found in the bone marrow of mice (Lits, 1934, 1936). Considerable congestion and a decrease in the number of nucleated cells are the consequence of this destruction. In some experiments, 20 percent of all the nucleated cells of the marrow were arrested at metaphase (Widmann, 1948, 1949, 1951, 1951). It was made clear by reticulocyte counts in the blood of rabbits that this decreases the output of young red blood cells. Normal animals and rabbits with phenylhydrazine-induced hemolytic anemia were utilized. A sharp but transient fall in the percentage of reticulocytes is a convincing demonstration of the inhibition of blood formation (Dustin, 1941, 1941). On the other hand, an injection of COL in rabbits and dogs was followed by a considerable increase in the number of circulating white blood cells. 12 hours after the injection, the bone marrow of rabbits appears empty of most of its nucleated cells. This is in agreement with observations of bone-marrow aplasia (Eigsti and Dustin, 1955).

At present, no clear relation can be discovered between the inhibition of mitotic growth. It has been discovered that in leukemic patients and in normal men a single dose of colchi-

cine (2 mg) may increase considerably the number of platelets. The bone-marrow megakaryocytes do not change in number, but there is evidence of a greater platelet-building activity by their cytoplasm (Keibl and Bichlbauer, 1949; Landolt, 1943). In essential thrombopenia, where megakaryocytes are present but appear to be unable to produce platelets, this effect of colchicine was not found. Some recent work attempts to relate the bone-marrow changes and leukocytosis. This is often preceded by a transient period of leukopenia, which appears to have no causal influence on the leukocytosis (Widmann, 1948, 1949, 1951, 1951). Bone-marrow studies in mice and rabbits all confirm the increase of arrested metaphases, which is about 15-fold in the rabbit after 15 hours. The erythroblastic cells become progressively more numerous than the granuloblastic; the increase is from 10-15 per cent to more than 60 per cent in mice. The immature cells increase in proportion because the adult cells leave the marrow. There is no visible relation between this phenomenon and the mitotic changes (Widmann, 1948, 1949, 1951, 1951). However, repeated daily injections of 12 mcg of colchicine increase considerably the number of leukocytes in the blood of mice (more than 250,000 per cmm.). It has been suggested (Widmann, 1948, 1949, 1951, 1951) that these changes may be the consequence of a central nervous stimulation of the bone marrow.

COL, side effect &toxicity

The subcutaneous and intramuscular injection of COL in the doses used was not accompanied by any general toxic symptoms. Many patients, however, showed evidence of local irritation manifested by local soreness and redness, and in some instances by paresthesia. While these symptoms all disappeared spontaneously, nevertheless the discomfort in some patients was of such degree as to make it desirable to use some other method for administering the drug (Oughterson *et al.*, 1937). There is insufficient data, owing to the small number of any one type of tumor, to draw conclusions as to the effect of COL on tumors in general. It is, however, apparent that the COL technique makes it possible in some instances to obtain a more accurate index of the rate of growth of the tumor than can be obtained by ordinary methods.

COL & liver toxicity

Liver and kidney damage. The mechanism of these changes is not clearly understood, but it certainly plays an important part in the general toxicity of the drug. Though bile secretion has been supposed to be increased, severe degenerative changes and necrosis have been described in the livers of mice, (Musotto and Di Quattro, 1941, 1947) especially after repeated injections (Lambers, 1951). In mice, the LD 50 dose induces liver cell steatosis in one hour (Rossi, 1950). Steatosis of heart muscle cells and kidney tubules was also noted. Female mice appear to be more resistant to this damage than males. Mitoses of liver cells have been described in human poisoning by colchicine. There are often arrested metaphases, even long after the drug has been administered, a fact which is explained by its slow excretion (Brues and Cohen, 1942, 1951). Three days after injection of colchicine in mice, normal mitoses also have been observed in liver cells. After several injections of colchicine, many arrested mitoses are to be seen. The stages of recovery lead often to bizarre nuclei which may resemble those of megakaryocytes. Cellular damage may not be evident at all, and the cause of these divisions is not clear. A hormonal stimulation related to stress and the adaptation syndrome is possible (Miszurski and Doljanski, 1949). In chronic intoxication of mice, after daily injections of 12 to 15 μg. for 20 to 30 days a great number of liver nuclei are irregularly shaped. More than 40 per cent of these contain spherical bodies resembling huge nucleoli. These are diffusely stained by acid dyes. They persist 13 days after the end of the injections. No mitoses were seen, a rather surprising fact (Lambers, 1951). Eigsti and Dustin, (1955) suggested that these intranuclear bodies result from arrested mitoses, and represent spindle material, similar to the hyaline globules and pseudospindles.

COL & hormones of fish, frog, mice

The idea of COL having some direct hormonal action led to some curious experiments which are important to consider because COL has often been used for the detection of hormone-stimulated growth. During the breeding season, the fish *Rhodeus amarus* displays brilliant reel "nuptial colours," which are related to the expansion of chromatophores and

to local hyperaemia. These colours appear in animals treated with male hormones. COL alone has the same effects (Havas, 1939, 1940; Havas and Kahan, 1948). Nuptial colours are displayed by fish subjected for 10 minutes to a 1.5/1000 solution, or for 35 minutes to a concentration of 0. 75 /1000 (Eigsti and Dustin, 1955). COL and hormones add their effects, and the full skin changes could be produced in 2 hours instead of 20 hours with hormone alone. The oxygen consumption of the animals was also increased (Eigsti and Dustin, 1955). In females of the same species, no increase in the size of the ovipositor was noted (Bretschneider and Duyvene de Wit, 1936). The changes of the male fishes, where vasomotor mechanisms play a great part, may have been either the consequence of a nervous action, or of the general toxicity of COL. The possibility of stimulating the action of pituitary hormones by the alkaloid was strongly suggested by experiments on the ovulation of isolated ovaries of *Rana pipens (frog).* This was considerably accelerated, both in whole animals and on isolated ovaries. The eggs were fertilizable, but none ever divided (Eigsti and Dustin, 1955). COL was believed to bring a "true potentiation" of the pituitary hormones controlling ovulation (McPhail, M. K., and Wilbur, K. M. 1943, 1944). In the rabbit, however, no potentiation of the action of pregnant mare's scrum, containing gonadotropic hormones, on the rate of ovulation could be detected (McPhail, M. K., and Wilbur, K. M. 1943, 1944). COL had no action on the weight of ovaries of mice similarly injected, or on the seminal vesicles of rats injected with testosterone. Neither do results of experiments on silkworms (Havas and Kahan, 1948) justify the conclusion that COL is "hormone-mimetic." The only possibility is that through nonspecific action, this toxic drug could stimulate the secretion of hormones by endocrine glands, in particular the pituitary (Eigsti and Dustin, 1955).

COL stimulation of cell metabolism

Dixon (1905) was probably the first to observe a stimulating effect of COL on nuclear division. Dixon and Malden (1908) were able to demonstrate hyperleucocytosis in the blood after exposure to COL in rabbits, rats, dogs, and humans. Dustin (1934) and his student Lits (1934) then investigated the effect

of this alkaloid on nuclear division and found in it a "poison caryoclasique" of similar but far stronger effect than cacodylate. During his investigations into the factors that act on the thymus gland and at the same time on the nucleoprotein metabolism, Dustin (1921) became aware of substances that exert a very specific effect on the nucleus. Substances that have marked inhibitory or excitatory effects, especially on the course of mitosis, are called "poisons caryoclasiques". In COL, an extremely violent karyoclastic poison of the cacodylate group has been recognized. When COL acts on the animal organism, a particularly strong karyokinesis wave first occurs after about hours, namely in the thymus, in the lymph glands and everywhere in the organism where there are young cells capable of division. This phenomenon is followed by a blocking of the nuclear divisions in metaphase, after some time, approximately after 36-48 hours, part of the mitoses degenerate, while the others form the achromatic figure and complete anaphase and telophase. Ludford (1936) also found a strong increase in karyokinetic figures when COL was applied to artificially cultured carcinoma and to mice. According to Dustin, COL, due to its karyokinesis-stimulating properties, represents a division-energy liberating factor. The alkaloid makes it possible to determine the readiness for mitosis (Imminance caryocinetique), because after a certain exposure time, every cell capable of division reacts by forming the nuclear division figures.

VBLS & ribosomes

Vinblastine, at concentrations of 10^{-5} M, produced complexes between polyribosomes and the crystals induced by the drug in L cell fibroblasts and lymphoblasts (Krishan and Hsu, 1971). Such complexes may well interfere with protein synthesis and lead to an inhibition in incorporation of amino acids as was observed by Ehrlich *et al.*, (1974). Ultrastructurally, the vacuolar appearance of the RER in vinblastine-treated preparations contrasted with the normal interconnecting canalicular structure of this organelle in COL-treated or untreated osteoblasts. The lack of stored material in the Golgi complex after vinblastine treatment, despite disruption of microtubules, may reflect concurrent inhibition of protein synthesis. The appearance of RER cisternae after exposure to vinblas-

tine is strikingly similar to changes previously observed in ascorbic acid deficiency *in vivo* (Ross and benditt, 1965) in which collagen synthesis is also inhibited (Ehrlich *et al.*, 1974).

Cytochalasin B & collagen

The effects of cytochalasin B on the synthesis and secretion of collagen by cranial bone cells have been difficult to evaluate. Light microscope autoradiographic examination revealed that the secretion of collagen by some cells in bones exposed to cytochalasin B was markedly inhibited, whereas other cells in close proximity were apparently unaffected. As a result, the significance of the quantitative difference observed in the proportion of grains retained intracellularly after cytochalasin B treatment was unclear. Ultrastructurally, a similar variability was seen in the appearance of both osteoblasts and fibroblasts treated with cytochalasin B. Heterogeneity in the response of baby hamster kidney cells to cytochalasin B, with respect to changes in cell shape and tendency to enucleate, was also reported (Goldman, 1972). Possibly both the configuration of intracellular microfilaments and the susceptibility to the drug may vary with different stages of the cell division cycle. No inhibition of incorporation of leucine was found after a relatively short incubation of cells in the presence of cytochalasin B (Prescott et al., 1972; Sanger and Holtzer, 1972), although another study by Yamada et al., (1970) observed a 20 percent reduction in protein synthesis by dorsal root ganglia over a 2-h period using a mixture of labelled amino acids. In a previous study (Ehrlich and Bornstein, 1972), cytochalasin B (5 mcg/ml) was found to have no effect on tryptophan incorporation into protein extracted from chick cranial bone with 0.5 M acetic acid and 0.1% Triton X-100 (Ehrlich *et al.*, 1974).

COL & plant cells

In 1937, Nebel and Ruttle began a study on induced polyploidy under the action of COL. They studied various plants including *Tradescantia reflexa*, marigold varieties (Gold Guinea, Dwarf, Crown of Gold), tomato varieties (John Baer, Oxheart and Rutgers Crop), snapdragon, white clover, pinks, poppy, and petunia. Their observation was that COL acts on every prematapha sic cell division in the stamen hair of *T. reflexa*

kept in culture solution to which the drug was added. This meant that whenever a cell was under the influence of COL while in early, mid-, or later prophase, its nucleus became didiploid after metaphase (Nebel and Ruttle, 1937). In this, COL differed markedly from all other agents used to induce polyploidy. They used a series of concentration experiments on stamen hair cells kept in culture solution for 14 hours. The concentrations they used were 10^{-1}, 8 x 10^{-2}; 6 x 10^{-2}; 4 x 10^{-2}; 2 x 10^{-2}; 8 x 10^{-3}; 8 x 10^{-4}; 4 x 10^{-4}; 2 x 10^{-4} molar (Nebel and Ruttle, 1937). No essential difference in effect was found within this extremely wide concentration range. Likewise, when these concentrations were observed over 24 hours, still no essential difference was seen.

Thus, they suggested that once a certain concentration is reached, COL destroys some link in the spindle-forming reaction. Another observation was that cyclosis or streaming of protoplasm continued as in the control even when the concentrations of COL were far above that necessary to inhibit anaphase (Nebel and Ruttle, 1937). Also, the effect of COL on nuclear divisions in stamen hair cells was not easily reversible whether they were immersed in culture solution for 1 hour and transferred to a pure culture solution or immersed in culture solution for 24 hours given that the effective concentrations of the drug were used (Nebel and Ruttle, 1937). Similar experiments carried out on the other plants by other groups. A study on inseminated eggs of *Arbacia punctuala* in 1937 showed that with the increasing concentration of COL above 10^{-5} x 2.8 M prevented cleavage, astral rays did not form, spindle reduced in size and finally obliterated (Nebel and Ruttle, 1937). Pseudo-division of nucleus continued. Prolonged periods resembling metaphase alternated with periods in which the chromosomes became vesiculated, forming abnormal resting nuclei. In the later experiments, the shoots of marigold varieties, tomato varieties, snapdragon, white clover, pinks, poppy, and petunia were immersed in 0.02 to 0.4 percent aqueous solution of COL from 1 to 24 hours or a thin film of 1 percent COL in lanolin was applied on the growing points (Nebel and Ruttle, 1937). The seedlings showed marked stunting and distinct thickening of hypocotyl. Many

seedlings failed to form shoots and died. If the injury was not too great, the main shoot continued development after some delay or often a cluster of shoots formed above the cotyledons of which some developed into main shoots. New leaves were thickened, ridged, sometimes fascinated and often had irregularly chewed-appearing leaf margins. As the leaves developed, chlorophyll chimeras were frequent in certain genera. Later, smooth normal-appearing leaved appeared and growth progressed as usual, or certain branches or even whole plants showed the characteristics of polyploidy typical of the species (Nebel and Ruttle, 1937).

The cytological examination of the meristematic areas of seedlings treated with 0.2 percent COL for hours showed clumped metaphases after fixation after 4 hours but there were no cells with chromosome number more than normal (Nebel and Ruttle, 1937). Those fixed 40 hours after the treatment showed irregularly shaped resting nuclei, giant cells with one large nucleus having 2 or more nucleoli, numerous clumped metaphases, restitution metaphases, two metaphases side by side in one cell, and cells in metaphase containing the tetraploid or, less often, apparently the octoploid chromosome number (Nebel and Ruttle, 1937). Some degeneration of chromosomes was also evident in certain cells.

COL & plant growth

In order to be able to compare the effect of COL on the animal organism with the influence of the poison on plants, Havas (1937 a), at the suggestion of Dustin, germinated *Triticum* in COL solution. As an influence of the COL on the growth of the wheat seedlings, Havas found a growth-promoting or growth-inhibiting effect, depending on the concentration, and he also noted a club-like hypertrophy on the roots and on the coleoptile. These experiments only gave a first orientation, while Dustin, Havas and Lits (1937) treat this new field in more detail. Eigsti (1938), inspired by the results of research on the influence of COL on animal cells, started investigations with COL on plants in 1937. He was mainly concerned with the cytological effect of COL on plant

cells, while the fundamental investigations of Blakeslee (1937) and especially Blakeslee and Avery (1937), the researchers he drew attention to the COL effect, attracted the attention of geneticists. The two researchers showed the possibility and the way to experimentally produce tetraploid plants by colchicination. Independently of the latter researchers, Nebel (1937) and Nebel and Ruttle (1938) also carried out studies with COL on various plants. Much of the subsequent work has been in the field of plant breeding, which has found in COL a reliable and largely useful agent for the production of polyploid crops.

COL & mitoses in plants

In cells under the influence of COL, the prophase of mitosis proceeds normally, the chromosomes differentiate, and the nuclear membrane disappears. The chromosomes are randomly scattered in the cell instead of being arranged in the equatorial plate. The chromosomes divide into chromatids. In the normal mitoses, at the beginning of the anaphasic movement, the attachment sites move polewards. In the abnormal mitoses, after treatment with COL, the mechanism that brings the individual chromatids apart is abolished. The effect of COL first inhibits the formation of the spindle figure and at the same time delays the division of the set site but does not affect the ability of the chromosomes to divide. After some time, a resting nucleus is reconstructed again. The restitution nucleus is tetraploid because each chromosome has previously divided into two chromatids. One finds a large restitution nucleus or two nuclei in a cell, which then often merge to form a tetraploid nucleus. The restitution nucleus or nuclei of the tetraploid cells remain dormant for a long time, during which the cell size increases considerably. With continued influence of the COL, such a cell can even repeatedly proceed to internal division, whereby the number of chromosomes is multiplied. The chromosome division always takes place normally, but the formation of the spindle fibers and the migration of the chromosome halves to the poles is always omitted. If such cells are removed from the influence of COL by placing them in pure water for a longer period, the subsequent mitoses proceed normally again.

The COL roots are characteristically thickened (swollen), especially in the part above the meristematic zone. The cause of the thickening is an increase in the volume of the cells. The meristematic cells are multinucleated, some of them have irregularly shaped restitutive nuclei, with lobes. Depending on the number of abnormal divisions in a tissue, nuclei of unequal size and thus cells of unequal size are produced. These cells with large or small nuclei contain many nucleoli of different sizes. In the following division in COL-free medium, the chromatin filaments are normally developed in the prophase nucleus.

In metaphase, the chromosomes have doubled and arrange themselves in the equatorial plate, accompanied by the spindle fibers. Now that the spindle is normal, in anaphase we see that the daughter chromosomes separate and migrate to the opposite poles. In this way, the nuclei, once abnormally divided, undergo normal mitosis again.

Since COL acts on the division itself and on cells that are ready to divide, meristems (root tips, vegetation points, germinating seeds) that are in lively division are particularly suitable for studying the COL effect.

Strong differences in the effectiveness of COL on individual plant species have already been noted by Nebel and Ruttle (1938). Attempts are made to explain these differences by a different behavior of the plasma of the cells concerned. Mostly, permeability differences of the plasma towards COL are held responsible for this. Experimental investigations have not been undertaken in this respect, since up to now mainly geneticists and karyologists have dealt with the question of COL effects and cytological investigations have in most cases only been carried out on fixed material.

Of the few botanical objects that allow live observation of nuclear division processes, only the stamen hairs of *Tradescantia* have been used for the observation of COL mites, first by Nebel and Ruttle (1938) and most extensively by Wada (1940). Nebel and Ruttle (1938) described the morphological changes

in the division of cells and came to the generally accepted conclusion that the toxin inhibits spindle formation. The realization of the material and morphological interrelationship of all division elements led Wada (1940) to study the effect of COL on the other division elements in living material. He was able to determine the speed of penetration into the cell, as well as the concentration limit of the poison on the living object. Furthermore, he investigated the special effect of the alkaloid on the individual division stages and the viability of the cells in different COL solutions.

Whereas up to now all researchers have regarded the morphological-physiological deviations in the appearance of colchicinated plants exclusively as a consequence of the doubling of the chromosome number, Levan (1942.a) has established that hypertrophy is completely independent of the doubling of the nuclei. Levan applied COL to X-ray irradiated Allium roots, and after a few days the roots showed the typical thickening of the nuclei, although no nucleus divisions took place in the cells concerned as a result of the strong X-ray irradiation. These experimental results prove that the formation of the characteristic COL lobes, i.e., the transversal cell elongation, occurs completely independently of the disturbance in the mitosis process.

Research in the field of biological COL effects has had the rare good fortune to have achieved an early success which must have captivated theorists and practitioners alike. The possibility of increasing the chromosome population by colchicination and producing tetraploid plants valuable for breeding has aroused justified interest, and the new path to polyploidy has since been followed by more and more researchers every year. It is understandable that the other results, which were already obtained in the first relevant studies, have received much less attention. Efforts to elucidate the nature of the COL effect also refer almost exclusively to the effect of COL as a mitotic poison: the inhibition of spindle formation and thus of the normal nuclear division mechanism was recognized partly in fixed material, but also partly through live observation of COL mitoses, and the reason for this was assumed to be physicochemical changes in the state of the nuclear divi-

sion figure. Thus, the opinion could arise, and has probably already arisen, that at least the basic features of the picture of the COL effect have already been correctly drawn. Boas was one of those to whom the effect of COL appears to be much more diverse; he pointed out, among other things, the already known morphological changes of COL-infused seedlings, especially the formation of the so-called club roots (COL tumors) as well as the spherical coleoptiles of the gramineae.

COL, plants, polyploidization & clubroots

The formation of clubroots was initially regarded as a secondary effect that occurs because of the polyploidization of the cells. However, later experiments by Levan (1942a) have shown that clubroots also appear during COL treatment when mitoses have been completely prevented by previous X-ray irradiation. The formation of clubroots with all the associated histological changes (Bhaduri 1939) was recognized as a COL effect independent of polyploidization, at least in so far as endomitotic processes may be regarded as excluded. Incidentally, this insight could have been derived earlier by analogy from the formation of spherical coleoptiles.

The growth of the gramineous coleoptiles is a pure elongation growth in which nuclear divisions play no role. The formation of the spherical coleoptiles triggered by COL can therefore not be the result of a mitotic disorder, it must rather be caused by influencing the "resting" cell. The disturbance of the septum formation in Cladophora is to be understood in the same sense; this effect also occurs independently of the mitosis process. The same applies to the formation of clubroot hairs; here, too, it is clearly a matter of cells undergoing elongation and not division growth. Finally, an influence of COL on the cytoplasm of resting cells is readily apparent from the results of the plasmolysis and time experiments, which reveal a change of state of the protoplast, especially of the outer boundary layer.

The COL changes the cytoplasm in a way that is not yet known. Depending on the state the cell is in, this change has very different effects. There are cell states in which the immediate impression of the cytoplasm by the COL does not result

in any visible consequences for the exercise of cell functions; we then get the picture of a colchicine-insensitive, resistant cell. However, there are also episodes in cell life in which the change of state of the protoplast triggered by COL results in profound disturbances of the cell processes just taking place. As far as is known, these colchicine-sensitive stages of the cell include mitosis readiness, mitosis itself, primarily the metaphase, and the cell elongation following mitosis. Outside these phases of cell life, changes are also triggered by COL, but they apparently have so little effect that they can only be detected by sensitive methods such as the plasmolysis time method.

That the cell, when it enters the critical phase of its life, mitosis, becomes particularly sensitive to external influences of various kinds, has been known for a long time (Fauré-Fremiet, 1925). Sensitivity to COL is not at all an isolated phenomenon in this respect; sensitivity to narcotics, to alkaline substances, to radiation of various kinds is also altered.

The effect of colchicine is thus, as already mentioned, particularly expressed when it affects cells that are in the pre-mitotic, mitotic, or post-mitotic stage of their life. Thus, the main sphere of influence of colchicine can be quite simply delimited and specified: Colchicine acts primarily on growing cells, regardless of whether this involves plasma growth or elongation growth. Colchicine is not unique in this respect, we know the same about the phytohormone auxin, it is not only a hormone of elongation growth, as was originally believed, but it can also influence division growth in a positive or negative sense. It is the same with colchicine. It not only interferes with division growth - as the name "mitotic poison" might suggest - but even more striking is its morphogenetic effect, which it achieves by changing the direction of elongation growth.

The similarity to a hormonal effect has already been noticed by many researchers and colchicine has repeatedly been referred to directly as a "phytohormone".

Mairold (1943) found that very similar looking clubs were obtained on lupins by Cholodny (1931) by putting coleoptile

tips on the roots. According to Cholodny (1931), it is the thereby mediated oversupply of auxin that leads to local swelling of the root in the elongation zone. Using heteroauxin, Czaja (1935) obtained clubroots in *Zea*. "Swelling" of the hypocotyl was obtained by Czaja (1935) and in repetition of the experiments by Schlenker (1937) on *Helianthus* seedlings. In all these and other cases, the effect of the "hormones" manifests itself in exactly the same way as with colchicine in an inhibition of elongation growth in the longitudinal direction of the organ and a promotion in the transverse direction. Habitually, the club roots look largely the same whether they are induced by auxin, heteroauxin or colchicine. Levan, who cytologically examined the colchicine tumors in 1938, was prompted by the "striking resemblance" of the hormone tumors to also study them in detail from a cytological point of view. The resultant finding that in hormone tumors the all-round cell enlargement is primary, but the inner division leading to polyploidy is secondary, while in colchicine tumors the conditions seem to be just the opposite, initially led Levan to the assumption that there was a fundamental difference between the mode of action of colchicine and that of the growth substances. However, since Levan himself later (1942a) found that colchicine tumors can also occur without previous polyploidization, and since Wada (1940) also suspects internal divisions (endomitotic processes) in colchicinated material, it is probably no longer possible to construct significant differences between the effect of colchicine and that of growth substances with regard to the culling effect.

Finally, it should be mentioned that, as far as polyploidization is concerned, this has been achieved by growth substances not only on roots (Levan 1939) but also on shoots (Greenleaf, 1937). So even the effect that is particularly characteristic of colchicine, the doubling of the chromosome set, can also be achieved by heteroauxin. Further literature on the polyploidising effect of phytohormones has been compiled by Bauch (1942). With regard to the swellings (tumors) caused by the growth promoter on the one hand and by colchicine on the other, the following should be emphasized: Jakes (1938) has achieved local distension of the lowest part of the hypocotyl in seedlings of *Raphanus sativus* by heteroauxin.

This corresponds to the normal tuber shape of the radish, but with heteroauxin treatment it is formed very early, namely already in the cotyledon stage. Heteroauxin thus also causes an enlargement of the cells in the transverse direction (primary thickness growth) on this object. This growth in thickness is strictly local; this is attributed to the fact that it takes place in the same zone that normally appears to be predestined for primary growth in radishes. In Jakes' further statements it is striking that the formation of the beet-like thickenings on the radish seedlings is more strongly promoted by heteroauxination at low temperature (16 degrees centigrade) than at higher temperature (30 degrees centigrade), whereas the effectiveness of colchicine is increased not only on animals but also on plants by increasing the temperature. However, it would not be appropriate to deduce from this a deeper difference in efficacy; the fact that in *Raphanus* the formation of heteroauxin nodules is promoted by lowering the temperature can be a correlatively conditioned, secondary phenomenon.

It is by no means only roots that are induced by colchicine to grow primarily thicker; very often the hypocotyl or epicotyl also take on a bloated habit. The same applies to the reaction of various plant parts to treatment with heteroauxin. Weißenböck and Stern (1939) were able to induce swellings in any part of the stem by injecting growth promoter solutions (heteroauxin) "depending on the location of the injection site", which are caused by an enlargement of the bark and pith cells. Jost and Reiss (1937) had earlier obtained clubbing on decapitated hypocotyls of *Phaeseolus* by the growth paste method. Thus, colchicine and heteroauxin cause swelling (tumor, i.e., primary thickness growth) in quite the same way on the most diverse organs capable of extension growth, colchicine seems to be even more effective in this respect insofar as it also allows this success to be achieved on coleoptiles, where "never even a hint of thickening" can be achieved with heteroauxin (Jost and Reiss 1937). The failure of heteroauxin on the columella has been associated with the fact that the normal development of this organ also does not result in any growth of thickness. However, the spherical coleoptile that occurs during colchicination shows that the ability to do so is also present in the coleoptile.

Epinastic leaf curvature can also be caused by heteroauxin and other "growth substances" (Schlenker 1937) as well as by colchicine (Havas 1937 b).

Jost (1942) was able to promote the formation of so-called vascular bridges by using heteroauxin paste. These bridges are formed when differentiated parenchyma cells are transformed into vascular elements and thus create a bridge connection between vessel ends that have been separated by a wound. An at least premature but probably also increased transformation of thin-walled cells into vascular elements with a typically thickened membrane structure is the characteristic histological feature of colchicine clubroot (Eigsti 1938). Bhaduri (1939) says the following about it: Colchicine treatment seems to accelerate the differentiation of vessels and sieve tubes; this differentiation not only occurs earlier, but the zone in which it occurs extends further towards the root tip, Bhaduri (1939) adds: "It appears from figures of Levan (1939) that premature differentiation of vascular tissue also takes place in roots treated with different growth substances".

Numerous researchers have shown that "rhizokalin" and other phytohormones, especially heteroauxin, stimulate root formation. According to Laibach and Fischnich (1935) this root formation is related to a stagnation of the growth substance. Havas (1937) promoted the formation of adventitious roots on *Impatiens balsaminea* stems by colchicine paste. According to his own observations, even the formation of normal lateral roots seems to be favored by colchicine and Jost (1942) thinks that lateral roots can only develop after accumulation of growth substance.

Finally, the tissue rupture caused by excessive transverse growth and turgor increase should be mentioned, which can be caused by ethylene (the laboratory air) and also by colchicine.

The many analogies in the action of colchicine on the one hand and heteroauxin and other substances called "growth substances" on the other prompted Havas (1938) to raise the question: "Is colchicine a phytohormone?" Since the pea test

method tested by Went (1934) for auxin gave negative results for colchicine, Havas was deterred from taking an unequivocal affirmative position on the above question. On the other hand, colchicine has also been regarded as a growth antagonist, since according to Havas (1937 b, c) it has an inhibiting effect on the growth of plant tumors as well as on the straight growth of decapitated coleoptiles (Avery and Sargent 1939). According to V. Guttenberg (1942), heteroauxin is not a growth substance but a growth activator. It "only results in increased auxin production and this is responsible for all further processes". It is quite probable that what v. Guttenberg has shown so convincingly for heteroauxin also applies to colchicine; on the path that v. Guttenberg has taken to prove his point, the validity of this assumption could be tested without any particular difficulty. Guttenberg also considers it possible that "heteroauxin also activates growth substance in the plant under natural circumstances. For Lefebre (you did not mention its year) has recently detected beta-indolylacetic acid in plants which show particularly conspicuous parenchyma growth, such as radishes, cauliflowers and others, and Linser has found unusually high amounts of growth substance in similar objects". Perhaps COL can also be considered as a natural activator of growth substances in some plants. According to Klein and Pollauf (1929), *Hemerocallis fulva* contains colchicine. The so-called storage roots of this plant are similar in their club-shaped form to the club roots into which the usual adhesive roots of *Hemerocallis* can be transformed when colchicine is added.

Of course, the assumption that colchicine is a growth substance activator does not satisfactorily solve all questions, since it is only a transfer of problems from the field of colchicine research to the already heavily burdened field of growth substance research; but perhaps a lot can be gained by inserting the more specific questions into the more general framework. For example, the transformation of longitudinal elongation growth by colchicine into transverse growth would be a special case, the "transverse reactions" of plants, for which Borgström (1939) knows how to cite numerous examples in the further development of the views of Czaja (1935) and Havas (1937 b).

The colchicine effect has also been understood as a disturbance of the normal polarity, whether it is the polarity of an organ such as the root or of a cell that is in the process of division and in which the daughter chromosomes are unable to migrate to the spindle poles. In all cases, says Walker (1938 b), the effect of colchicine is the same: "an alteration of the normal polarity of the cell".

Whether the transverse elongation is really caused by a redirection of the growth substance (transverse elongation), or by a mere accumulation, increase in concentration of the hormone, remains a special question which cannot be dealt with here; the increase of individual cells such as pollen tubes, root hairs, stamen hairs speak rather for the latter possibility (Mairold, 1943).

V. Guttenberg (1942) did not comment on the way in which heteroauxin causes the activation of auxin, and it is not possible to form a more precise idea of this even today. "It is not strange for the physiologist that one and the same effect, e.g., the triggering of cell elongation, can be caused by quite different substances. We often find such phenomena; they are understandable precisely when catalytic causes are involved. We constantly come across such cases in stimulus physiology". Bünning and Jost (1937) (this reference is not available in the translated file of Mairold's paper) agree with Fitting (1936), who interpreted phytohormones as "stimulants". What applies to the phytohormones, however, applies no less to their activators; they, too, are to be understood as triggers that trigger whole chains of reactions in the living protoplasm and determine the direction of this process.

If, taking into account the macroscopically visible growth disturbances (clubbing, etc.) that colchicine triggers, its effects are seen as those of a catalyst, Bhaduri (1939) had already come to the same conclusion earlier on the basis of his study of the microscopically visible cytological colchicine effects. He too starts from the striking fact that analogous mitotic disturbances and the polyploidisation triggered by them can be triggered by a number of quite different agents as well as

by manifold physical influences such as cold, heat, centrifugation, X-rays, injuries. In addition, there are abnormal mitoses, which according to Milovidov (1938) are achieved by desiccation (withering). According to Bhaduri (1939), the essence of colchicination is "to bring about specific chemical reactions as a result of which very specific physical changes in the state of the protoplasm take place. The components involved in this chemical reaction are always present in the cell, but probably cannot react with each other under normal conditions. Colchicine seems to act as a catalyser of this reaction". Bhaduri further points out that other agents can cause the same reactions but with different degrees of effectiveness, as is generally known for catalytic reactions. It is therefore not at all surprising that in mitosis, too, quite different chemical and physical influences result in the same effect.

What Bhaduri says about mitosis can also apply to the formation of transverse walls according to his own observations of algal cells. Not only is it disturbed by colchicine and the formation of incomplete and irregularly shaped transverse walls is triggered, but also by narcotics, cold and centrifugation. In all its effects, in the inhibition of spindle formation, in polyploidisation, in abnormal transverse wall formation, in the suppression of elongation growth in the longitudinal direction and the promotion of primary thickness growth, in the promotion of root formation, in the causation of tissue ruptures, the stimulus of colchicine can also be represented more or less equally well by other triggers. Depending on its current state of life, the life path of the protoplasm is deflected by different occasions in a similar direction via unfamiliar chains of reactions.

As with the stimulus processes, so also with the colchicine effect, the question comes to the fore as to which is the primary and which are the further links in these aberrant reaction chains. In recent decades it has become fashionable to look for and find the primary effect of external influences, the first invisible precursor of the visible stimulus reaction, in a change in the viscosity of the protoplasm. This custom is partly due to external factors, but it is also partly justified. The external reason is that viscosity is the physical property that can

be determined most easily and reliably on living protoplasm by several methods. However, research into the viscosity of protoplasm is justified by the fact that internal friction is "the ideal indicator of internal changes of state". If it is to be shown whether changes occur in the living substrate under certain interventions, viscosity determinations are certainly appropriate. However, it must not and should not be forgotten: "Today, an unambiguous conclusion cannot yet be drawn from changes in viscosity to the nature of the changes in state revealed by them" (Weber 1917). Since rhythmic viscosity changes play a role in normal mitosis and colchicine causes an unusual reduction in the viscosity of the atractoplasm or cytoplasm in dividing cells according to various authors, it was obvious to see this viscosity influence not only as a symptom of the colchicine-induced changes in the state of the protoplasm, but as the primary effect itself, which is responsible for the disturbance of the mitosis process.

Important in this question is the finding of Wilbur (1940) that the viscosity of the non-fertilised, i.e., non-dividing Arbacia egg is not influenced by colchicine. "That is, colchicine seems to have no primary viscosity effect on protoplasm". Rather, Wilbur imagines that certain chemical processes occurring during mitosis, which normally lead to an increase in the viscosity of the protoplasm, are inhibited by colchicine. However, the biochemical effects of colchicine are too incompletely known to be able to say anything about the nature of the inhibition, "but perhaps enzymatic changes are involved".
It is very possible that the link in the chain of reactions that is of particular importance for the disruption of the normal mitosis process is that which is based on a change in viscosity during colchicination, whereas for other colchicine effects, changes in viscosity may be of no significant importance. The colchicine effects are too diverse to be understood by a single transformation in the complicated reaction mechanism of the protoplasm (Mairold, 1943).

COL & volvocales
Shyam and Sarma, (1974) found that the effects of COL have been extensively studied on several higher plants, and in

recent years to some extent on algae. But no reports seem to exist concerning COL effects on any member of Volvocales. Therefore, they conducted a first-of-its-kind study on one of the members of colonial Volvocales (Chlorophyceae), *Gonium pectoral* and studied the effects of COL on their cell division. They found that 0.5 percent of COL did not produce any effects, but 1 percent concentration caused contraction of chromosomes, accumulation of metaphase plates, polyploid nuclei, multinucleate cells, and cell enlargement (Shyam and Sarma, 1974). The most remarkable effect at this concentration was the increase in chromosome number (partial doubling, diploidy, and/or tetraploidy). The affected cells generally did not to divide further instead, they died. Shyam and Sharma (1974) noted that COL did not affect all cells because normal cells showing normal chromosome number were also observed in the treated colony.

Anti-tubular agents & collagen

In a study by Ehrlich *et al.*, (1974) embryonic chick cranial bone was cultured in the presence of the antimicrotubular agents - COL and vinblastine - and with several other compounds known to affect the cellular handling of collagen. Collagen exists within cells as a higher molecular weight precursor, procollagen, which contains additional sequences at the NH2-termini of all three chains of the macromolecule. The subcellular mechanisms involved in the transport of procollagen from its site of synthesis on the rough endoplasmic reticulum (RER) to the extracellular space are not well understood. Thus, in contrast to the process in exocrine glands, the role of the Golgi complex in the secretion of collagen and other connective tissue proteins has not been firmly established (Ehrlich *et al.*, 1974). There is evidence that secretion of a fraction of collagen by fibroblasts may be achieved by bypassing the Golgi complex, possibly by intermittent communication via vesicles or direct communication of the cisternal space of the RER with the exterior of the cell. Communication between cisternal elements or vesicles and the extracellular space may be dependent on the ability of microtubules to assist in the translocation of these structures to positions subjacent to the plasma membrane. COL and vinblastine were found

to inhibit secretion of procollagen by fibroblasts, and these compounds, as well as other antimicrotubular agents, retarded the conversion of procollagen to collagen by cranial bones in culture (Ehrlich *et al.*, 1974). Secretion of procollagen, quantitated by light microscope autoradiography, was correlated with the extent of conversion of procollagen to collagen and with rates of collagen and noncollagen-protein synthesis. COL inhibited procollagen secretion and conversion to collagen and specifically inhibited collagen synthesis. Cells exposed to COL revealed an increased number of dilated Golgi-associated vacuoles and vesicles, some of which contained parallel aggregates of filamentous structures (Ehrlich *et al.*, 1974).

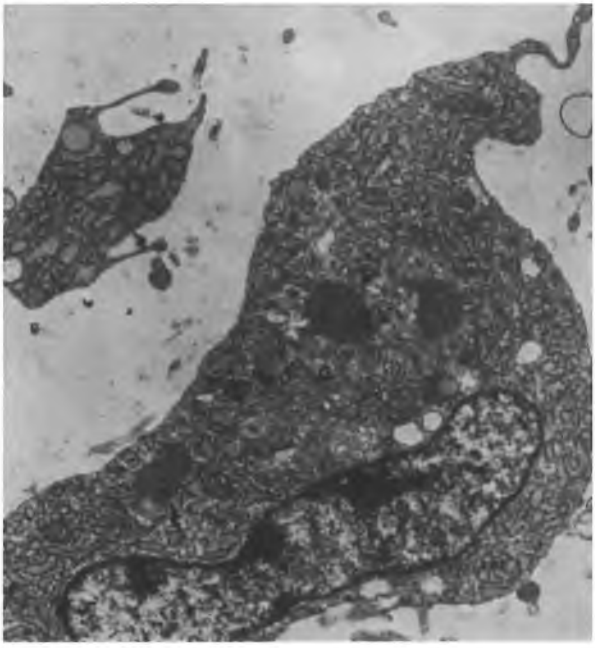

FIGURE 4: *This electron micrograph represents a periostal fibroblast after treatment with VBLS. Several membraneous aggregates (arrows) can be seen. In addition, the cisternae of the rough endoplasmic reticulum appear to be vacuolar and to have lost their usual three-dimensional canalicular appearance. The Golgi complex (G) appears unaltered in the micrograph x 14.000 (from Ehrlich).*

Their observations suggest that the pathway of at least a fraction of procollagen secretion by osteoblasts includes the Golgi complex. Disruption of microtubules may interfere with the movement of Golgi-derived vesicles, and the resulting accumulation of collagen precursors in the Golgi complex may lead secondarily to an inhibition of synthesis (Ehrlich *et al.*, 1974). Although vinblastine also inhibited both procollagen secretion and conversion to collagen, the observed reduction in general protein synthesis and striking changes in the ultrastructure of the rough endoplasmic reticulum complicated interpretation of the effects.

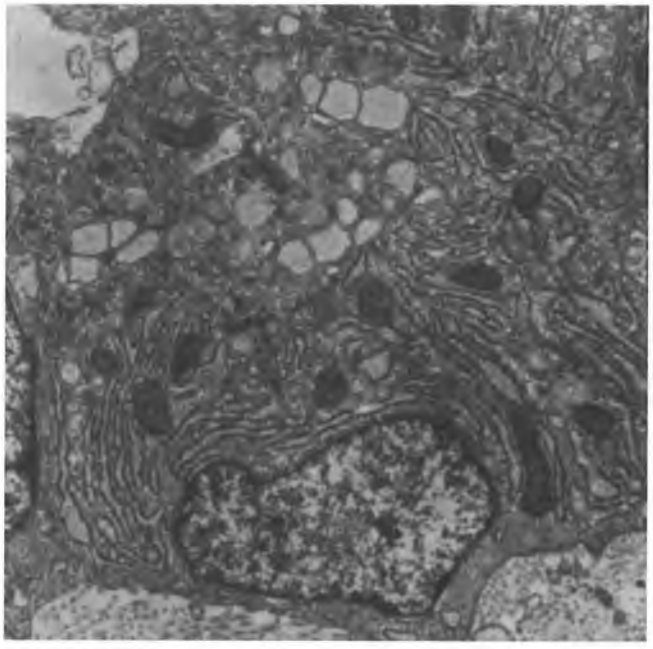

FIGURE 5:
This a relatively low power electron micrograph of several osteoblasts in bone treated with COL. The marked enlargement of Golgi-associated vacuoles can be seen. The rough endoplasmic reticulum in this cell is not altered in its appearance. In a number of the Golgi vacuoles filamentous structures are seen. The appearance of increased numbers of large Golgi-associated vacuoles was the most striking finding after COL treatment, x 14,0000, from Ehrlich.

Interpretation of the effects of cytochalasin B was limited because the cellular response in cranial bone was markedly heterogeneous and the drug caused an inhibition in the incorporation of radiolabeled amino acids into both collagen and noncollagen protein (Ehrlich *et al.*, 1974).

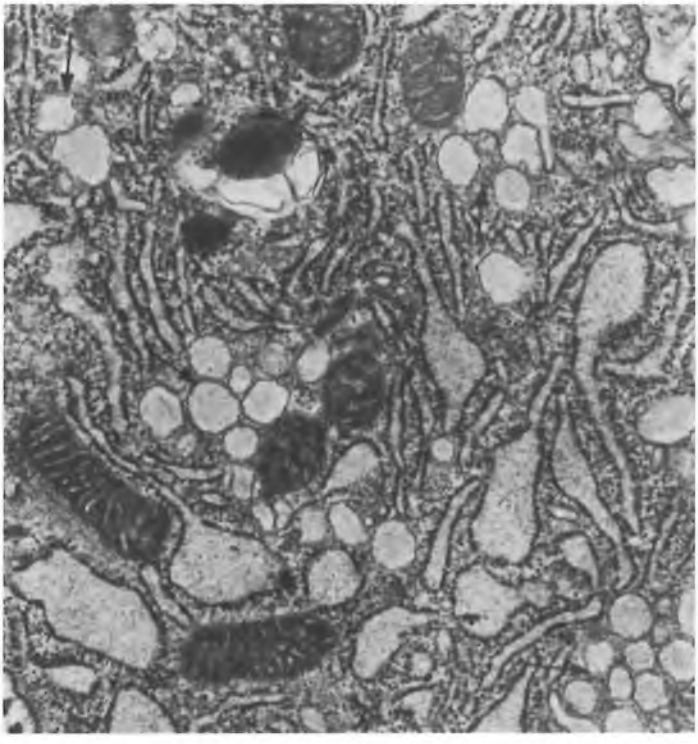

FIGURE 6: *This electron micrograph demonstrates part of osteoblasts from COL treated one. The characteristic appearance of increased numbers of enlarged Golgi-associated vacuoles can be seen. There are at least four foci of enlarged Golgi vacuoles (arrows) present. The rough endoplasmic reticulum of this cell demonstrates the usual three-dimensional canalicular array characteristic of this organelle, x 29,000, from Ehrlich.*

The effects of vinblastine on cranial bone cells at the concentrations used in their study are complex, as indicated by an inhibition of both collagen and noncollagen-protein synthesis.

6. Chapter: Colchicine and mitosis

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Summary: Biochemistry of COL
COL is an alkaloid compound first isolated by Zeisel in 1882 from the plant *colchicum autumnale*. For a long time, the chemical structure was based on speculations, due to the complex spatial formation of several organic ring structures. The data collected by Sharma and Sharma (1980) implies that: (a) at least one methoxy group in ring A is necessary for colchicine action; (b) ring C must be 7-membered, and the hydroxyl group should preferably be replaced by an amino group; (c) esterification of amino group in ring B increases the activity; and (d) isocolchicine and its derivatives are less active. A proper distance should be maintained between esterified side chains of rings B and C. This last statement is based on the fact that in isocolchicine, where the position of the methoxy group is reversed in ring C, the decrease in efficacy has been considered to be due to the presence of hydrogen bonds between the side chains of rings B and C (Sharma and Sharma, 1980). In the case of "colchicine", the weak action is the result of the iso-form of this molecule. Though these data indicate the reactive groups responsible for colchicine action, the exact reaction involved in spindle inactivation is not yet fully clear.

Effect of COL on mitosis
Brues (1936) found that colchicine, produces arrest in metaphase of a maximum number of mitoses when injected subcutaneously in aqueous or oily solutions but these results are obtained only within certain limits of dosage. Larger doses prevent cells from entering mitosis, but it has only partial effects in a smaller dosage. Colchicine, octahydrocolchicine, N-acetylcolchinol and four derivatives of the latter all produce similar effects to colchicine, but in a considerably higher dosage (Brues, 1936). Dimethyl- and trimethyl-colchicinic acids are ineffective in any sublethal or lethal dosage. In another study Deysson (1944) found that colchicine (0.005%) applied to *Allium* root tips for 46 hours increased

the percentage of tropokineses, which is first sign of spindle disturbance.

Effect of COL on mitosis

Eigsti and Winn (1949) mentioned that after repeated injection in mammals, plentiful mitotic figures were occasionally observed in smears of bone marrow. They studied the karyokineses of the epithelial and endothelial cells in the mucosa of stomach and intestine, in experimental gastroenteritis following colchicum poisoning in the gastrointestinal mitoses in two dogs given, respectively, 10 and 15 g tincture of bulbs of colchicum, and dying 24 and 48 hours later (Eigsti and Winn, 1949). They found an extraordinary great number of dividing cells in the stomach. Furthermore, within the Lieberkühn glands of the intestine nearly all cells were engaged in indirect division. Endothelial mitoses could be seen nearly in all vessels (Eigsti and Winn, 1949). They also noticed that some of the mitotic figures apparently underwent destruction which they speculated was a necrosis of the cells, giving rise to some sort of pseudo-karyokinetic forms. Benitez *et al.*, (1954) selected the mammalian fibroblast as specimen and colchicine as a mitotic inhibitor whose effects are clear-cut study the mode of action of mitosis poisons. They observed the morphology of controls and colchicine-treated tissue from 4 to 72 hours and established numerical relationships between resting and dividing cells. In treated fibroblast cultures from young male rats, the peak of metaphasic accumulation was reached after 20 hours and this level remained steady for a short time and thereafter declined. They also investigated other agents, chemically related to colchicine, or reputed to be similar in effect. Among these, tropolone, tropolone methyl-ether, 4, 5-tetramethylene tropolone (simple cycloheptene compounds), and gammexane and protoanemonin produced no significant metaphase arrest in this biological system while aminopterin appeared to have a transient effect (Benitez *et al.*, 1954). Another aspect they studied was the reversal or antagonism of the colchicine effect by other compounds. Three agents, widely different in chemical structure, were found to diminish the accumulation of mitoses greatly when administered simultaneously with colchicine. Mesoinositol performs this function very specifically, which means that under similar conditions, related compounds such as muco-inositol, inositol lipide, dextro-inositol, epi-inosose, meso-inosose, and common sugars have no such effect (Benitez *et al.*, 1954). Adenosine

triphosphate (ATP) significantly reduced the mitotic coefficient, when employed with colchicine, at five times the lowest concentration for shortening resting-fibroblast models, but it does not alter the percentage of metaphases arrested (Benitez *et al.*, 1954). The most potent antagonist of colchicine was tropolone, one of its simpler structural analogs, which not only produced a spectacular decline in the percentage of metaphases but, when administered alone, shifted the phase percentages in a direction opposite to that induced by colchicine (Benitez *et al.*, 1954). These findings cast doubt on the probability that any single principle will provide an explanation of the mitotic mechanism.

Effect of COL on mitosis

Benitez *et al.*, (1954) used a time-honored approach to investigate the mechanism of mitosis from the standpoints of cell physiology and of cell chemistry by using diversified chemical agents designated (because of this action) as mitotic poisons. More recent investigations have included compounds which will inhibit or reverse the effects of the mitosis inhibitors. They selected the adult rat fibroblast (from subcutaneous areolar tissue) as physiological material, and colchicine as a mitosis inhibitor whose action as a spindle poison, producing prolonged metaphase arrest, is clear-cut. Benitez *et al.*, (1954) postulated a variety of routes by which a mitotic poison might operate chemically, such as by preventing the production or utilization of an essential metabolite, by interfering with one or more enzymes necessary for the completion of a metabolic process, or indirectly, by depriving the cell of an energy source necessary for one of these processes. The route or routes by which colchicine produces its effects are still unknown, and the methods in which these three agents antagonize them are correspondingly open to conjecture. The results of their experiments, however, carried out in a quantitative manner, with a variety of agents and under comparable conditions in the same controlled and standardized biological system, emphasize the dangerous inadequacy of a monistic explanation of mitotic mechanics.

Effect of COL on mitosis

Robbins and Gonatas (1964) found that the mammalian cells of the HeLa (S3) strain, when exposed to spindle inhibitors, undergo several morphological transformations during interphase as well as during mitosis. During their histochemical and ultrastruc-

tural study, they found that the lysosomes, represented by the multivesicular bodies in HeLa cells, form clusters and become circumferentially disposed instead of occupying the polarized juxtanuclear position characteristic of these organelles (Robbins and Gonatas, 1964). Under the electron microscope they showed to have acquired a dense osmiophilic core that is separated from the bounding unit membrane by an electron lucent halo.

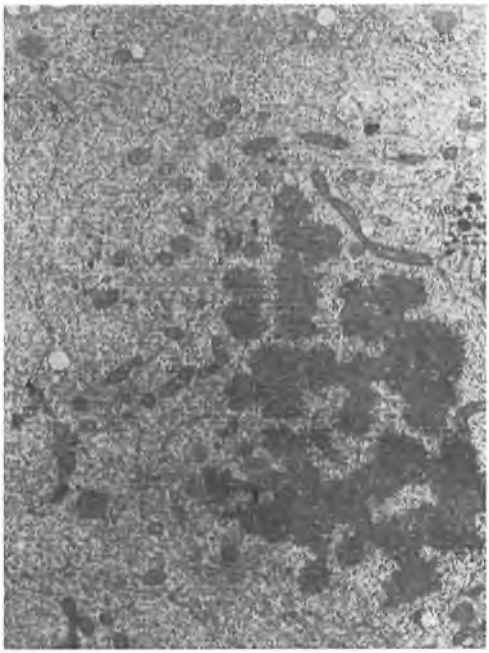


FIGURE 1: *Part of COL treatment of fibroblasts treated in metaphase. Moderate osmiophilic spherical bodies and cisternae of the endoplasmic reticulum, the latter being in close proximity to the fused chromosomes (M= mitochondrium, Chr = chromosome, SB = spherical bodies; ER = endoplasmic reticulum, x 15.000) Courtesy of The Rockefeller University Press.*

The Golgi apparatus fragments under the influence of spindle inhibitors and takes up a circumferential distribution in a pattern similar to that of the lysosomes (Robbins and Gonatas, 1964). On the ultrastructural level, no significant modifications in this organelle were observed by them. In addition, they also noted numerous 60-80 angstroms fibrils in the interphase cell, coursing

through the cytoplasm as well as a paucity of spindle microtubules. Also, a striking similarity was seen between the behavior of the lysosomes in the drug-treated interphase cell and the untreated, normal mitotic cell (Robbins and Gonatas, 1964).

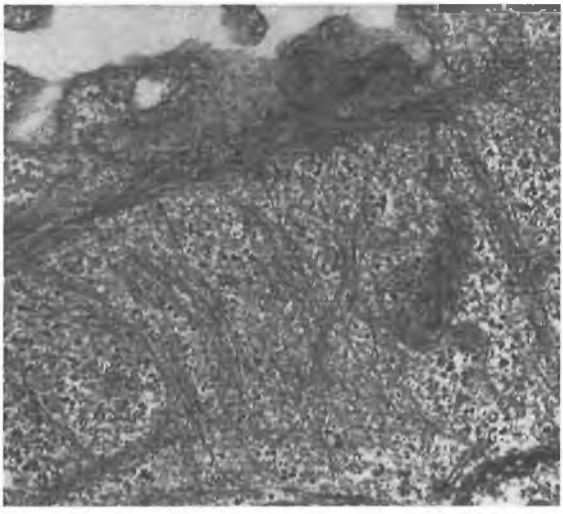


FIGURE 2: Portion of COL treated interphase cell, showing extensive 60 to 80 angstroms fibrils in the exoplasma. Courtesy of The Rockefeller University Press

Effect of COL on mitosis

Taylor (1965) prepared H^3-colchicine of high specific activity (2.5 curies per mM) to study the mechanism of colchicine inhibition of mitosis in cultures of human cells, strain K.B. No direct effects on the duration of the cell cycle or macromolecular synthesis were demonstrable at a concentration of colchicine which completely inhibited mitosis. The radioactive compound was bound to the cells at a rate proportional to colchicine concentration. The binding appeared to be reversible since the radioactivity of the cells reached a maximum value for a given concentration and was slowly lost after resuspension of the cells in fresh medium (Taylor, 1965). A suitable exposure to colchicine produced accumulation of metaphase-blocked mitoses after the colchicine was removed from the medium. An exposure of 6 to 8 hours at 10^{-7} M was sufficient to block essentially all the cells in metaphase, thus indicating that colchicine is bound to most interphase cells Taylor (1965).

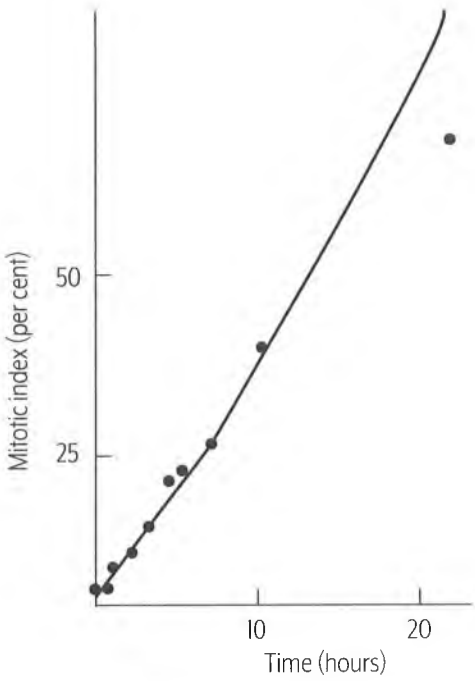


***FIGURE 3:** Accumulation of mitoses in the presence of COL 10^{-7} M. The solid line is theoretical and based on mathematical calculation. Courtesy of The Rockefeller University Press*

The data are in quantitative agreement with a mechanism involving reversible binding of colchicine to a set of cellular sites. Based on the correlation between the time of first appearance of blocked mitoses and the radioactivity per cell, it is suggested that if a critical fraction (3 to 5 percent) of the sites are complexed, the cell is unable to form a functional mitotic spindle Taylor (1965).

Effect of VLBS on mitosis

Cutts (1961) found that the alkaloid, vincaleukoblastine (VLBS), produced C-mitosis in cells of L1210 ascites tumor and in normal rat bone marrow *in vivo*. The affected cells were arrested in metaphase, resulting in the complete disappearance of post-metaphase, but the Prophase stages were not affected. He suggested that the effect of VLBS in producing metaphase arrest could be modified by the administration of glutamic acid or tryptophan.

Effect of VLBS on mitosis

Vinblastine can induce the self-association of tubulin *in vitro*, and at very high concentrations (10^{-3} M) the drug is able to precipitate tubulin and several other proteins including actin and neurofilament proteins form solution (Wilson *et al.*, 1982). This activity appears to be nonspecific, and due to the strong cationic character of vinblastine and may result from the binding of the drug to large numbers of nonspecific ionic sites on the surfaces of various proteins (Wilson *et al.*, 1982). There are two specific vinblastine binding sites per molecule of tubulin and its binding affinities for the sites ranges from 8 x 10^6 liters/mole at 37 degrees centigrade to 2.3 x 10^4 liters/mole at 25 degrees centigrade (Wilson *et al.*, 1982). In addition to blocking the formation of microtubules at low concentration, vinblastine appears to disassemble microtubules directly by causing splaying and peeling of protofilament strands at the end of microtubules. Wilson *et al.*, (1982) investigated the binding of ^{3}H-vinblastine to steady-state bovine brain microtubules at different drug concentrations to correlate the affinity and stoichiometry of drug binding to the microtubules with the ability of the drug to clock microtubule polymerization substoichiometrically, and with the ability of vinblastine to induce the unravelling of protofilaments at microtubule ends. Their results showed that vinblastine prevents the net addition of tubulin to steady-state bovine brain microtubules *in vitro* by binding rapidly, reversibly, and with high affinity to a very limited number of molecules of tubulin at the net assembly ends of the microtubules (Wilson *et al.*, 1982).

Anti-tubular agents & collagen and cell matrix

Moskalewski *et al.*, (1975) isolated epiphyseal chondrocytes from fetal guinea pigs enzymatically, aggregated them, and maintained the aggregates in organ culture. Light and electron microscopy revealed that the culture produced a typical cartilaginous matrix, but no calcification occurred. There is evidence that COL and VBLS can impair the secretion of hormones such as insulin and thyroid hormones, responsible for calcification of the cartilaginous matrix. Moskalewski *et al.*, (1975) used crystalline modification of COL from MERCK, which still contains up to 20% w/w of chloroform. The time of incubation with the anti-tubulin agents was 40 min and 240 min in their experiments. Exposure of aggregating cells, or

preformed aggregates, to colchicine or vinblastine at 10^{-5} M concentration led to the disappearance of the microtubules, dissociation of the Golgi complex into dictyosomes, and clustering of lysosomes (Moskalewski *et al.*, 1975). Surprisingly, a considerable amount of microfilaments have been observed in the chondrocytes treated for a few days with either compound in concentrations of 10^{-5} M. Under the influence of VBLS x 10^{-5} M an increased process of autophagocytosis have been observed in the treated chondrocytes after 40 min of exposure. Further incubation with VLBS revealed an accumulation of cytoplasmatic granular fragments in the lysosomes after 240 min in the chondrocytes, which were due to portions of the endoplasmic reticulum connected thru microtubules to the Golgi complex and ribosomes. An accumulation of phagosomes and polyphagosomes was conspicuous. Exclusively with VBLS, the appearance of single, parallel aligned microtubular proteins, especially individually separated tubulin, has been observed. Macrotubulins have been first observed after 60 min and 120 min of VBLS exposure. Later, the macrotubulins dissociated into the individual microtubules and separated tubulin proteins. The macrotubulins had a diameter of 350 angstroem to 400 angstroem. Surprisingly, no paracrystals were found in the examined chondrocytes, consisting of VBLS-tubulin complexes, as in fibrocytes incubated with VBLS. The fragmentation of the complex consisting of microtubules and Golgi vesicles has been described by Robbins and Gonatas (1964), who also observed the early disappearance of cytoplasmic microtubules of HeLa tumour cells within 60 min of exposure to anti tubulin agents. Similar pictures have been shown by De Brabander (1975) on dissociated and further on fragmented Golgi vesicles scattered in the cytoplasma of fibrocytes. In fibroblasts cells of cranial bones treated with COL for 60 min to 180 min the appearance of vacuoles in Golgi vesicles have been observed followed by their fragmentation. For VBLS, these vacuoles in Golgi vesicles have not been observed in chondrocytes during the same time period as COL exposure. Common to both anti tubulin agents was a cytolasmic increase of lysosomes in the chondrocytes. An interesting observation was that the cytoplasmic morphological changes were reversible under both anti tubulin agents.

Another aspect in the studies on the influence of anti-tubulin agents on chondroblasts was the formation and secretion of matrix substances, especially porteoglycans. These are formed at the polyribosomes of the rough endoplamatic reticulum and are transported out of the cell via tubulin bridges. The attachment of sugar components to the formed protein chain takes place at the Golgi complex, from where the transport into the intercellular space also takes place. There is evidence from studies on fibrocytes that COL-induced fragmentation of Golgi vesicles and their separation from tubulin proteins suppresses the formation and secretion of proteoglycans. The reduced production of intracytoplasmic proteoglycans may secondarily lead to a slowdown of protein synthesis at the rough endoplasmic reticulum. Electron microscopic studies by Moskalewsky et al (1975) have shown that the intercellular matrix of chondrocytes does indeed have a reduced concentration of microfibrillar proteins and proteoglycans, possibly due to their impaired or slowed transport along the Golgi vesicle via the Golgi vacuoles and tubulin transport proteins to the cytoplasmic membrane.

This was in contrast to the action of ethanol, hexylene glycol and deuterium oxide, which have been shown to stabilize such structures. Thus, in anti-tubulin treated cells the dictyosomes with accompanying vesicular structures were dispersed throughout the cytoplasm, whereas they were localized in a well-defined juxtanuclear region in controlled cells. Further to that, it was found, that COL impaired both, the synthesis and production of collagen in vitro, whereas VBLS inhibited synthesis and secretion of collagen as well as other proteins. Both anti tubulin agents have been shown to impair the secretion of glycosaminoglycanes, which are filling out the cartilaginous matrix in chick embryo chondrocytes, together with dicationic minerals. As a result of this, the Golgi complexes showed marked changes.

The number and size of cisternae forming a dictyosome were often reduced. Cells treated with vinblastine displayed macrotubules and an increased number of phagosomes. Both drugs reduced the deposition of intercellular matrix. In cells first

exposed to either of the drugs for 2 to 5 days and then transferred to fresh medium for 3 to 6 days, the microtubules reappeared, the Golgi complex regained its normal appearance, and the amount of matrix increased (Moskalewski *et al.*, 1975).

VBLS crystals in cells

Another study by Krishan and Hsu (1971) used Earl`s L929 mouse fibroblasts in order to study the influence of COL on the binding to VBLS-protein crystals and their association with polyribosomes. They worked with colchicine-^{3}H labeling of vinblastine- and vincristine-induced crystals in tissue culture cells and found the presence of colchicine-binding microtubular proteins in these structures. VBLS, which they purchased from E. Lily Indianapolis (Velban and Oncovin) cause the hypertrophy organelles of L929, i.e., the rough granular endoplasmic reticulum together with the accumulation of proteinaceous cytoplasmic crystals, similar to cultured cells from the CNS of animals, and platelets. Electron microscopic observations revealed longitudinally structured formations of proteins with a wide diameter of 280 angstroms. Other studies have revealed long structures of polyribosomes formed in the cytoplasm of L929 under the influence of VBLS and associated with the VBLS protein crystals. Further investigation on the precipitation of COL binding protein by VBLS from cells of various tissues, e.g., nerve, muscle, connective tissue suggests that there may be a similarity in structure in the cytoplasmatic proteins to COL-induced protein. The electrostatic attraction of positively charged VBLS with the anionically charged RNA of polyribosomes or soluble cytoplasmatic proteins could not be excluded. Their study raised the consideration that whether the protein forming the intracellular cytoplasmic crystals are similar to colchicine-binding microtubular proteins and the proteins precipitated by vinblastine and vincristine from the supernates of the cell homogenate.

Locomotion of Sarcoma cells

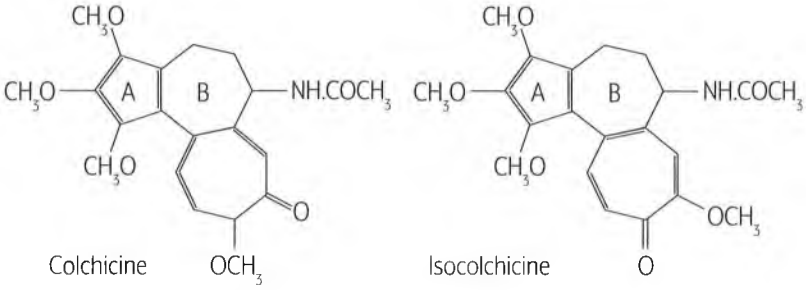
Abercrombie and Ambrose (1958) described the movement and interrelations in tissue culture of chick heart and mouse muscle fibroblasts, and of the rounded cells of mouse sarcoma 37 and sarcoma 180 from time-lapse colour films taken with an inter-

ference microscope. They found in their study that a fibroblast moving on a plane surface with its leading pole free is preceded by a ruffled membrane. Other, smaller, ruffled membranes may occur elsewhere around the periphery of such a cell. The large, ruffled membranes are associated with pinocytosis. They showed that when a ruffled membrane of one fibroblast touched any part of the peripheral surface of another fibroblast, it usually formed a close adhesion to it. The ruffling then stopped, the membrane if growing ceased to expand and the whole cell if moving in the direction of the membrane soon ceased to do so (Abercrombie and Ambrose, 1958). A lateral membrane may then lead the cell in a new direction. Adhesions are usually temporary. The adhesion and cessation of activity of a leading ruffled membrane appear to be the visible expression of the contact inhibition previously reported. A somewhat similar cessation of activity may occur in a ruffled membrane leading a cell, in the absence of contact with other cells, producing a change of direction when another membrane takes over the leadership (Abercrombie and Ambrose, 1958). In the light of the findings of Dustin (1934) who studied the action of colchicine on sarcoma transplant, Crocker type, of mice, it was noted by Abercrombie and Ambrose (1958) that a rounded sarcoma cell had a very actively moving surface, but not in the form of a ruffled membrane. Little or no adhesion or change in surface activity, or in locomotion, was seen in a sarcoma cell when it met another, or met a fibroblast, or conversely in a fibroblast when it met a sarcoma cell (Abercrombie and Ambrose, 1958).

Main Body: Biochemistry of COL

In recent years intense interest has been aroused by the remarkable biological properties of colchicine, which were first revealed by in 1934 and 1938 (Sharma and Sharma, 1980). One study observed that Colchicine is the methyl ether of an enolone containing three additional methoxy groups, an acetylated primary amino group and three non-benzenoid double bonds. A later study noted that the threshold regions of colchicine-mitotic activity are identical for both crystalline and amorphous forms; chloroform exerts no appreciable effect (Sharma and Sharma, 1980). One of the significant findings in *Allium cepa* is that whenever isocolchicine is used instead of

colchicine, no C-mitotic action is observed. The latter differs from the former only in minor details of structure. The data collected by Sharma and Sharma (1980) implies that: (a) at least one methoxy group in ring A is necessary for colchicine action; (b) ring C must be 7-membered, and the hydroxyl group should preferably be replaced by an amino group; (c) esterification of amino group in ring B increases the activity; and (d) isocolchicine and its derivatives are less active. A proper distance should be maintained between esterified side chains of rings B and C.



This last statement is based on the fact that in isocolchicine, where the position of the methoxy group is reversed in ring C, the decrease in efficacy has been considered to be due to the presence of hydrogen bonds between the side chains of rings B and C (Sharma and Sharma, 1980). In the case of "colchicine", the weak action is the result of the iso-form of this molecule. Though these data indicate the reactive groups responsible for colchicine action, the exact reaction involved in spindle inactivation is not yet fully clear. Pertinent suggestions have been made based on results obtained with spindle poisons of different chemical structure (Sharma and Sharma, 1980).

Effect of COL on mitosis

The effects of the alkaloid colchicine as a mitotic poison were first described in 1934 and the nature of its action has been further studied in 1936.

It is essential to select as a test-object a tissue with a high normal rate of mitosis in which the abnormal arrested metaphase can be unequivocally distinguished from the normal metaphase.

Experimental tumors fulfil the first of these criteria but owing to the frequency of abnormal mitotic figures it is often impossible to say whether a given mitosis shows the toxic effects of a mitotic poison or not (Brues, 1936). Moreover, in tumors many cells in apparently normal later stages of division are seen after effective doses of the drug have been given and, at least in the case of certain transplanted sarcomata (probably owing to inadequate blood supply), the effect may be confined to the borders of the tumor and in some sections missed altogether.

The most satisfactory tissue for the study by Brues (1936) was liver in the process of restoration following subtotal hepatectomy. In the cane of the rat, the average mitosis rate during the period of rapid regeneration was nearly as great as that in most experimental tumors; and histological study was facilitated by the large size of the hepatic cells and by the highly characteristic distribution of the chromosomes under the influence of a mitotic poison. In the normal hepatic cell mitosis, the chromosomes form a very compact group shortly after the disappearance of the nuclear membrane during prophase and remain so throughout the division - except in prophase it is impossible to distinguish and count the individual chromosomes (Brues, 1936). On the other hand, after parenteral administration of a suitable dose of colchicine, the chromosomes scatter widely throughout the cell shortly after disappearance of the nuclear membrane, as if they repelled one another, and it is often possible to count them. In addition, the cell in this arrested stage has the rounded border characteristic of a cell in mitosis and in most cases the cytoplasm stains much more lightly with eosin (Brues, 1936). Except under the influence of a very small dosage, these figured were abnormal and in the same section with normal metaphases and later stages of mitosis, making it possible to distinguish the abnormal from a normal one. An appropriate dose of sodium cacodylate gives essentially the same picture in this organ.

The effects of the alkaloid COL as a mitotic poison were first described by Lits (1934) and Dustin (1934) and the nature of its action has been further studied by Ludford (1936). In common with several apparently unrelated substances, notably the

cacodylates, it has been shown to arrest cell division during metaphase and to maintain cell thus affected in this phase of mitosis for several hours following administration; this occurs both in tissues *in vivo* and in explanted tissue (Brues, 1936). It is, of course, most readily seen in tissues in which the mitosis rate is normally high, notably in the glands of the intestine, and it occurs as well in malignant tumors and in explants from them. In the case of the regenerating rat liver (in which the normal rate of cell division is known with reasonable accuracy), under suitable conditions, the number of mitoses seen in arrested metaphase after administration of COL over a given length of time is equal to the number of mitoses which would normally have occurred and gone on to completion during that time (Amoroso, 1935). Brues (1936) carried out a study to determine the effects on mitosis of various compounds derived from COL, to assess the importance of the various molecular groupings of COL and to determine whether similar mitotic affects may be shown with COL derivatives which are devoid of the high toxicity of the parent alkaloid.

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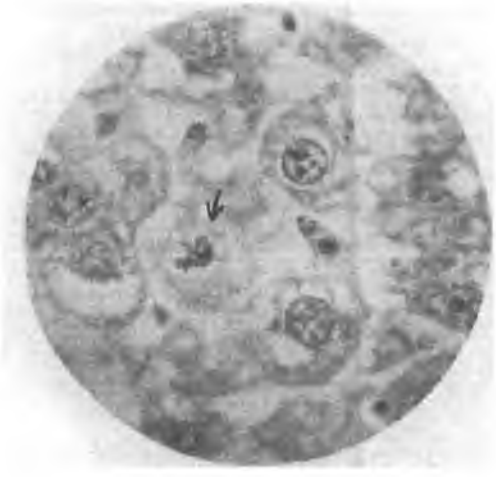

FIGURE 4: *Normal metaphase as indicated by arrow in regenerating liver 30 hours after operation, x 600 (from BRUES).*

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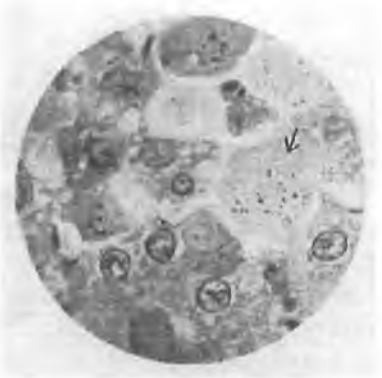


FIGURE 5: *Abnormal mitoses in regenerative liver 2 days after operation and 8,5 hours after Treatment (100 mcg s.c) One abnormal FIG In indicated by arrow, x 600, from BRUES.*

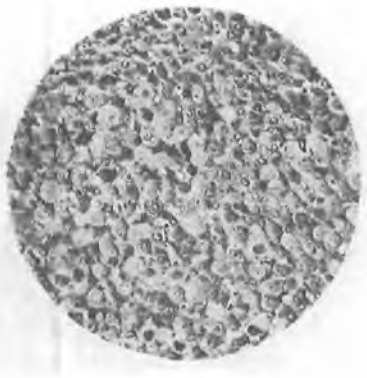


FIGURE 6: *Low power view of the liver, showing COL large numbers of arrested mitoses, x 95, from BRUES*

In addition, the cell in this arrested stage has the rounded border characteristic of a cell in mitosis and in most cases the cytoplasm stains much more lightly with eosin (Brues, 1936). Except under the influence of a very small dosage, these figured were abnormal and in the same section with normal metaphases and later stages of mitosis, making it possible to distinguish the abnormal from a normal one. An appropriate dose of sodium cacodylate gives essentially the same picture in this organ.

Although the COL molecule contains a variety of molecular groupings (e.g., methoxyl, methoxymethyleneketone, acetyl-

amino) it cannot be said from the results of the investigation carried out by Brues (1936) that any single group is essential for the mitotic inhibiting action of COL. The compounds examined in that study included COL derivatives in which the methoxymethylene group had been first hydrolyzed to hydroxymethylene, then replaced by iodine, and finally completely eliminated, the last two stages being accompanied also by modification of the ketonic group (conversion into a phenolic hydroxyl group). Also, some of the compounds examined had the acetylamino-group intact, others had this group hydrolyzed to the free amino-group, while in yet another case the amino-group was replaced by a hydroxyl. All of these derivatives showed activity (Brues, 1936). The inactivity of dimethyl- and trimethyl-colchicinic acids is of interest. In the former case the suppression of activity was attributed to demethylation of one of the three methoxyl groups in ring I, but trimethyl-colchicinic acid appeared to be anomalous, for the only modifications in the COL molecule were the hydrolysis of the methoxymethylene group to hydroxymethylene and the hydrolysis of the acetylamino-group to the free amino group, whereas neither of these changes is necessarily accompanied by loss of activity (Brues, 1936). Possibly the inactivation of trimethylcolchicinie acid is associated with the presence of the basic groups and also the strongly acidic hydroxymethylene group in the same molecule. The lack of specificity suggested by these results appeared to warrant the examination of synthetic compounds of analogous structure.

No substances are effective on mitosis in the small doses required in the case of COL itself, and the toxicity roughly follows the same relative dosage with different compounds, except in the case of the colchicinic acids (which are ineffective on mitosis), and possibly in the case of the nitrogen-free carbinol, in which the lethal dose is yet undetermined (Brues, 1936). The colchicine employed in these experiments was obtained from the Hoffman La Roche Laboratories. Since this substance contains about 25 percent chloroform of crystallization, a few rats were injected with amorphous colchicine, which contains no chloroform, with entirely similar results as far as the cytological picture was concerned (Brues, 1936).

Colchicine was dissolved in 0.9 percent saline solution before administration. When large quantities were used, it was first dissolved in a minimum amount of alcohol, to facilitate solution in water. In order to control the use of alcohol in administration of this and other substances, comparable amounts of alcohol alone have been injected into animals during hepatic regenerations, without any resulting abnormalities of mitosis being detectable (Brues, 1936).

However, the fact that the lethal effect of COL appears only after several hours, when the mitotic effect is beginning to wear off, suggests that the two effects may be dissociated, and this question requires further investigation. There is obviously a wide gap between the effective and lethal doses of COL (and its salicylate) and those of the other substances. Some of the other differences of dosage may, however, depend upon variations in solubility and absorbability. Several derivatives of COL have been investigated (Brues, 1936) with regard to their effects as mitotic poisons. The test object was the regenerating rat liver (Brues, 1936), in which the arrested mitosis is conspicuous and is easily distinguished from the normal mitosis.

Effect of COL on mitosis

The description of the effects of colchicine on mitosis by Dustin (1934) marked the beginning of a period of active investigation. During the next 15 years, publications dealing with this alkaloid averaged about 100 titles per year. Eigsti and Winn (1949) mentioned that after repeated injection in mammals, plentiful mitotic figures were occasionally observed in smears of bone marrow. They studied the karyokineses of the epithelial and endothelial cells in the mucosa of stomach and intestine, in experimental gastroenteritis following colchicum poisoning in the gastrointestinal mitoses in two dogs given, respectively, 10 and 15 g tincture of bulbs of colchicum, and dying 24 and 48 hours later. They found an extraordinary great number of dividing cells in the stomach. Furthermore, within the Lieberkühn glands of the intestine nearly all cells were engaged in indirect division. Endothelial mitoses could be seen nearly in all vessels. Moreover, it was rare to see the

latest stages of division. Eigsti and Winn (1949) considered that the cellular elements may have been directly excited and stimulated by the tincture of colchicum. They also noticed that some of the mitotic figures apparently underwent destruction which they speculated was a necrosis of the cells, giving rise to some sort of pseudo-karyokinetic forms. They suggested the relation of the observed images to true mitosis. The illustrations showed gastric and Lieberkühn glands, one with "*quasi tutti gli elementi in cariocinesi*, " and divided equatorial plates, but no true anaphases or telophases were represented (Eigsti and Winn, 1949).

Effect of COL on mitosis

Benitez *et al.*, (1954) used a time-honored approach to investigate the mechanism of mitosis from the standpoints of cell physiology and of cell chemistry by using diversified chemical agents designated (because of this action) as mitotic poisons. More recent investigations have included compounds which will inhibit or reverse the effects of the mitosis inhibitors. They selected the adult rat fibroblast (from subcutaneous areolar tissue) as physiological material, and colchicine as a mitosis inhibitor whose action as a spindle poison, producing prolonged metaphase arrest, is clear-cut. Their experiment consisted of the four groupings: (1) colchicine alone; (2) colchicine plus antagonist; (3) antagonist alone, (4) saline. This antagonism of apparently antimetabolic nature between the two structurally related compounds colchicine and tropolone was of great interest. It is not known whether compounds such as colchicine and tropolone exist in animal cells, but it is known that 7-membered rings exist in plant cells. Benitez *et al.*, (1954) speculated that it may be pertinent that the Syrian hamster, which in its native habitat normally feeds on *Colchicum autumnale*, is more than five times as resistant to colchicine poisoning as the laboratory rat. With doses of 10 milligrams per100 grams. the hamster gained weight and was fertile; at 2 milligrams per 100 grams. The rat died of acute colchicine poisoning (Orsini and Pansky, 1952). The possibility seems to exist that colchicine may act to produce metaphase arrest by functioning as an antimetabolite in a chain of reactions required for nuclear division. The evidence gath-

ered within this same biological system lends no support to theories postulating the intervention of ATP in spindle-fiber contraction. When this compound is present in the medium at a concentration of 5×10^{-3} M.

The cytoplasm of resting cells and dividing cells alike contracts and remains contracted for several hours. Division seemed to be prevented by mechanical means from taking place. The internal cohesiveness of the cytoplasm as a whole is decreased by colchicine (Baray and Palis, 1952). ATP, administered along with colchicine, at first restored or even exaggerated the normal viscosity, and as a result, the cytoplasmic movement was retarded, and the mitotic coefficient was lower than for the controls. However, the phase percentages were unaffected; the same percentage of the figures were in metaphase whether ATP was present with colchicine or not (Benitez *et al.*, 1954). It seems unlikely therefore that ATP is antagonizing the action of colchicine by restoring spindle-fiber contraction.

If ATP induces over-all contraction of the cytoplasm as a whole, it is difficult to see how this contraction could affect the ordered movement of the chromosomes unless the material of the spindle fiber is presumed to be substantially more sensitive in its response than the general cytoplasm, or unless the production of ATP in the cell is sharply localized in the spindle area (Benitez *et al.*, 1954). There seems to be no present evidence that either of these conditions exists. Though the efficacy and the specificity of meso-inositol in antagonizing colchicine have been repeatedly demonstrated in this biological system over a period of several years, the nature of its action is still obscure. Like ATP, it reduces the mitotic coefficient without greatly changing the phase percentages but, unlike ATP, its effects are delayed until about the beginning of the next mitotic wave (Benitez *et al.*, 1954). This fact would seem to indicate that it is metabolized. Specific relationship to the spindle remains undemonstrated. The fact that chemical agents of such diverse nature as the cyclohexene derivative inositol, the ribonucleotide ATP, and the cycloheptene derivative tropolone will reverse in

substantial degree the metaphase arrest produced by a single agent - colchicine - confirms that the mitotic process is chemically exceedingly complex. Also, only one of these agents was able to bring about complete recovery in the presence of colchicine, though they were used at 100-fold its strength, or more. Benitez *et al.*, (1954) postulated a variety of routes by which a mitotic poison might operate chemically, such as by preventing the production or utilization of an essential metabolite, by interfering with one or more enzymes necessary for the completion of a metabolic process, or indirectly, by depriving the cell of an energy source necessary for one of these processes. The route or routes by which colchicine produces its effects are still unknown, and the methods in which these three agents antagonize them are correspondingly open to conjecture. The results of their experiments, however, carried out in a quantitative manner, with a variety of agents and under comparable conditions in the same controlled and standardized biological system, emphasize the dangerous inadequacy of a monistic explanation of mitotic mechanics.

Effect of COL on mitosis

In 1934, Dustin reported that the alkaloid, colchicine, produced a metaphase "explosion" when growing cells were exposed to the drug. A short time later it was determined that rather than increase the number of cells that divided, colchicine inhibited all those that entered metaphase from proceeding any further in the division cycle. Chromosomes in the cell that was arrested in metaphase displayed varying degrees of disorientation in contrast to their usual characteristic alignment on the metaphase plate, and conventional cytological techniques demonstrated that this phenomenon was due to inhibition or destruction of spindle fibres. Because of the striking morphological transformations which colchicine induces in the dividing cell, it is not surprising that the effects of this drug and other similarly acting compounds on the nondividing cell have received little attention. Eigsti and Dustin, (1955) in their extensive monograph covering several thousand references list an interesting graph dealing with the drug response of cells in mitotic stages.

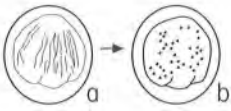
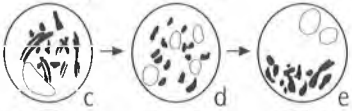
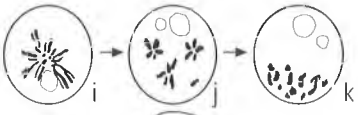
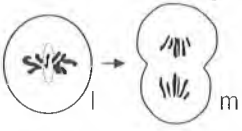
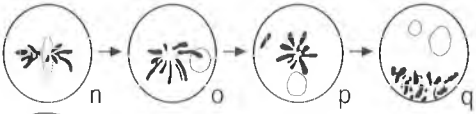
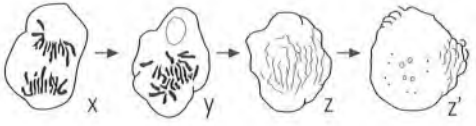
Mitotic treated	Colchicine x10^{-6} molar	SUCCESSIVE CHANGES
late prophase 	50.25	
	2,5	
	1,9	
prometaph 	25.2,5	
	0,2	
metophase 	25	
	2,5	
anaphase   	50.25	
	50.25	
	50.25	

FIGURE 7: *: Mitotic stages when treatment of fibrocytes began with COL, shown in the right column. Concentrations are expressed in molarity. Successive stages are lettered a to z'.* ***a and b:*** *prophase reversions occurring 10 to 20 minutes after treatment with this strong concentration. Chromatin resembles early prophase.* ***c to e:*** *chromosomes lie at random, no spindle formed, exploded c-metaphases, chromosomes continue to shorten, then clump together in groups at bottom of cell, hyaline globules formed in* ***d*** *rise to top of cell* ***f to h:*** *the evolution of,* ***a,*** *star metaphase.* ***i to k:*** *star metaphase that becomes- increased to multiple star and lost chromosomes.* ***l to m;*** *weak solutions do not fully inhibit spindle but reduce the size.* ***n to q:*** *the metaphasic spindle is reduced, hyaline globules form in* ***o,*** *chromosomes settle to bottom and globules rise in cell.* ***r*** *cell divides when concentration is too weak to destroy spindle completely, compare figure* ***r and c,*** *that received same concentration, but applied at different stages. Anaphase spindles are reduced if concentration is 25 x10^{6} M or more. Chromosomes fuse and intermingle in* ***t and v,*** *hyaline globule forms in stages* ***t, v, and y.*** *Four nucleoli in w' and z' indicate a, tetraploid restitution nucleus. These stages show the interaction of concentration, stage of mitosis, and length of exposure. (Diagrams adapted from M. Gauiden and J. Carlson, Experimental Cell Research 1957). Courtesy of The Rockefeller University Press*

In most instances this response, such as intranuclear precipitation and degeneration has been induced with COL concentrations between 10^{-3} M and 10^{-6} M or less which is effective in the induction of metaphase arrest (Haas, 1941). Robins and Gonatas (1964) in their study emphasized some of the histochemical and ultrastructural transformations in interphased cells following exposure to low concentrations of spindle inhibitors and illustrated that these transformations are extensive. HeLa cells (strain S3) were used throughout their studies. Robins and Gonatas (1964) stated that colchicine and similarly acting compounds are regarded primarily as spindle inhibitors.

Their main effect is purported to be the actual destruction of the spindle fibres if they are already formed at the time of drug administration, or prevention of their formation if the cell is exposed before entering mitosis (Robins and Gonatas, 1964). While this effect on the spindle fibre is real, the drug has profound effects on the interphase cell as well. Despite these effects, however,

it appears that treated interphase cells in which the described transformations have taken place still retain the capacity to enter mitosis. This follows from the fact that the histological disturbances of the Golgi apparatus and lysosomes are present in most cells 10 hours after exposure to colchicine, while the number of cells arrested in metaphase continues to increase up to about 25 hours (Robins and Gonatas, 1964). Thus, many modified interphase cells must be able to commence nuclear membrane breakdown and chromosome condensation.

The consensus has been that colchicine reacts with a molecular system of spindle precursors (Levan and Ostergren, 1943). Although the lysosomal transformations indicate that this cannot be the entire action, it is possible that one facet of this transformation, namely, the spatial depolarization of these organelles in the interphase cell, is an effect secondary to interference with microtubule (spindle tubule) structures. It is now known that these structures are present in most if not all cell types (Slautterbach, 1963), and it has been suggested that they partake in the direction of protoplasmic streaming (Ledbetter and Porter, 1963). If this is true, then the disintegration, in interphase, of microtubular arrays would disrupt this streaming, and conceivably the structural depolarization of the organelles would result simply by virtue of ever-present Brownian movement.

The internal ultrastructural transformations and clustering of the lysosomal particles in interphase in a fashion wholly comparable to that noted only in the normal mitotic cell is less readily explained (Robins and Gonatas, 1964). It is interesting to note, however, that in normal prophase the disappearance of the diffuse network of normally present cytoplasmic microtubules (not to be confused with those arising from the centriole at this phase of division), and lysosomal clustering, occur simultaneously (Robins and Gonatas, 1964). It may be that these two phenomena are linked in a complex chain of events, and one follows the other whether mitosis ensues or not.

Effect of COL on mitosis

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the mitotic apparatus of colchicine-treated sea urchin eggs (Sauaia and Mazia, 1961). This inhibition by colchicine presumably occurs either through a direct or indirect effect of the compound on the spindle. An example of indirect action would be the activation of an enzyme which attacks the spindle while a direct action might involve binding of colchicine to spindle fibres causing them to dissociate into protein subunits (Taylor, 1965). Since colchicine in concentrations less than 10^{-7} M completely inhibits mitosis in cultures, the association constant of an assumed colchicine-protein complex should be sufficiently large to enable the complex to be isolated (Taylor, 1965). These considerations led Taylor (1965) to prepare tritium-labelled colchicine of high specific activity to facilitate a study of the mechanism of action. He studied the effects of colchicine on cellular metabolism, on the kinetics of inhibition of mitosis, and on the uptake and binding of radioactive colchicine.

The results he obtained were consistent with reversible binding of colchicine to a set of sites within the cell and lead to the conclusion that mitosis will be inhibited if a critical fraction of these sites is occupied by colchicine.

All experiments performed before were with suspension cultures of human cells, strain K.B. (Eagle, 1955). The cultures were maintained in logarithmic growth with a doubling time of 24 hours. The experimental findings showed that at a colchicine concentration of 10^{-7} M there was no measurable effect on the rates of DNA, RNA, and protein synthesis nor on the rate of progress of cells around the mitotic cycle, for some hours after mitosis was completely inhibited (Taylor, 1965). A plot of T_L, the time lag before increase in the mitotic index, against 1/C had an inflection point at about 2×10^{7} (Taylor, 1965). At concentrations greater than 2×10^{7} M, cells in metaphase at $t = 0$ were blocked. At somewhat lower concentrations the original metaphases and anaphases completed mitosis while prophase cells were blocked (Taylor, 1965).

FIGURE 8

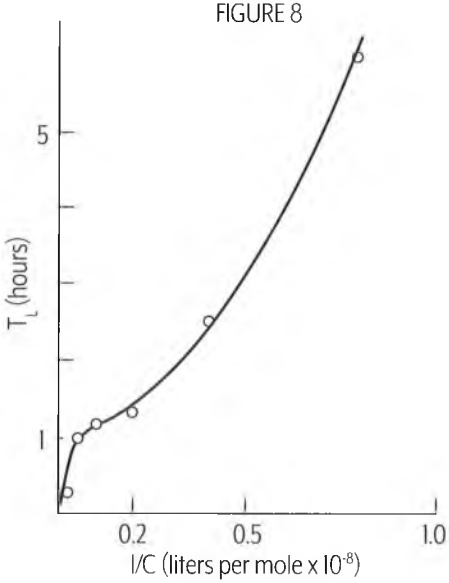

***FIGURE** 8: The time lag before increase in mitotic index T_L versus the reciprocal of the colchicine concentration. Values of T_L were obtained by linear extrapolation of calculated values to the initial value of the mitotic index*

Exposure of the culture to colchicine for increasing periods of time led to increasing periods of further accumulation of mitoses after the inhibitor was removed from the medium. An exposure of 6 to 8 hours at 10^{-7} M was sufficient to block essentially all the cells in the culture as they reached metaphase (Taylor, 1965). Colchicine penetrated the cells rapidly and equilibrated with external colchicine in less than 15 minutes and was present in the cells in a bound form which was only slowly lost when cells were resuspended on colchicine-free medium (Taylor, 1965). The rate of binding of colchicine was proportional to concentration.

Bound radioactivity reached a maximum value per cell for a given concentration of colchicine. The time to attain the maximum radioactivity depended on concentration and could be reduced to 4 to 5 hours at very high concentrations (10^{-5} M) (Taylor, 1965). Since colchicine does not interfere with cellular metabolism or macromolecular synthesis, its action is on a target unique to mitosis. Therefore, Taylor (1965) assumed that the cell contains

a set of sites capable of binding colchicine and that a normal mitotic spindle cannot be formed if a critical fraction of the sites is occupied by colchicine. Since an exposure to colchicine of 6 to 8 hours is sufficient to block essentially all the cells in the culture (doubling time, 24 hours), colchicine must be bound to interphase cells (Taylor, 1965). The binding sites are probably present in all the cells although the amount of colchicine bound per cell may depend on its position in the mitotic cycle. Ignoring this possible heterogeneity in binding (which may be only a factor of two), it was inferred from the uptake of radioactivity at the time of first appearance of blocked metaphases that an uptake of about 500 CPM per 3×10^6 cells was sufficient to block mitosis (Taylor, 1965). A similar value (400 to 800 CPM per 3×10^6 cells) was obtained from measurements of the accumulation of mitoses and retention of radioactivity after dilution of the culture. Taylor (1965) estimated that inhibition would occur if about 3 to 5 per cent of the sites were complexed with colchicine.

FIGURE 9

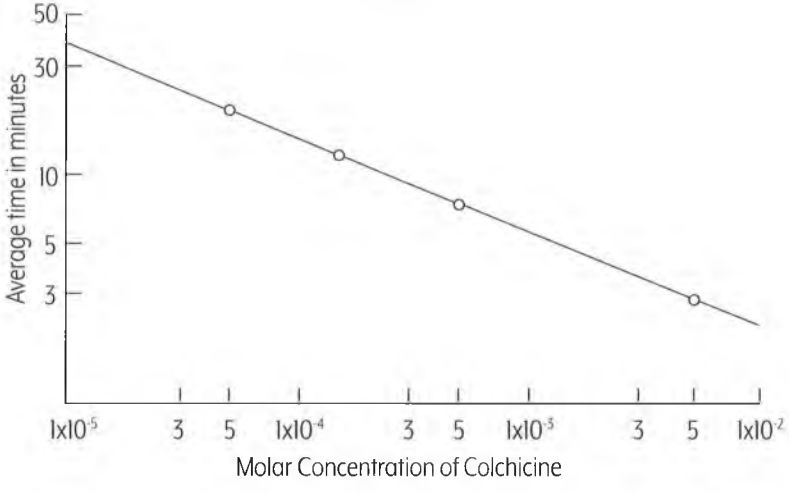

FIGURE 9: The average time for disappearance of metaphasic spindle of Chaetopterus egg, disappearance measured by polarized light pattern. The stronger the concentration, the shorter the time for complete disappearance of spindle. Temperature of sea water 25 degrees centigrade (adapted from INOUE, 1952, Experimental Cell Research Supp., Vol 2; pp 305-318. *Courtesy of The Rockefeller University Press*

The time of first appearance of blocked mitoses was approximately proportional to the reciprocal concentration, i.e. $(\text{concentration})^{-1}$ (Taylor, 1965). Since the rate of incorporation is proportional to concentration the time to block a critical fraction of the sites would also be proportional to $(\text{concentration})^{-1}$ (Taylor, 1965). It is therefore probable that the rate limiting step in inhibition of mitosis is the formation of the colchicine-site complex. If other steps are involved, such as enzymatic activation of colchicine they are presumably much faster than the binding step. The kinetic laws obeyed by most enzyme reactions (Michelis-Menten kinetics) require that the $(\text{rate})^{-1}$ be proportional to $(\text{substrate concentration})^{-1}$ (Taylor, 1965). The colchicine binding does not follow this prediction but rather the rate is proportional to concentration which is the result expected for a first order reaction (Taylor, 1965). The kinetics of inhibition of mitosis and of uptake of colchicine are consistent with binding to a structural protein of the spindle, but saturation of the target at times short compared to the generation time may present a difficulty. If spindle precursor protein is the target site, it would be concluded that the amount present in a culture in exponential growth is a large fraction of the total amount in a culture in which most of the cells are blocked at metaphase (Taylor, 1965). Since the fate of spindle protein after division and the period in the mitotic cycle of new spindle protein synthesis is unknown this conclusion need not be inconsistent with the hypothesis that the target site is a spindle component (Taylor, 1965). It is also possible that colchicine may bind to microtubule elements of the spindle and this component is present at all stages of the mitotic cycle since it has other functions.

Effect of VLBS on mitosis

Isolation of the alkaloid, vincaleukoblastine (VLBS), from the plant Vinca rosea Linn was reported in 1958 by Noble, Beer, and Cutts. This material, or crude extracts containing it, had been shown to produce a profound and apparently specific bone marrow suppression and peripheral granulocytopenia (Cutts, 1958; Cutts, 1957). In other studies, VLBS was found to affect some experimental tumors in animals (Cutts, 1960; Johnson et al., 1959, Johnson et al., 1960; Noble et al., 1958),

and preliminary clinical trials have indicated that it may also modify the growth of various tumors in humans (Hertz et al., 1960; Hodes et al., 1959; Hodes et al., 1960; Warwick et al., 1960). Various studies have attempted to elucidate the mode of action of VLBS. Cutts (1961) focused on the changes in mitosis induced by VLBS *in vivo* in the L1210 and Ehrlich ascites tumors and in normal rat marrow. Palmer and his colleagues (Johnson et al., 1960) observed arrested metaphase in cell cultures, and Johnson and coworkers (Johnson et al., 1960; Johnson et al., 1960) reported reversal of VLBS by amino acids, in vitro and in vivo. A similar *in vivo* effect has been found in the experiments carried out by Cutts (1961). Vincaleukoblastine appears to be a spindle inhibitor and as such produces C-mitosis in dividing cells. Like colchicine, VLBS does not arrest the initial phase of division but halts it at metaphase. Unlike colchicine, the arrest of mitosis can be modified by administration of amino acid. VLBS appears to be a more efficient spindle inhibitor than colchicine in that it prevents completely the passage of dividing cells into anaphase and telophase. At the same doses of colchicine post-metaphase stages could always be found. The effects of VLBS depend to some extent upon the dosage used. There may be a blocking of the spindle at low concentrations, with doubling of the chromosomes but failure of chromatid separation, or formation of macro- and micronuclei with lagging chromosomes in the interpolar region. Higher concentration may have a direct action upon chromosomes, resulting in their shrinkage and coalescence into a dense pyknotic mass. Cells which survive large doses of VLBS show various abnormalities, formation of multipolar spindle, bridging, etc. If a second dose of VLBS is given before the arrested cells are able to complete division, the action is cumulative (Cutts, 1961).

Effect of VLBS on mitosis

The vinca alkaloid, vinblastine, is a potent mitotic inhibitor, and appears to prevent cell growth by disruption of microtubule function. Though much has been learned about the interaction of the vinca alkaloids with tubulin and microtubules, the precise mechanism of action of this class of drugs remains to be elucidated. In addition to inhibiting the formation of mi-

crotubules and to destroying preformed ones, vinblastine possesses the ability to induce the formation of highly birefringent uniaxial crystals in cells, composed of tubulin complexed with vinblastine (Schochet, et al., 1968; Wilson et al., 1978). Vinblastine can induce the self-association of tubulin *in vitro* (Na and Timasheff, 1980; Na and Timasheff, 1980), and at very high concentrations (10^{-3} M) the drug is able to precipitate tubulin and several other proteins including actin and neurofilament proteins form solution (Wilson et al., 1970; Mori and Kurokawa, 1979). This latter activity appears to be nonspecific, and due to the strong cationic character of vinblastine and may result from the binding of the drug to large numbers of nonspecific ionic sites on the surfaces of various proteins (Wilson *et al.*, 1982). There are two specific vinblastine binding sites per molecule of tubulin and its binding affinities for the sites ranges from 8×10^6 liters/mole at 37 degrees centigrade to 2.3×10^4 liters/mole at 25 degrees centigrade (Wilson *et al.*, 1982). In addition to blocking the formation of microtubules at low concentration, vinblastine appears to disassemble microtubules directly by causing splaying and peeling of protofilament strands at the end of microtubules (Warfield et al., 1974). Wilson *et al.*, (1982) investigated the binding of ^{3}H-vinblastine to steady-state bovine brain microtubules at different drug concentrations. Their purpose was to correlate the affinity and stoichiometry of drug binding to the microtubules with the ability of the drug to clock microtubule polymerization substoichiometrically, and with the ability of vinblastine to induce the unravelling of protofilaments at microtubule ends. Their results showed that vinblastine prevents the net addition of tubulin to steady-state bovine brain microtubules *in vitro* by binding rapidly, reversibly, and with high affinity to a very limited number of molecules of tubulin at the net assembly ends of the microtubules (Wilson *et al.*, 1982).

The most likely location of the approximately 16-17 high affinity vinblastine binding sites on steady-state microtubules is at one or both ends of the microtubules. The rates of vinblastine exchange at these sites were too rapid to measure with the methods used in their study (Wilson *et al.*, 1982). At least half of the sites are suggested to be at the assembly ends, so they

hypothesized that half (approximately 8) sites are located at the assembly ends and the other half are located at the disassembly ends (Wilson *et al.*, 1982). However, an uneven distribution of sites is also equally likely due to the possible unique geometries at the microtubule ends and the polar orientation of the tubulin molecule in the microtubule surface lattice. The quantity of vinblastine bound per microtubule under conditions of half-maximal inhibition of assembly rate was approximately 1 molecule of vinblastine per molecule (Wilson *et al.*, 1982). If an equal distribution of high affinity sites exists at the two microtubule ends, then at 50% inhibition of assembly, approximately 0.5 molecules of vinblastine would be bound at each microtubule end, or one vinblastine molecule bound at the net assembly end for every two microtubules (Wilson *et al.*, 1982). In this situation, the binding of a single vinblastine molecule to the net assembly end of a microtubule would be able to reduce the rate of tubulin addition to near zero. If all the high affinity binding sites were located at the net assembly ends, then the binding of only two vinblastine molecules per microtubule would be sufficient to block assembly. Wilson *et al.*, (1982) argued that the binding of vinblastine to the microtubules they titrated in the high affinity range did not reflect any copolymer formation between vinblastine or vinblastine-tubulin complexes and free tubulin, since the bound vinblastine was rapidly and completely exchangeable. If vinblastine were able to form copolymers, much higher stoichiometries would be observed (Wilson *et al.*, 1982). Their findings also reveal that colchicine inhibits the rate of tubulin loss at the assembly ends of steady-state microtubules, as well as the gain, thus producing a kinetic cap at this microtubule end. They investigated whether vinblastine could induce an assembly end kinetic cap and found that microtubules pulse-labeled at their assembly ends with ^{3}H-guanine nucleotide and treated with assembly-inhibiting concentrations of vinblastine lost their assembly end label (and, therefore, lost tubulin from the assembly ends) at a rate that was indistinguishable from that of non-drug-treated microtubules upon 3-fold dilution of the micro tubule suspension. We conclude that if vinblastine is able to induce a kinetic cap, the cap is substantially weaker than the one induced by colchicine.

The observation that vinblastine can bind rapidly and directly to the high affinity sites on the micro tubules may be highly significant. The difficulty in understanding the mechanism of substoichiometric poisoning of microtubule assembly by colchicine arose because of evidence which indicated that the drug bound first to soluble tubulin, and it was the complex which most effectively blocked assembly (Wilson *et al.*, 1982). Since the ratio of free tubulin to colchicine-tubulin complex was high, it was difficult to imagine how the drug would work other than by kinetically slowing the rate of tubulin loss as well as the rate of gain at the microtubule assembly end (Wilson *et al.*, 1982). An assembly end kinetic cap is not required with vinblastine, if it interacts directly with the assembly ends of the microtubules as the data suggests. It is likely that the mechanism of inhibition of microtubule assembly by vinblastine is simpler than that of colchicine. The kinetically rapid binding of vinblastine directly to a limited number of high affinity binding sites at the net assembly end of a microtubule could effectively block further tubulin addition regardless of the ratio of free tubulin to tubulin-vinblastine complex, so long as sufficient free drug was available to ensure that the binding sites at the microtubule assembly ends were constantly occupied by drug. This raised the question of how only one or two vinblastine molecules bound to the growing end of a microtubule prevent (s) further polymerization. Wilson *et al.*, (1982) speculated that polymerization occurs normally through a helical, stepwise addition of tubulin, with the addition of an incoming tubulin molecule at each growth (start) point dependent upon the proper three-dimensional placement of the previous tubulin molecule. Vinblastine is a large molecule and the binding of vinblastine to tubulin at just one or two growth points must be capable of producing sufficient misalignment of incoming tubulin molecules by steric hindrance to prevent further assembly of the polymer.

Anti-tubular agents & collagen and cell matrix

Some antimicrotubular agents like COL, vinblastine, and vincristine (Olmstead and Boristy, 1973; Wilson eta l., 1974), cause a disruption of the microtubules, whereas others such as ethanol, hexylene glycol (Kane, 1965; Kirkpatrick, 1969) and

deuterium oxide (Gross and Spindel, 1960; Tilney and Gibbins, 1969) stabilize these organelles. They impair the secretion of many cell products, e.g., hormones such as insulin (Lacy et al., 1968; Malaisse et al., 1971) and thyroid hormone (Nevé et al., 1972; Williams and Wolff, 1972). More recently, microtubules have also been implicated in the secretion of the macromolecules of connective tissue matrices. It has also been found that COL and vinblastine, as well as other antimicrotubular agents, inhibit both secretion and synthesis of collagen in vitro (Dehm and Prockop, 1972; Ehrlich et al., 1974). COL and vinblastine decrease the rate of secretion of collagen by cells isolated from chick embryo tendons by about 70 percent, and the amount of intracellular collagen concomitantly increases about two-fold (Dehm and Prockop, 1972). In a biochemical and ultrastructural study on bone cells, Ehrlich *et al.*, (1974) found that COL, in addition to the effect on secretion, also depressed collagen synthesis, whereas vinblastine inhibited synthesis of collagen as well as other proteins. These authors suggested that the reduction in collagen formation by COL is due to an accumulation of collagen in the Golgi vacuoles, with secondary inhibition of its synthesis. Likewise, COL and vinblastine depress both secretion and synthesis of glycosaminoglycans by isolated chick-embryo chondrocytes, but, contrarily, do not greatly reduce the synthesis of collagen by cartilaginous tibia anlagen (Jansen and Bornstein, 1974). Based on these findings, it is usually assumed that microtubules are involved in the intracellular translocation of secretory vacuoles, and that the primary effect of antimicrotubular agents in connective tissue cells is on secretion. Both short- and long-term effects and the ability of the cells to recover after prolonged exposure to these drugs were studied by Moskalewski *et al.*, (1975). During the experiments, they found that the antimicrotubular agents caused marked changes in the Golgi complex. As an attempt to determine whether these alterations were directly due to the absence of microtubules or were secondary to inhibition of the synthesis of matrix components, the effects of puromycin, a potent protein synthesis inhibitor, on cellular fine structure was also examined (Moskalewski *et al.*, 1975). They used chondrocytes isolated from cartilaginous epiphyses of guinea pig fetuses as experimental system, aggregated

them, and maintained them in organ culture. Because of the potential usefulness of this system for other studies on the function and metabolism of cartilage, the morphology of the chondrocyte aggregates was recorded for up to 25 days, i.e., beyond what was necessary for the experimental purposes proper (Moskalewski *et al.*, 1975).

It is generally accepted that the Golgi complex is involved in the synthesis and secretion of matrix components in connective tissue cells. There is considerable morphological and biochemical evidence indicating that the formation of both collagen and proteoglycans is initiated in the granular endoplasmic reticulum, and then completed during transport through the cisternae of the reticulum to the Golgi complex, as well as within this latter system, where the molecules finally accumulate in vacuoles before being released to the extracellular space by exocytosis (Moskalewski *et al.*, 1975). During recent years antimicrotubular agents have been found to interfere with both secretion and synthesis of collagen in cultured bones and fibroblasts (Dehm and Prockop, 1972, Ehrlich et al., 1974), and of glycosaminoglycans in isolated chondrocytes (Jansen and Bornstein, 1974). Based on these findings, it has been suggested that disruption of microtubules interferes with the movement of Golgi vacuoles to the cell surface, and this results in accumulation of secretory products in the Golgi complex, which then secondarily leads to an inhibition of synthesis. In their study, aggregates formed and cultured in the presence of COL and vinblastine showed a reduced content of intercellular matrix (Moskalewski *et al.*, 1975). This reduction comprised both collagen fibrils and matrix granules, the latter demonstrated to contain proteoglycans by ruthenium red staining (Luft, 1971; Thyberg et al., 1973). Considerable amounts of intercellular matrix were laid down in the treated aggregates. The chondrocytes retained part of their capacity for synthesizing and secreting extracellular macromolecules despite the disappearance of the microtubules and the structural alterations of the Golgi complex (Moskalewski *et al.*, 1975). It is evident from their results that the reduction of matrix deposition in aggregates treated with COL and vinblastine could be due to a primary dysfunction of

the Golgi complex as well as to a disturbance in the transport of secretory vacuoles.

VBLS crystals in cells

Besides their strong mitosis-arresting effect (Noble et al., 1958; Palmer et al., 1960; Cutts, 1961; George et al., 1965; Krishan, 1968), the vinca alkaloids, vinblastine sulfate and vincristine sulfate cause hypertrophy of the granular endoplasmic reticulum and annulate lamellae in cultured mammalian cells (Krishan et al., 1968), and the accumulation of large proteinaceous cytoplasmic crystals in tissue culture cells (Bensch and Malawista, 1969), in cells of the central nervous system (Schochet et al., 1968), and in platelets (White, 1968). In electron micrographs, longitudinally cut crystals show a substructure of parallel, electron-opaque lines with approximately 280 angstroms periodicity while in cross-sections a honeycomb-like arrangement of tubules, 280 angstroms wide, is seen (Krishan and Hsu, 1971). Association of large arrays of ribosomes and polyribosomes with the crystals has been recently demonstrated (Krishan and Hsu, 1969; Krishan, 1970). In cells reintubated in fresh medium after the formation of the vinblastine-induced crystals, large masses of 50-80 angstroms filaments are seen instead of the crystals, suggesting that the crystals and the filaments are interconvertible (Krishan and Hsu, 1969). Precipitation of a colchicine-binding protein, isolated and characterized earlier (Borisy and Taylor, 1967), from the high-speed supernates of homogenized tissue culture cells and pig brain, and from isolated microtubular protein, by vinblastine has been reported (Marantz et al., 1969; Bensch et al., 1969).

The precipitation of structural proteins other than the colchicine-binding microtubular proteins by vinblastine was demonstrated by previous studies (Wilson et al., 1970). Their study raised the consideration that whether the protein forming the intracellular cytoplasmic crystals are similar to colchicine-binding microtubular proteins and the proteins precipitated by vinblastine and vincristine from the supernates of the cell homogenates (Marantz et al., 1969; Bensch et al., 1969). They worked with colchicine-^{3}H labeling of vinblastine- and

vincristine-induced crystals in tissue culture cells and found the presence of colchicine-binding microtubular proteins in these structures.

Locomotion of Sarcoma cells

In the sarcoma cells on which observations have so far been made by Dustin (1934) who was working on the action of colchicine on sarcoma transplant, Crocker type, of mice, it was noted by Abercrombie and Ambrose (1958) that they were of the more rounded kind which in culture are most actively moving and most actively invasive of a contiguous colony of fibroblasts. It is probably a mechanical consequence of the shape of these cells that they move over the surface of the flattened fibroblasts and never vice versa (Abercrombie and Ambrose, 1958). The style of movement of the sarcoma cells is very different from that of fibroblasts. They have intense and continuous activity over their whole visible surface. There is little corresponding to a leading ruffled membrane, though the activity at their surface seems often to be of greater amplitude at the leading end, and to take place on a major projection of the cell which might be called an incipient pseudopodium (Abercrombie and Ambrose, 1958).

Nothing like the contact phenomena observed between two fibroblasts occurs when a sarcoma cell meets another sarcoma cell or a fibroblast. There is no cessation of the sarcoma cell's surface activity corresponding to the cessation of ruffling, and, at least when a sarcoma cell meets a fibroblast, its movement is not checked (Abercrombie and Ambrose, 1958). This is in agreement with the immunity of these sarcoma cells to contact inhibition by fibroblasts (Abercrombie et al., 1957). The sarcoma cell must adhere in some degree to a fibroblast, or it would not be able to move over its surface; and the fibroblast does not appear to be exactly equivalent to any other substratum, since these sarcoma cells gave the impression of becoming a little distorted in general shape when on a fibroblast's surface (Abercrombie and Ambrose, 1958). But the distinction that a fibroblast makes between another fibroblast and a glass or fibrin substratum is not made by a sarcoma cell; both are available for the latter's movement.

7. Chapter: Colchicine and microtubules and microfilaments

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Summary:
Microtubules and microfilaments are two main parts of the cytoskeleton of the cell. The main difference between them is that microtubules are long, hollow cylinders made up of tubulin protein units, whereas microfilaments are double-stranded helical polymers made up of actin proteins. Tubulin protein is derived from many sources with a molecular weight of 110,000 Daltons and a sedimentation velocity of 6S. Tubulin can be separable in sodium dodecyl sulphate electrophoresis or other denaturing solvents into two different peptides known as alpha and beta-tubulin. Tubulin in the 6 S form binds 1 mole of colchicine per mole of protein. Several investigators have demonstrated the enzymatic activity of tubulin. Tubulin has been implicated as both an ATPase and a protein kinase. Tubulin possesses enzyme activity only because hydrolysis of the terminal phosphate of GTP is related to its polymerization (McIntosh, 1974).

Furthermore, microtubules are considered a class of proteinaceous, intracellular fibres present in many organisms and a common constituent of eukaryotic cells. They first appeared around one billion years ago, together with the nucleus, signifying the transition from prokaryotes to eukaryotes (Stephens, 1971). In terms of morphology, a microtubule is defined as a long, unbranded, apparently hollow tube of estimated 210 to 250 angstroms in diameter with a 150-angstrom hole (McIntosh, 1974). Its wall consists of globular subunits, and these subunits align into 11 to 13 longitudinal strands, and the tips of tubules tend to splay out into their respective 40 angstrom strands. It is believed that protein is derived from microtubules and has been separated from doublet fibrils of cilia,

flagella, mitotic apparatus, and extract of nervous tissues. Another author investigated the microtubular and microfibrils structure in lymph node macrophages. He argued that the microfibrils in reticuloendothelial cells is universally 6 nm and appearance-wise, the microfibrils are contractile while the microtubules are skeletal in functions (Carr, 1972).

Microtubules are commonly present in cilia and flagella, the mitotic apparatus, nerve processes, the cortex of meristematic plant cells and elongation cells (during the formation of lens or spermatogenesis of certain insects). They are also found in selected structures including the axostyle of parasitic flagellates in protozoa, the axoneme of *Eobinosphaerium*, the fibre systems of *Stentor*, and the cytopharyngeal basket of *Nassula*. Different comparative and experimental studies have shown that the main functions of microtubules are to help in compartmentalizing cytoplasmic movement, chromosome movement in cell division, in the distribution of cytoplasmic activities, and support of cellular structure or maintenance of anisometric cell form. The control of microtubule distribution in cells is the dynamic equilibrium that exists between the polymer and its monomers. In addition, the control of the distribution of microtubules in the cells also involve loci that affect the nucleation of microtubules. These loci included the basal body, the centriole, satellites associated with the basal body and centriole, the kinetochore, and a host of other dense particles, many of which membrane-associated. In addition, bridges have been associated with almost microtubule-associated motile mechanisms (Fulton, 1971; Tinley, 1971; Porter, 1966). Microtubules are also involved in ciliary beating, secretion, neuroplasmic flow, and mitosis, among other cell motions (Dustin, 1978). Furthermore, microtubules alone could only give position changes by their growth, such as pushing apart the polar structures in mitosis, and maybe disassembling, leaving space for other structures to be displaced. Sometimes, microtubules also help in displacement of large molecules including secretory or pigment granules (Dustin, 1978).

Ruffled membrane

A ruffled membrane serves as the locomotory organ of fibro-

blasts. The waxing and fading of ruffled membranes at different areas of the fibroblast's edge allow it to migrate with changes in direction. Individual cells have competitive ruffled membranes. They can be inhibited or have their lives extended by the cell environment, most notably through contact inhibition and contact guidance. Mutual contacts produce long-lasting adhesions that bind fibroblasts together in a meshwork (Abercrombie, 1961). Moreover, fibroblast cytokinesis results from a local contraction, which is set to work in the appropriate stage of mitosis by an uneven distribution of the cellular relaxing system between polar and the equatorial regions of the cell (Hoffmann, 1964).

Initially, it was thought the function of microtubules in the cell of plants and animals depended on the locality of these elements inside the cells or parts of cells. Most tissue cells, regardless of their innate capacity for active pseudopod-directed motility, must remain limited to the borders of their parent tissues and organs for the duration of the organism's existence. Contact inhibition of motility could be a way to immobilise cells in tissues and promote positional stability. Spherical aggregates of chick heart, sclera, and skin fibroblasts were joined with tritiated thymidine-labelled aggregates of the same cell type to examine the efficiency of contact inhibition in immobilising cells in 3-dimensional tissue-like masses. The two aggregates cohere and broaden the area of mutual contact after being placed in touch, forming a single aggregate with a planar interface. The movement of marked cells across the interface and into the unlabelled aggregate was used to detect cell mobility. Under ideal conditions for directed migration, fibroblasts travelled up to 17 cell diameters and 36 percent as far in solid tissue masses as they did in monolayer culture, according to measures of distances traversed in day-old aggregate pairs (Armstrong and Armstrong, 1978).

Later, chick heart, sclera, and skin fibroblast aggregates were joined with tritiated thymidine-labelled aggregates of the same cell type. The two aggregates cohered and expanded the contact area to create a single aggregate with a planar interface between the labelled and unlabelled halves after being

placed in contact. The motility of cells in the aggregate was determined by detecting the movement of tagged cells across the barrier into the unlabelled half. The three-dimensional aggregates showed a considerable reduction in fibroblast motility after exposure to therapeutic concentrations of antimicrotubular drugs (Armstrong and Armstrong, 1979).

Microtubules

Microtubules are disrupted by standard osmium or acrolein fixation. These tubules may be depolymerized in the presence of low temperature. In addition, the plant alkaloids, colchicine, vinblastine, and podophyllotoxin observed to be rather specific in their capacity to act reversibly at low concentrations to disassemble microtubules. The most specific microtubule poisons such as colchicine have been used to treat gout and used as a therapeutic drug with interesting results (Dustin, 1978). For instance, microtubule breakdown is detected in the presence of 5 or 40 micrograms per millilitre of colchicine in BHK-21/C13 fibroblast-like cells (Goldman, 1971). Microtubules rapidly disappear when exposed to colchicine. As a result, different morphological and physiological changes occurred in the absence of microtubules: (a) the cells change from being fibroblast-like to being epithelial-like, (b) the typical intracellular birefringence pattern is disrupted, and a juxtanuclear cap of birefringent filaments forms, (c) even though membrane ruffling persists in colchicine-treated cells, and time-lapse cinematography shows that cell movement is suppressed. Further, colchicine-induced breakdown of microtubules suggests that they play a critical role in determining fibroblast cell-shape, formation of major cell processes, and cell motility (Goldman, 1971). Other studies reported that microtubules are durable components that are resistant to colchicine and cytoplasm.

In contrast, another study reported that colchicine and various other chemicals are useful for studying how a microtubule protein is assembled into tubules. However, colchicine is known to be sensitive to slow morphogenetic movements involving microtubule polymerization. The direct creation of force in mitosis could be attributed to assembling a microtubule protein into mitotic spindle microtubules. Furthermore,

structural changes in microtubule protein may be responsible for the chemosensitivity of the neural systems of a higher animal. Tubulin proteins interact with tiny molecules to explain touch, hearing, olfaction, chemotaxis, and the formation of action potentials (Bourne and Margulis, 1973).

The study of microtubules has been linked to the study of mitosis, even before the discovery of spindle "fibres" as microtubules and the involvement of microtubules in accessory structures such as centrioles. For many years, the importance of colchicine poisoning of mitosis covered more fundamental issues, which could only be solved with the isolation of tubulin and evidence of colchicine's unique affinity to this protein. In addition, spindle poisons have been used in the research of mitotic growth, cytogenetics, and the development of polyploid or amphidiploid plants for nearly four decades. However, the scope of this research was limited to the fundamental issues surrounding the involvement of microtubules in mitotic motions (Dustin, 1978). Colchicine treatment interferes with chromosomes' normal alignment and separation on the mitotic spindle (Inoue, 1952).

Furthermore, contraction ceased when microtubular function inhibitors like vinblastine and colchicine were given topically to actively contracting wounds. The findings suggest that wound contraction is linked to the function of microtubules in fibroblasts within the wound and that it is occurring at its fastest rate. However, these findings do not support the idea that wound contraction is mediated by the microfilament components of cells (Ehrlich, et al, 1977).

In addition, a study by manipulation reveals that the chromosome shows a stronger resistance toward movement when birefringent structures of microtubules are greater and become loose by the action of colchicine or birefringence disappears (Begg and Ellis, 1978).

Main Body: Structure and function of Microtubules
Microtubules are a class of proteinaceous, intracellular fibres found in a wide variety of organisms (Stephens, 1970). They

were originally defined by their characteristic morphology as seen in the electron microscope where they appear as tubes of 250 angstroms outside diameter with a 150-angstrom hole. They are unbranched and may run for many micrometres. Since the widespread use of glutaraldehyde as a fixative for electron microscopy, microtubules have been found in all eucaryotic cells examined (McIntosh, 1974).

The sites in a cell where microtubules are commonly encountered include cilia, flagella, nerve processes, the mitotic apparatus, the cortex of meristematic plant cells (Ledbetter and Porter, 1963) (Full reference unavailable), elongating cells such as during the formation of the lens (Byers and Porter, 1964) (Full reference unavailable) or during spermatogenesis of certain insects (Moses et al., 1968) (Full reference unavailable), and selected structures in protozoa such as the axostyle of parasitic flagellates (Grimstone and Cleveland, 1965) (Full reference unavailable), the axoneme of *Echinospherium* (Tilney and Porter, 1965), the fibre system of *Stentor* (Bannister and Tatchell, 1968), and the cytopharyngeal basket of *Nassule* (Tucker, 1968) (Full reference unavailable). In all there examples the microtubules are associated spatially with movement. Moreover, in all these cases the microtubules are aligned parallel to the asymmetry of the cell or cell process and thus could be associated with its support. These two functions, support and movement, cannot always be separated (Fulton, 1971; Tinley, 1971).

Structural studies under favourable conditions have revealed that the microtubule wall as seen in transverse view is composed of 13 subunits (Ledbetter and Porter, 1974; Tilney et al., 1973). Longitudinal view of the tubule wall using a variety of negative stains shows strands of globular subunits called protofilaments which run parallel to the tubule axis. Optical diffraction (Grimstone and Klug, 1966) and computer-facilitated analysis (Erickson, 1974) have shown that the microtubule is constructed from morphological units which appear as a two-lobed structure packed into a 40 X 51 A unit cell (short dimension parallel to the microtubule axis). The morphological units are arranged in parallel rows (the protofilaments) and placed slightly out of register to make a 3-start helix.

Colchicine binding subunit in microtubules

In 1967 two groups independently reported the use of colchicine-binding activity in cell lysates as an assay for a specific and ubiquitous cytoplasmic protein that was available in quantity from tissues rich in microtubules (Wilson and Friedkin, 1967). Detailed study in these and other laboratories has confirmed that the colchicine binding protein is a subunit of microtubules. The protein, named tubulin, has been isolated from numerous sources and characterized with a variety of techniques. The protein has a molecular weight of 110,000 Daltons and a sedimentation velocity of 6 S. There is circumstantial evidence to support the view that the 6 S molecule is made from one copy of alpha and one of beta tubulin (Bryan and Wilson, 1971), but there are conflicting views and evidence in this matter (Witman et al., 1972; Meza et al., 1972; Stephens, 1970). The amino acid compositions of alpha and beta tubulin are similar but not identical, and an investigation of the primary structure of the first 24 amino acids at the N terminal end of the two peptides shows them to be sufficiently similar to support the suggestion that they arose from a common ancestor. Comparison of this same region of the tubulins from embryonic chick brain and from sea urchin sperm tails reveals a single amino acid substitution in the beta chains and no differences in the alpha, indicating a high degree of conservation over evolutionary time (Luducna and Woodward, 1973).

Tubulin in the 6 S form binds 1 mole of colchicine or podophyllotoxin per mole of protein. These two alkaloids compete for a single binding site (Wilson et al., 1974). Vinblastin, which induces a non-physiological aggregation of tubulin rather than inhibiting assembly, does not compete with the other drugs; the stoichiometry of its binding is currently in dispute (Bryan, 1972; Berry and Shelansky, 1972). The 6 S dimer also binds two molecules of GTP, one with high and one with low rate of exchange (Berry and Shelansky, 1972; Stephens et al., 1967). The vinblastin and GTP binding sites are not independent, and there is some evidence that the bound nucleotide is involved in the control of tubulin polymerization (Berry, 1972; Olmsted and Borisy, 1973).

Functions of microtubules

The functions of microtubules have been investigated in a variety of systems. Comparative and experimental studies have implicated microtubules as causal agents in the development and maintenance of anisometric cell form (Porter, 1966). The literature abounds with reports of microtubules found within cells changing their shapes. There are experimental conditions that block microtubule formation or disrupt existing tubules: the plant alkaloids colchicine, podophyllotoxin, and vinblastine seem to be rather specific in their capacity to act reversibly at low concentrations to disassemble microtubules (Wilson et al., 1974; Margulis, 1973). These treatments have been applied to many elongate and elongating cells to provide a wealth of circumstantial evidence that assembling and assembled microtubules contribute to the definition of the cell shape. The role of microtubules in the shaping of spermatozoa is evident and the multiple variants of the male sex gametes are composed, structurally, of a few organelles – acrosome, nucleus, mitochondria, flagella – the last being the organ of motility. The shaping of this highly specialized cell, throughout the animal and vegetal kingdom, results form a gradual condensation and deformation of nucleus, which is often related to the location pf microtubules (Dustin, 1978). Microtubules are also associated with some organelles which contribute to eucaryotic cell motility (Porter, 1966). Flagellar microtubules have been shown to slide over one another as the flagellum bends (Satir, 1968). Movement of whole cells, like ameboid movement, is the result of complex interactions between cytoplasm, contractile proteins (actin, myosin, and tropomyosin) and microtubules (Dustin, 1938). Several studies with colchicine indicated that the destruction or disassembly of microtubules could change the aspect of cell motion without suppressing the ameboid activity (Porter, 1966). In HeLa cells treated with 10^{-5} M colchicine, saltatory motion is arrested although membrane motion, and the formation and extension of microvilli and are not modified (Freed and Lebowitz, 1970). Fibroblasts and macrophages, under the effect of microtubular poisons, do not become immotile, but lose the polarized aspect of their motion: their cytoplasm maintains its contractility, locomotion in a definite direction is not seen

anymore. Ruffling becomes evenly distributed on the entire cell surface, instead of being mainly active at the leading edge (Bhisey and Freed, 1971; De Brabander, 1977; Gail and Boone, 1971). Still others see the tubules as passive structural components and suggest that a completely different system, such as actomyosin, is the causal agent in motion (Forer and Behnke, 1972). Studies on cytological effects of colchicine and vinblastine have implicated microtubules in processes of secretion (Wolff and Williams 1973; Lacy and Malaisse, 1973) and in the redistribution of cell surface molecules (Berlin et al., 1974), but little is yet known about the direct involvement of tubules in these processes.

While studying the orientation and function of centrioles, microtubules, and microfilaments during chemotaxis, Malech et al. (1977) evaluated the human neutrophils in a gradient of chemoattractant (5% *Escherichia coli* endotoxin activated serum) was evaluated by electron microscopy. Similar experiments are planed with human fibroblasts under the influence of a chemoattractant and anti-tubulin agents. Purified neutrophils (Hypaque-Ficoll) were placed in the upper compartment of chemotactic chambers.

Malech et al., (1977) found that a gradient of chemoattractant induces a polarization of neutrophil structure and function that resembles that of an actively migrating cell even when migration is impeded by a physical barrier. This involves an increase of microfilaments at the leading end, with restriction of pseudopod formation to the leading end toward the chemoattractant. In addition, the centriole with associated microtubule array orients in the cell to the side of the nucleus toward the chemoattractant, and the nucleus is significantly shifted to the rear of the ceil. A relatively rapid reorientation occurs when the direction of the chemoattractant stimulus is changed. They also defined some of the structural differences between the three types of neutrophil migration: random migration (cells in buffer), activated random migration (cells in uniform concentration of chemotactic factor), and directed migration or chemotaxis (cells in a gradient of chemotactic factor). The most striking morphological changes distin-

guishing the types of migration involve overall cell shape and orientation of specific intracellular structures, rather than the sudden appearance of any new structures.

When neutrophils were in buffer (random migration), many cells were rounded or moderately spread on the surface of the filter without evidence of pseudopod formation. In the presence of a uniform concentration of activated serum (activated random migration), an increased number of cells had a motile morphology, and the population of cells migrated farther into a filter than randomly migrating cells, thus confirming our earlier analysis of the effect of activated serum on the morphology of cells in suspension (Gallin and Rosenthal, 1974). Therefore, activated serum converted the cell population from a resting to migratory state. In addition, when applied as a gradient, activated serum oriented almost the entire population of cells. These observations do not necessarily imply that all chemotactic stimuli must increase the rate of locomotion; indeed, there is evidence that with some stimuli the individual cells orient but do not migrate faster when exposed to a gradient of chemotactic factor additional speculation regarding the role of the various structural elements in stabilizing the direction of migration. It has been noted by several observers that most neutrophils migrating on a solid substrate respond to changes in the direction of a chemoattractant by describing a curve and maintaining the same leading end (Allison, 1974). Ramsey (1974) has noted that neutrophils subjected to a rapid change in the direction of chemoattractant to a position opposite the initial migration appear to resorb the leading pseudopod and produce a new one at what was previously the tail. Although the cell stops its forward motion immediately, Ramsey's report indicates that it takes 45 seconds of reestablishment of locomotory morphology in the opposite direction before migration is resumed in the new direction. The nature of the shift in the direction of pseudopod extension is not known. However, the initial paradoxical downward movement of the centriole away from the new source of the chemotactic gradient in our studies may reflect an inertial phenomenon with respect to the inside of the cell in its initial attempt to reorient toward the stimulus. In fact, this response

was so strong that early after reversal, some cells spread out laterally just beneath the filter surface, with the entire cell, including the nucleus, enmeshed in the filter pores. In other cells lying on the filter, the centriole and nucleus were parallel to the filter surface as the nucleus relocated toward the bottom of the cell and the centriole relocated toward the top. The increase of microfilaments early after reversal in the side of the cell toward the chemoattractant was noted only when the centriole had completed relocation to a position above the nucleus. Pseudopod formation and upward migration of the cells into the fluid medium also was not seen before the time when relocation of centriole was evident in most cells. Thus, the observations of Malech et al., (1977) not only support the concept that microtubules are required for maintaining directed migration but, in addition, that the position of the centriole and its associated array of microtubules are involved in establishing the direction of migration after a change in location of the stimulus.

Cytochalasin B and colchicine were used as chemical probes to further evaluate the relative roles of the cellular contractile apparatus and microtubules in neutrophil orientation and pseudopod formation during chemotaxis. Cytochalasin B at 3 mcg/ml prevents neutrophil migration (Becker et al., 1972; Gallin and Rosenthal, 1974; Hartwig and Stossel, 1976; Zigmond and Hirsch, 1972), and in the current study it affected the number of microfilaments seen but did not prevent the maintenance of the internal orientation of the neutrophil in response to a gradient of chemoattractant. Thus, a normally functioning microfilament apparatus is not required for the orientation process. However, impairment of pseudopod penetration into the filter resulted from loss of microfilament function. Therefore, pseudopod formation appears to require normal microfilament function.

Malech et al., (1977) suggested that microtubules function to maintain orientation in migrating neutrophils. Our studies with colchicine and lumicolchicine were designed to further explore this possibility. Initial experiments showed that the internal orientation of the neutrophil, in response to a

gradient of chemoattractant, was eliminated by colchicine treatment, and in the presence of colchicine, the nucleus and centriole occupied random positions within the cell. The dose-response studies demonstrated that these effects of colchicine correlated with concentrations previously shown to inhibit microtubule assembly (Hoffstein et al., 1977), and interestingly, in the study of Malech et al., (1977), they occurred at concentrations of colchicine that can be obtained *in vivo* (Ertel et al., 1976). Lumicolchicine, an agent possessing many of the properties of colchicine, except tubuling binding activity (Sagorin et al., 1972), had no effect on these processes. Thus, microtubules are important in the initiation and maintenance of the internal structural orientation of the neutrophil. In untreated cells, the radial array of microtubules may prevent the random drift of the nuclear lobes by locking them into position on the side opposite the source of chemoattractant.

Colchicine also affected pseudopod formation, but in a somewhat complex manner. In the absence of colchicine, when the direction of the gradient of chemoattractant was reversed, neutrophils responded by extending pseudopods up into the fluid medium as they withdrew from the filter. In addition, when neutrophils were suspended in a chemotactic factor, without a substrate, they were also able to form pseudopods. Thus, in the absence of colchicine, pseudopod extension and orientation are not dependent on the location of a solid substrate. In the current study, colchicine, in concentrations that inhibited microtubule assembly, prevented the formation of pseudopods by cells in suspension, even in the presence of a strong stimulus such as activated serum. However, upon adherence to a substrate, colchicine-treated cells could form pseudopods, although they were narrow and elongated. However, unlike control cells, pseudopods did not develop on the non-substratum side of the cell when the stimulus source was reversed and placed above the cells. The formation of pseudopods and their orientation in the absence of microtubules appeared dependent upon a suitable surface for cells to adhere. The dense bundles of microfilaments noted in untreated cells at sites of attachment to the filter surface were unaffected by colchicine treatment. This microfilaments system probably

contributes to the formation and orientation of pseudopods when a filter substratum is provided as an exoskeleton to guide cell movement. However, the microfilament-filter substrate combination is an imperfect substitute for the microtubule skeleton and, in the absence of microtubules, pseudopod formation and activated random migration, as well as directed migration, are significantly impaired. Microtubules appear to be the primary organizers of the internal contents of the neutrophil. They modulate pseudopod formation by providing a cytoskeleton within the core of the pseudopod that is linked to the internal portions of the cell body by the nature of the radial structure of the microtubule-centriole apparatus. In this way, pseudopod extension is possible in the absence of adhesion to a substrate, and its location on the cell body is stabilized.

During chemotaxis, the maintenance of pseudopod orientation is probably critical for efficient migration toward the stimulus. However, this data does not suggest that enhanced forward migration under conditions of chemotaxis is entirely eliminated by colchicine treatment. There is also a small impairment of activated random migration by colchicine, the explanation for which may be found in previous observations of various cell types treated with colchicine. Randomly migrating cells treated with colchicine show an increased tendency for eccentric and more frequent direction changes, and also a decreased rate of peripheral spread of a cell population (Allison, 1974). Thus, stabilization of the internal structure and consistent organization of the leading pseudopod may also be necessary for efficient random migration.

Based upon the findings in this study, Malech et al., (1977) proposed a model in which microtubules modulate pseudopod formation and orientation and enhance migration in a single direction. However, these events are probably also under the influence of other systems, including the microfilament network.

Carr (1972) found unusual microtubular complex in the reticuloendothelial cells which established the ubiquity of microfibrils and microtubules. While considering the role of micro-

fibrils in macrophages, Carr postulated that the contractile mechanism involves elongated structures similar to those seen in other motile cells. Also, the same elongated structure is likely to be involved in all the types of cytoplasmic movement which occur in macrophages; that is, in translational movement, in movement of a mass of cytoplasm without translational movement, in the movement of cytoplasmic processes, and in organelle movement. The 6 nm microfibrils he observed were in a position which suggests that they may be responsible for most forms of movement in macrophages, though not quite enough are seen around pinocytic vesicles to account for their movement (Carr, 1972). Micropinocytic vesicles do not appear to have 6 nm microfibrils related to them; possibly because their movements do not appear to be energy dependent. The function of microtubules, which are common in cells, is less clear. If it be assumed that the lymph node macrophages studied by Carr (1972) are static but have moving processes, then a firm cytoskeleton would be required. The microtubular complex is in the right position for this. On this hypothesis such freely moving cells as peritoneal cells would not need a developed microtubular apparatus.

Locomotion in fibroblasts

In 1961, Abercrombie studied fibroblasts in terms of their locomotory behaviour and proposed a few hypotheses on which much of the pattern of movements of whole populations can be based. For individual fibroblasts, it was proposed that the main locomotory organ of the fibroblast is its ruffled (undulating) membrane (RM). The size, shape and activity of RMs vary a good deal within one population of fibroblasts and vary more between populations of different sorts of fibroblasts. They precede moving cells of other types, such as Schwann cells and nerve fibres (Abercrombie, 1961). Another hypothesis was that an isolated fibroblast on a plane surface moves with changes of direction. A chick heart fibroblast isolated from other fibroblasts, on a glass surface which has no large-scale regularity of structure, usually moves about, though with stationary intervals. For at least 12 hours or so it does not need any contact with its fellows in order to move (Abercrombie, 1961). It changes its direction at very irregular intervals,

of the order of several hours. This occurs by the waning of an existing RM and the waxing of a new one. It is difficult to be sure that the former always precedes the latter, but it usually does. The third hypothesis was that the ruffled membranes on an individual cell are competitive (Abercrombie, 1961). A growing or a large active established AM seems to prevent the development of a second RM; a previously ruffled membrane that has ceased its ruffling or is regressing seems to permit it. An incompatibility of two RMs on one cell is suggested by the high frequency with which dells with a single RM are observed on a glass surface. That this is due to mutual interference, and not to the operation of some central controlling agent, can be shown experimentally by discouraging the appearance of any large RM, when multiple small membranes result; and conversely by encouraging the emergence of a large RM, when the small ones do not appear. Fourth hypothesis was that the individual ruffled membranes can be inhibited or promoted by heterogeneities of the environment (Abercrombie, 1961). Naturally, because of competitive interaction, selective inhibition of one membrane promotes development of another membrane, and vice versa, and the categorization of agents as inhibitors or promotors is somewhat arbitrary. It can be concluded that contact inhibition selectively inhibits and contact guidance selectively promotes the life of a RM. Finally, the contacts that form between fibroblasts are adhesions which usually persist for a considerable time. When one RM is undergoing contact inhibition, it and its cell cease moving soon after contact has been made. The RM may then retract from the cell it has contacted, perhaps because of the light contraction of the RM (Abercrombie, 1961).

Fibroblasts and other non-muscular cells (Hoffmann-Berling, 1954), thrombocytes (Bettex-Galland and Luscher, 1961), amebae (Hoffman-Berling, 1956), and slime mould plasmodia (Nakajima, 1960) make use of the same general mechanochemical principle involved in muscular contraction for generating movement. Muscle and other biological systems deliver mechanical work by alternating between activity and rest. A contracted muscle relaxes, a fibroblast cell rounded up in mitosis, returns to former expanded state. To study whether

the relaxation processes if also common to both muscle and non-muscular cells, Hoffmann studied the vesicular elements of the skeletal muscles (referred to as grana) and found that inhibition of contraction in fibroblasts and in muscle is due to grana of an identical physical nature. Also, the mechanisms of granar action in muscle and fibroblasts correspond. Next, the cells contract locally. And finally, he found that fibroblast cytokinesis results from a local contraction, which in the appropriate stage of mitosis is a set of work by an uneven distribution of cellular relaxing system between the polar and the equatorial regions of the cell (Hoffmann, 1963).
The motility of fibroblasts in monolayer cultures is inversely related to cell density (Abercrombie & Heaysman, 1953; Elsdale, 1968; Gail & Boone, 1971; Martz, 1973). Abererombie & Heaysman (1953) attributed this reduction in motility to contact inhibition. The present study extends the observations on fibroblast motility in 2-dimensional monolayers to the situation of 3-dimensional tissue masses. One of the cell types used by Armstrong and Armstrong (1978), the chick embryo heart fibroblast, was also used by Abercrombie & Heaysman in their studies of contact inhibition. Armstrong and Armstrong (1978) observed that motility in aggregates was significantly less than maximal. The extent of reduction ranged from almost 100 percent for the stellate-shaped interior cells of heart fibroblast aggregates to 64 percent for skin fibroblasts. As with cells in confluent monolayers, motility in aggregates, although much reduced from maximal, was in most cases still considerable. Average maximal movements of 94-227 mcm in day were observed (Armstrong and Armstrong, 1978). Based on these observations, it was concluded that the motile performance of fibroblasts in confluent monolayers and in cell aggregates is similar: the extent of motility is reduced from maximum, but in most cases is still considerable. It can be suggested that contact inhibition contributes to the reduction of motility (Armstrong and Armstrong, 1978).

Effects of antimicrotubule agents on locomotion of fibroblast
Later, in 1979, Armstrong and Armstrong worked on the chick heart fibroblasts, sclera epithelium, and skin fibroblasts to study the effects of antimicrotubular agents on cell motility

in fibroblast aggregates. The culture media they used was 9 parts Dulbecco-modified Eagle's medium (Gibco powdered medium) plus 1 part heat-inactivated chicken serum (Gibco). Medium contained 100 units per millilitre penicillin, 100 microgram per millilitre streptomycin and 1 microgram per millilitre fungizone. The gas phase was 5 percent Carbon dioxide and 95 percent air. Cells were labelled during growth of the initial monolayers with [*Me*-^{3}H] thymidine (1 microcurie per millilitre). Antimicrotubule agents added to the medium at the time of fusion of the paired aggregates included Colchicine (Sigma, 10^{-5} M), Colcemid (CIBA, 1 microgram per millilitre), Vinblastine sulphate (Sigma, 10^{-6} M), and R17934 (nocodazole, Aldrich, 1 microgram per millilitre, diluted from a 5 milligram per millilitre stock solution in DMSO) (De Brabander et al., 1976). Cultures exposed to the antitubule agents were shielded from light to avoid photoinactivation of the drug.

Primary monolayer cultures of fibroblasts were prepared from trypsin-dispersed 10-day chick embryo heart ventricle, back skin, and sclera. The sclera was separated from the pigment epithelium following treatment of 10-day chick embryo eyes in 3 percent Difco 1:250 trypsin dissolved in $Ca2^+$-, $Mg2^+$-free saline (37 degrees centigrade, pH 7.3, 35 minutes). Cells were plated at 12-20 x 10^6 cells in 90-millimetre Falcon tissue culture dishes. Non-fibroblastic heart and skin cells were removed by decanting the unattached cells after 0.5-1.5 hours at 37 degrees centigrade. Fresh culture medium containing [^{3}H] thymidine was added, and the cultures were grown to confluency (requiring 2-3 days). The confluent monolayer was then scored with a rubber policeman to divide it into squares 0.5-1.0 centimetre per side. The pieces were gently scraped from the dish and were allowed to round up into compact spherical aggregates by overnight culture in shaker flasks (gyratory shaker, 25 millilitre flask, 90 rounds per minute, 37 degrees centigrade). Shaker flasks were gassed with 5 percent carbon dioxide, 95 percent air, then sealed with silicone rubber stoppers (Bellco, Vineland, N.J.) (Armstrong and Armstrong, 1979).

Suspensions of heart myocytes freed of most of the contaminating mesenchymal cells were obtained as the cells which did

not attach when dispersed 10-day chick embryo heart ventricle cell populations were incubated in 90-millimetre Falcon tissue culture plastic dishes at 37 degrees centigrade for 1.5 hours. Coherent myocyte tissue was prepared by pelleting the cells by gentle centrifugation in 16-millilitre screw-cap test tubes (Pyrex No. 9826), followed by stationary incubation for 3-6 hours at 37 degrees centigrade. The coherent pellets were removed and chopped into smaller aggregates with small knives made from sewing needles. The aggregates were then allowed to round up during an incubation overnight at 37degrees centigrade in 25 millilitre Ehrlenmeyer flasks placed on a gyratory shaker (Armstrong and Armstrong, 1979).

Pairs of spherical aggregates (one labelled, one unlabelled) were placed in hanging drops of culture medium (Abercrombie, 1973; Niedderman and Armstrong, 1972) to which the antimicrotubule agent was added or omitted in the control experiments. The aggregates came into contact at the bottom of the hanging drop and within 3 to 6 hours adhered tightly enough to enable them to be removed and transferred to shaker flasks for further culture. The antimicrotubule agents had no apparent effect on the adhesion of the aggregates. The antimicrotubule agents were added to the medium in the shaker flasks in the appropriate cases. The culture medium was supplemented with unlabelled thymidine (1 micromole per millilitre) for rounding up of both labelled and unlabelled monolayer aggregates for hanging drops, and for subsequent aggregate pairs. This reduced the incorporation into unlabelled cells of labelled thymidine transferred from labelled cells (Armstrong and Armstrong, 1979).

Aggregates to be used for light microscopy and auto radiography were fixed in Bouin's fixative, embedded in paraffin, and sectioned at 5 micrometres. During embedding, the aggregate pairs were oriented in the paraffin block so that the plane of sectioning would pass approximately perpendicular to the plane of fusion of labelled and unlabelled aggregate. Proper orientation was easily determined since most aggregate pairs were still ellipsoidal at the time of fixation. All aggregates were serially sectioned, so any aggregate pairs that were

not so oriented could be readily identified and were excluded from the analysis. Measurements of the distances of cell migration in aggregates were made on the sectioned, paraffin-embedded material. For each aggregate pair, the section with the widest diameter was taken to be the section passing through the middle of the aggregate pair (the "mid-sagittal section"). Measurements of the distances moved by labelled cells into the unlabelled aggregate were made for each pair on a total of 10 sections centred on the mid-sagittal region, with one or two sections between each used for measurement to ensure that different cells were seen each time. The distance of migration was taken as the distance between the plane of fusion of the two aggregates (determined by the plane in the interior of the aggregate where little mixing occurred) and the furthermost labelled cell in the unlabelled aggregate. Measurements were performed with an ocular micrometre previously calibrated with a stage micrometre (Armstrong and Armstrong, 1979).

Their results showed that the upon exposure to antimicrotubule agents like colchicine, colcemid, and vinblastine sulphate, the lamellipodial extension and retraction continued, indicating that microtubules are not involved in these processes (Armstrong and Armstrong, 1979). The cells, however, did lose their elongated shape and the leading lamella which, in untreated cells was restricted to a limited portion of the cell's perimeter, broadened until it encompassed most or all of the cell's border. Based on their study and the literature, Armstrong and Armstrong (1979) suggested that microtubules function to maintain cell polarity by stabilizing the leading lamella, restricting lamellipodial activity to a limited portion of the cell's margin. BHK-21/C13 (Stoker and Macpherson, 1964) cell motility has been functionally associated with the presence of three cytoplasmic fibres: microtubules, filaments, and microfilaments (Goldman and Follett, 1969; Goldman and Follett, 1970). Cytoplasmic microtubules have been implicated in the normal motile processes of a variety of cells, from the movement of heliozoan axopods to pigment granule migration in Fundulus melanophores (Tilney and Porter, 1965; Tilney et al., 1966; Bickle et al., 1966). The

drug colchicine, which reversibly breaks down microtubules, has been used to define the motile functions of microtubules in many cell systems (Bhisey and Freed, 1971; Freed and Lebowitz, 1970; Wessells et al., 1971). This drug presumably acts by binding to protein subunits of the microtubule walls (Borisy, 1967). Goldman (1971) utilized colchicine to determine the possible functions of microtubules in normal BHK-21 cell motility. This study has also helped to define possible functional relationships between the three types of cytoplasmic fibres found in BHK-21 cells. The results indicate that microtubules are involved in the formation and maintenance of fibroblastic shape in normal cells. Microtubules rapidly disappear after exposure to colchicine, resulting in epithelial-like cell morphology. In colchicine-treated cells, the 100-120 angstrom filaments were arrested in a juxtanuclear position, indicating an inability to move away from the nucleus in the absence of microtubules. Goldman's results suggest that microtubules play important roles in the determination of fibroblastic cell-shape, the formation of major cell processes, and cell locomotion. Filament distribution is also affected by the absence of microtubules which suggests a functional relationship between the two types of cytoplasmic fibres. Microfilaments, however, seem to be independent of the microtubule-filament complex and perhaps play important roles in cell spreading and membrane ruffling.

Effect of colchicine on secretary function of microtubules
In a study by Ehrlich et al., (1977) embryonic chick cranial bone was cultured in the presence of the antimicrotubular agents, i.e. colchicine,vinblastine, and cytochalasin B which are known from previous studies to affect the cellular handling of collagen. Secretion of procollagen, quantitated by light microscope autoradiography, was correlated with the extent of conversion of procollagen to collagen and with rates of collagen and non-collagen-protein synthesis. Colchicine inhibited procollagen secretion and conversion to collagen and specifically inhibited collagen synthesis. Cells exposed to colchicine revealed an increased number of dilated Golgi-associated vacuoles and vesicles, some of which contained parallel aggregates of filamentous structures (Ehrlich et al., 1977).

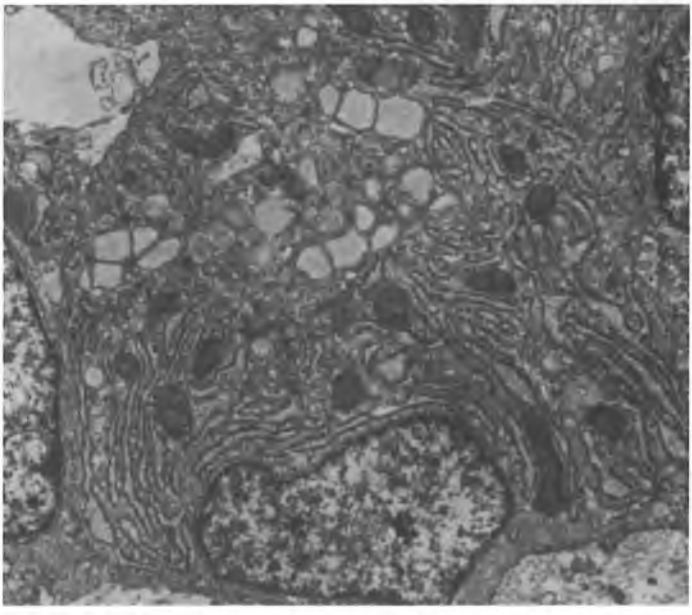

FIGURE 1: *This is a relatively low-power electron micrograph of several osteoblasts in bone treated with COL 10^{5} M. The marked enlargement of Golgi associated vacuoles can be seen. The rough endoplasmic reticulum in this cell is not altered in its appearance. In a number of Golgi vacuoles filamentous structures are seen, which could be a sign of procollagen secretion. The appearance of increased numbers of large Golgi associated vacuoles was most striking after COL treatment. Courtesy of The Rockefeller University Press*

These observations suggest that the pathway of at least a fraction of procollagen secretion by osteoblasts includes the Golgi complex. Disruption of microtubules may interfere with the movement of Golgi-derived vesicles, and the resulting accumulation of collagen precursors in the Golgi complex may lead secondarily to an inhibition of synthesis. Although vinblastine also inhibited both procollagen secretion and conversion to collagen, the observed reduction in general protein synthesis and striking changes in the ultrastructure of the rough endoplasmic reticulum complicated interpretation of the effects (Ehrlich et al., 1977).

8. Chapter: Vinblastine and metaphase arrest

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Summary:
Inactivation of the structure and function of the mitotic apparatus, particularly mitotic spindles, after reacting with some external agents leads to metaphase arrest and consequently inhibition of the cell division.

The configuration of the mitotic apparatus and its functional mechanism is considered to consist essentially of a polarized and oriented array of fibrous proteins (Schmidt 1937; Swann 1952) the spindle, of which at least the chromosomal fibers in some cells are contractile and are perhaps under some sort of control by the kinetochores or centromeres and by the centrioles of centrosomes, if available. The other fibers of the spindle and the asters, which radiate from the spindle poles in some cell types, appear to have some characteristics of contractile proteins.

Periwinkle (*Vinca*) alkaloids, are useful drugs owing to their therapeutic efficiency against neoplastic diseases, identified to affect cell division by different approaches (Palmer et al. 1960). For example, over dosage of these therapeutic drugs damage the organs rich in microtubules, for instance, neurons (Uy et al. 1967). Preceding studies illustrated that these compounds, including vinblastine, showed the rapid decline and disappearance of birefringence of mitotic spindle after inoculation of these drugs in eggs of the marine annelid; in addition, an electron microscope clarified the disappearance of microtubules after exposure to such alkaloids (Malawista et al. 1968). The same

drugs, when used in a study of microtubules of polymorphonuclear leukocytes, resulted in the disappearance of microtubules and generated cells intracellular bodies of singular regularity which introduced the definition of a crystal (Bensch and Malawista, 1968). Periwinkle alkaloids in very low concentrations (VBLS = 2.5×10^{-5}M) cause an intra-cytoplasmic conversion of microtubule protein in the shape of regular microtubular bodies. The length of these crystals is normally up to 8 mcm; in L-strain fibroblasts *in vitro* their size increases with time when exposed to the alkaloids. Likewise, human leukocytes *in vitro* comprise the same crystalline structure after exposure to these substances. Neither colchicine nor puromycin can stop the formation of this type of structure; however, the latter compound can delay crystal growth (Bensch and Malawista 1969).

Likewise, vinblastine (VLBS), an alkaloid derived from *Vinca rosea*, has been found to play a role in metaphase arrest during mitosis of cells in tissue culture (Berrah and Konetzka 1962). The VLBS has been used as a therapeutic substance against the many types of cancers in human beings, exhibited as an inhibitor of cell division (Hodes, Rohn et al. 1960; Warwick, Darte et al. 1960). Palmer et al. (1960) studied the effects of VLBS at the cellular level during the cell multiplication in cell culture and determined that accumulation of VLBS arrested the metaphase through inhibiting the spindle formation, consequently stopping cell division. Another study compiled similar results of metaphase arrest after *in vivo* application of vinblastine (Cutts 1961).

Another work reported the effects of vinblastine sulfate on the colony-forming ability of L cells *in vivo*. L cells were exposed to vinblastine sulphate (VLBS) during the exponential growth phase in cell culture for different lengths of time and verified their ability to form macroscopic colonies and to growth through the phases of the cell-division cycle. L cells were found to inhibit the proliferation at 10^{-7} gm/ml concentrations of VLBS and started accumulation of mitotic structures which reached to the maximum stage after doubling time approximately (Bruchovsky, Owen et al. 1965). The VLBS treated cells in culture displayed a continued loss of colony-forming

function, which was closely related to the gathering of mitotic structures. When cells were prohibited from going to mitosis by the implication of a reversible inhibitor of cell growth, such as phenethyl alcohol, they displayed no such continued loss of colony-forming function even in the existence of VLBS. Incompletely synchronized cell cultures, which have the colony-forming ability only in the late G1 phase, were tested against VLBS. The results showed that no colony-forming function was lost until the existence of VLBS inhibited the cell division. The time relationship studies during different phases of cell cycle in VLBS treated culture did not displayed significant inhibiting effects on the growth of L cells through different phases of cell cycle except mitosis (Bruchovsky, Owen et al. 1965).

Another study reported the cytological effects of alkaloid drugs on Earle's HeLa epidermoid carcinoma cells (EHE) of the cervix and J96 extracted from the blood of monocyte leukemia. They compared the level and time interval of metaphases arrest in cell culture by methiodide, leurosine and vinblastine; colchicine and demecolcine were used for reference purposes. All four drugs produced a similar cytological effect, but vinblastine was most active and continued active for the longest duration. For this purpose, they cultivated cells in a cell culture medium with 10 percent horse serum for 24 hours under standard conditions of cell culture. After 24 hours, the culture medium was replaced with fresh medium and treated with different concentrations of vinblastine sulphate and grown according to standard laboratory procedures. Afterwards, cells were trypsinized, fixed in ethanol-acetic acid (3:1), stained with aceto orcine, and observed under phase microscopy for effective mitosis, *i.e.,* 2000 cells at each concentration of VBLS added were scored for dividing cells/ mitosis or metaphase arrests. They found that VBLS is more effective in producing cell arrests in metaphases at lower concentrations in EHE cells. Most effective results were at approximately at 0,001 microgram VBLS/ml EHE cells tested = 1 nanogram VBLS/ mL cells tested, with 452 percent increase in metaphase arrests (Palmer et al., 1963). At approximately 0.01 microgram VBLS/ ml EHE cell suspension 200 percent

increase in metaphase arrest was noted. At approximately 0.1 microgram VBLS/ ml EHE cells suspension tested = 100 nanogram VBLS/ mL cells tested, 129 percent increase in metaphase arrests were noted (Palmer et al., 1963). At higher concentrations (1 microgram VBLS/ml cell suspension = 60 percent inhibition of metaphase; 5 microgram VBLS/ mL = 80 percent increase of metaphase inhibition) the metaphases are of the "ball type" in EHE cells, which is evidence of the toxicity of the drug at high concentrations (Eigsti and Dustin, 1957), i.e., 5 microgram VBLS / ml EHC-cells produced 0.8 increase of metaphase arrests. VBLS makes metaphases for the long time period compared to colchicine, such as VBLS take 36 hours while colchicine takes 24 hours. Thus, vinblastine is considered more effective than other alkaloids; however, a higher concentration of leurosine and methiodide is needed to create c-mitosis (Cutts, 1961; Palmer et al., 1963).

There are two types of mitosis arrest; oriented and un-oriented, both be determined by several variables prevailing during therapy or during a recovery from the drug. The concentration of the drug, mitotic stage at time of action, time duration of exposure, recovery procedures, type of cell, and conditions favorable to mitosis, all play an essential role in the production of the particular arrested metaphase, whether oriented or unoriented. Spindle disorders can be classified in three categories: (1) full inactivation, stathmokinesis, (2) partial inactivation, merostathmokinesis, and (3) slight disturbance in orientation, tropokinesis (Eigsti and Dustin, 1955). All these types are produced by alkaloid drugs. Scientists make comparative studies with other chemicals known to influence mitosis; well-defined cytological standards of judgment are needed to classify reactions as either disturbed or normal. If the reaction is disturbed, it is important to distinguish the type according to velocity or strength of the reaction. The most reliable criteria appear to be those based upon tests at telophase, rather than at earlier stages (Östergren 1950). The isolation of the mitotic apparatus in bulk quantity by process of "selective solubilization" from dividing sea urchin eggs by (Mazia and Dan 1952), was a great step forward toward the biochemical characterization of the apparatus, the structure of

the mitotic apparatus could be recovered fairly free of other cytoplasmic material, thus, may clearly provide proof of its existence and disappearance.

Nuclear mitosis and the complete procedure of cell cycle are not synonymous, as the nuclear and cytoplasmic processes are reserved together during cell division. Actually, the karyokinesis (nuclear mitosis) and cytokinesis (cytoplasmic developments) are highly incorporated and are closely coordinated processes (Eigsti and Dustin, 1955). One cannot always mark the separation between the processes. For this reason and perhaps others, scientists use the term mitosis as entirely identical with cell division, when mitosis is only one feature of a dividing cell (Hughes 1949). If vinblastine acts during a division, the significant difference between mitosis and cell division becomes clear. The multiplication of chromosomes continues in the occurrence of the drug at a certain concentration, whereas the totally absence of spindle fibers avoids the transport of chromosomes to the particular poles.

Main Body:
Nuclear mitosis and the completed process of cell division are not synonymous because the nuclear processes and the cytoplasmic processes taken together make up cell division. Every mitotic cycle builds anew the spindle fibers. Cytoplasmic separation, a function of cytokinesis, is closely coordinated with the fiber and spindle functions (Vilter, 1944). Abnormal chromosomal distributions many be caused by spindle dis-

FIGURE 1: *Four large crystals (arrow) in metaphase arrested L-fibroblasts. 1 mcm thick diameter, 8 mcm longitude, plastic-embedded material observed under phase microscope, 10^{5} M VLBS, 8 hr incubation, x 2000 (curtesy of Dr Narmunescu).*

turbances in three degrees: multipolar, apolar, and unipolar (Eigsti and Dustin, 1955). When three or more groups of chromosomes join to form discreet groups, partial spindle disturbances are obvious. When anti-tubular agents act during the cell division, the significance of what has been noted for mitosis and cell division becomes apparent. The multiplication of chromosomes continues in the presence of the drug at a certain concentration, whereas the total absence of spindle fibers prevents the movement of chromosomes to the respective poles (Eigsti and Dustin, 1955). Inhibition of fibers has one drastic effect on the cytoplasmic phases of cell division: the cytokinetic processes are completely eliminated.

Effect of Vinca alkaloids on microtubules

Bensch and Malawista (1969) found that the periwinkle alkaloids in very low concentrations cause an intracytoplasmic sequestration of microtubule protein in the form of symmetrical, microtubular bodies. These crystals, which may measure up to 8 mcm in length, appear within 30 minutes in L-strain fibroblasts *in vitro,* but they increase in incidence and size with time of exposure to the alkaloids.

Similarly, if exposed to these compounds, human leukocytes in vitro contain identical crystalline structures. Neither colchicine nor puromycin prevents the formation of these bodies, the latter compound, however, retards crystal growth. The results of the experiments in which only the periwinkle alkaloids were used were essentially identical, with little difference in effect between the two periwinkle compounds or the different cell types (Bensch and Malawista, 1969).

The same alkaloids, when used in a study of microtubules of polymorphonuclear leukocytes, again made the microtubules disappear but produced in these cells the intracellular bodies of singular regularity which fulfilled the criteria for definition of a crystal. This observation led to a systematic examination of the effects of the *Vinca* alkaloids vinblastine and vincristine sulfate on microtubules in mammalian cells. In another study, human leukemic lymphoblasts exposed to velban (vinblastine) showed prominent proteinaceous crystals and large complexes

of ribosomes (which are arranged in clusters and helices) in association with fine granular, electron dense material. This granular material showed relatively greater electron density than the crystals and lacked the prominent linearity of the crystals. In some cross sections tubular profiles were seen interspersed between the granular material and the ribosomes. It was suggested that the ribosomes are involved in the synthesis of the electron dense material which is subsequently organized into the prominent crystals.

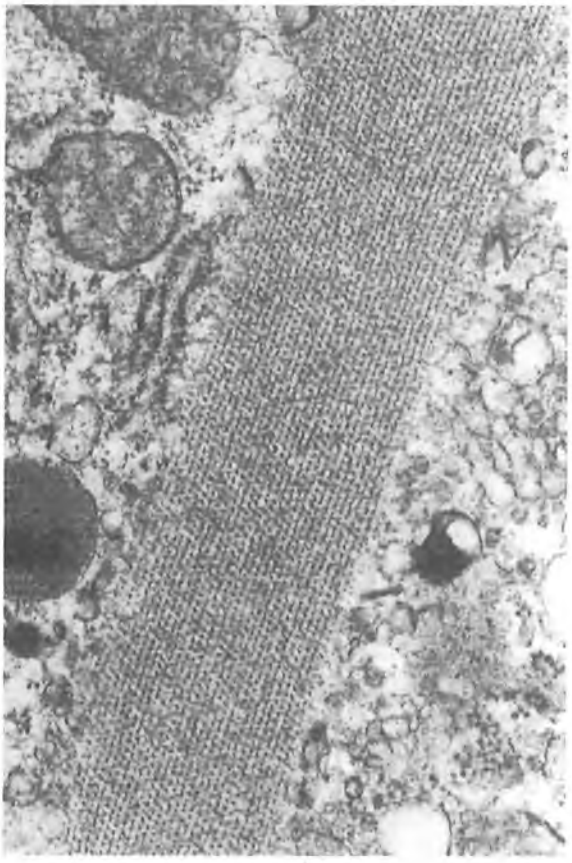

***FIGURE** 2: A longitudinally sectional crystal shows clearly rows of dots with fine interconnections between the latter; 10^{-5} M VLBS, 4 hours of incubation, x 81.000 (curtesy of Dr Narmunescu).*

Colchicine & mitoses

Mazia (1956a) found that COL attacks the secondary bonding of the gelated spindle protein into fibers, but not the initial formation of the gel. This led to his speculation that perhaps COL acts through the mitotic centers and kinetochores. Lettre (1952) showed that the effect of COL on mitosis was the inhibition of a reaction between ATP and a contractile system of actomyosin type. COL in concentrations to about 2.5×10^{-8} M (which is near or below the usual low threshold of COL mitotic activity) brought about a 25 to 30 percent decrease in the rate of the viscosity of heart actomyosin gels induced by ATP (Barany and Palis, 1952). He also found that actomyosin threads treated with COL no longer contracted when exposed to ATP. Sacroblast ribbons grown in tissue culture were disrupted by COL at 10^{-8} M or more, apparently because of disorientation of a system of extended protein micelles, which were probably protofibrils of actomyosin (Godman, 1955). The effect of COL on mitosis is characteristic. It produces a picture dubbed "COL-mitosis" or "c-mitosis" by Levan (1938). Since similar effects are produced by many other agents, the use of the term is by no means restricted to cell division influenced by COL. In distributed c-mitosis occurring in onion roots exposed for 8 hours to COL, D' Amato (1948a) found chromosomes in groups of 10 and 6, 11 and 5, and 12 and 4, commonly; in groups of 13 and 3, 14 and 2, and 9 and 7 less often; and only occasionally in groups of 8 and 8. Tropolone at 10^{-4} M reversed the metaphase arrest cause by 2×10^{-6} M COL in a sudden effect that appeared after 16 hours of simultaneous exposure of the fibroblasts to the two agents (Benitez at al., 1953).

Effects of alkaloid drugs on tumor

Vinblastine sulfate (VLBS) is an inhibitor of cellular proliferation, which has been used in the treatment of a number of human cancers (Hodes et al., 1960; Warwick et al., 1960). Its action at the cellular level has been studied by Palmer et al. (1960) with 2 lines of proliferating cells in cell culture. These workers observed an accumulation of metaphases in VLBS-treated cultures and concluded that VLBS acts by inhibiting spindle formation, resulting in metaphase arrest. A similar conclusion was reached by Cutts

(1961), who studied the mitotic arrest induced by VLBS in vivo. However, these investigations did not establish to what extent the inhibitory effects of VLBS are reversible, nor was it clear whether the progress of cells through the phases of the division cycle other than mitosis was affected by the presence of VLBS. In the work reported in by Bruchovsky et al. (1965), the effects of VLBS on colony-forming ability of L cells in culture were examined as a function of the position of the cells in the division cycle. The cell line used, termed L60TM, was derived from L60, a subline of Earle's L-cells.

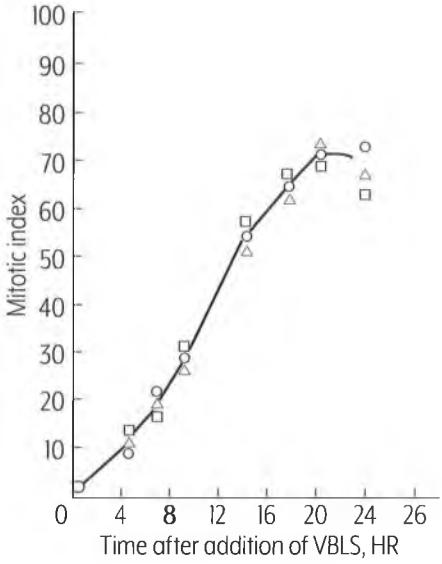

FIGURE 3: *Accumulation of mitotic figures in the presence of different concentrations of VLBS. Multinucleated cells were included in the mitotic index; Growth curves for L cells treated with various concentrations of VLBS,* △*5 x* 10^{-8} *mg/ mL;* ○10^{-7} *mg/ mL,* □*2 x* 10^{-7} *mg/ mL*

The results obtained indicate that both retardation of progress through the cycle and irreversible loss of colony-forming ability due to the action of VLBS occurring in cells at or near mitosis. For cells in the remainder of the cell cycle, neither colony-forming ability nor the rate of progress through the cell cycle is detectably affected by the

action of VLBS. These properties make VLBS very suitable as an agent for studying the cell cycles of proliferating cell populations both *in vitro* and *in vivo* (Bruchovsky et al., 1965). The time required to suppress the proliferative capacity of cells randomly distributed around the cell cycle would be expected to provide an estimate of the generation time of the cells.

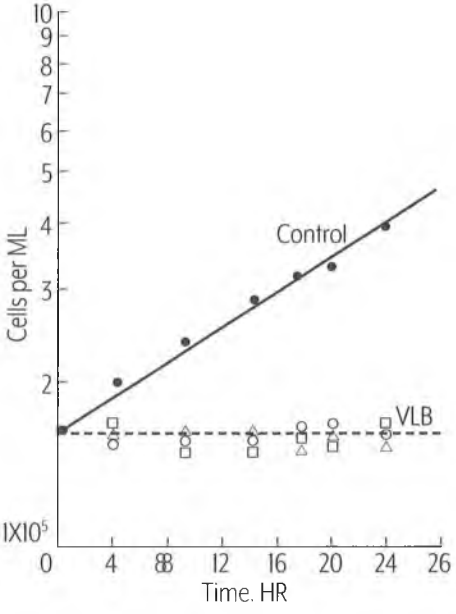

FIGURE 4: *Growth curves for L cells treated with various concentrations of VLBS,* • *untreated (doubling time 18hours),* △*5 x 10⁻⁸ mg/ mL;* ○*10⁻⁷ mg/ mL,* □*2 x 10⁻⁷ mg/ mL*

Another study reported the cytological effects of alkaloid drugs on Earle's HeLa epidermoid carcinoma cells (EHE) of the cervix and J96 extracted from the blood of monocyte leukemia (Palmer et al., 1963). They compared the level and time interval of metaphases arrest in cell culture by methiodide, leurosine and vinblastine; colchicine and demecolcine were used for reference purposes. All four drugs produced a similar cytological effect, but vinblastine was most active and continued active for the longest duration. For this purpose, they cultivated cells in a cell

culture medium with 10 percent horse serum for 24 hours under standard conditions of cell culture. After 24 hours, the culture medium was replaced with fresh medium and treated with different concentrations of vinblastine sulphate and grown according to standard laboratory procedures. Afterwards, cells were trypsinized, fixed in ethanol-acetic acid (3:1), stained with acetoorcine, and observed under phase microscopy for effective mitosis, *i.e.*, 2000 cells at each concentration of VBLS added were scored for dividing cells/ mitosis or metaphase arrests.

They found that VBLS is more effective in producing cell arrests in metaphases at lower concentrations in EHE cells. Most effective results were at approximately 0,001 microgram VBLS/ml EHE cells tested = 1 nanogram VBLS/ mL cells tested, with 452 percent increase in metaphase arrests (Palmer et al. 1963). At approximately 0.01 microgram VBLS/ ml EHE cell suspension 200 percent increase in metaphase arrest was noted. At approximately 0.1 microgram VBLS/ ml EHE cells suspension tested = 100 nanogram VBLS/ mL cells tested, 129 percent increase in metaphase arrests were noted (Palmer et al. 1963). At higher concentrations (1 microgram VBLS/ml cell suspension = 60 percent inhibition of metaphase; 5 microgram VBLS/ mL = 80 percent increase of metaphase inhibition) the metaphases are of the "ball type" in EHE cells, which is evidence of the toxicity of the drug at high concentrations (Eigsti and Dustin, 1957), i.e., 5 microgram VBLS / ml EHC-cells produced 0.8 increase of metaphase arrests. VBLS makes metaphases for the long time period compared to colchicine, such as VBLS take 36 hours while colchicine takes 24 hours. Thus, vinblastine is considered more effective than other alkaloids; however, a higher concentration of leurosine and methiodide is needed to create c-mitosis (Cutts 1961; Palmer et al. 1963).

9. Chapter: Colchicine and vinblastine sulphate on monocytes & fibroblasts

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Summary
Patients with familial Mediterranean fever (FMF) who were part of a double-blind trial of daily colchicine as prophylaxis for their disease had leukocyte functions studied while receiving colchicine or placebo. Leukocytes taken from these patients while on prophylactic doses of colchicine produced normal quantities of leukocytic pyrogen, ingested bacteria normally, and migrated normally in chemotactic chambers. In addition, these patients had normal numbers of circulating T and B lymphocytes as well as normal blastogenic responses of their peripheral lymphocytes to mitogenic stimuli. The patients on colchicine, however, had significantly fewer neutrophils and monocytes accumulating at skin-window sites 24 hours after the initial abrasion. Because the early phase of the skin-window response was normal in these patients, the decreased late response may be related to a failure to amplify the initial inflammatory reaction. The reduced capacity to generate a normal inflammatory response may account for the failure of these patients to develop full attacks while taking colchicine.

Main Body:
Daily colchicine has recently been shown to be highly effec-

tive in preventing the attacks of familial Mediterranean fever (FMF) in patients with this disorder (Dinarello et al., 1974; Zemer et al., 1974). Although there is evidence that colchicine interferes with cell migration and the metabolic processes of phagocytosis when the drug is added in vitro to human leukocytes Caner, 1964; Goldfinger et al., 1965; Malawista and Bodel, 1967), the mechanism of colchicine's action in FMF remains unknown. (Gallin and Rosenthal, 1974 have shown the dependence of migration of human granulocytes on calcium ions in the presence of in concentrations of COL, which can block their directed movement. They concluded, that directed movement of granulocytes would be dependent on assembly of microtubules and intracytoplasmic liberation of calcium ions. During a double-blind trial using colchicine (0.6-1.8 mg/day) in patients with FMF, the patients' leukocytes were studied while they were taking either colchicine or placebo to determine any functional change induced by the drug in this disease.

The results of this study for pyrogen production were as follows. Eight patients taking two or three colchicine (0.6 milligram) tablets per day for 2 weeks to 1 year were studied. Leukocytes obtained from these 8 patients while on colchicine produced normal amounts of pyrogen. In 34 rabbit assays the mean (±SEM) of the peak fever (rise above baseline temperature) in the colchicine group was 0.64 degrees centigrade ±0.05, and in 50 determinations in the control group the mean was 0.65 degrees centigrade ±0.08. Five patients were also studied while participating in a double-blind trial in which patients served as their own control (Dinarello et al., 1974). The mean peak fever from supernates of leukocytes from patients taking colchicine was 0.49 degrees centigrade ±0.06 and from patients taking placebo 0.65 degrees centigrade ±0.05. When a paired t test was used, this difference was not significant ($P > 0.5$).

The results for phagocytosis were as follows. Six patients on colchicine therapy for periods of 2 weeks to over 1 year were studied. Phagocytic uptake of labelled *S. aureus* by leukocytes from patients on colchicine was not different from that of normal controls. The mean (±SEM) percent uptake of *S. aureus* after 20 minutes was 54.3 percent ±4.0 in patients taking

colchicine and 51.9 percent ±2.5 for concurrently studied controls. This difference was not significant. Lymphocyte studies showed that the FMF patients receiving colchicine had total lymphocyte counts, proportions, and total numbers of circulating T and B lymphocytes that were no different from controls. The mean (±SEM) *in vitro* lymphocyte blastogenic responses expressed as difference between stimulated and unstimulated (control) cultures (Δcpm) in the colchicine group were 202,442 ± 34,747 cpm, 191,713 ± 24,712 cpm, and 94,136 ± 17,947 cpm for PHA, Con A, and PWM respectively. The values in the normal control group were 204,013 ± 20,764 cpm, 150,127 ± 20,118 cpm, and 78,477 ± 11,971 cpm for phytohemagglutinin (PHA), concavalin A (Con A), and PWM respectively. There were no differences between the two groups with any of the mitogens studied ($P > 0.2$). In 2 patients who underwent lymphocyte studies both before and 1 to 3 months after initiation of colchicine therapy, there were no significant differences in any of the above lymphocyte studies.

Leukocyte migrations in 9 patients taking colchicine were compared to those of 15 control subjects, including 4 patients taking placebo. After 3 hours of incubation, no cellular abnormality of neutrophil chemotaxis or random migration was detected in cells obtained from FMF patients taking placebo ($P > 0.05$, t test). Similarly, there was no detectable defect in the kinetics of in vitro random or directed neutrophil migration in the chemotactic chambers after 1 or 2 hours (Dinarello et al., 1974).

Skin-window responses were studied in 11 patients with FMF both on and off colchicine. Skin window technique is used in immunology where the top layer of skin is scraped off making it possible to identify the immune response that would occur with a diminished physical barrier in the host and observe mobilization of leukocytes. The patients on colchicine had been on this therapy for at least 1 week. Compared to normal subjects, FMF patients off colchicine had normal skin-window responses. In FMF patients on colchicine the first 9 hours of the response were similar to those in normal controls; however, after 9 hours the response was reduced. The geometric mean number of neutrophils and monocytes at 24 hours was

significantly reduced ($P < 0.05$) when compared to both normals and FMF patients off colchicine.

Attacks of FMF have clinical characteristics very similar to those of an acute inflammatory process, and this fact has been confirmed histologically with tissues removed at the time of the attack (Brick and Cajigas, 1951). Although there has been some speculation that FMF is an inborn error of metabolism (Sohar, et al., 1967), the inducing agent or mechanism of the acute attack remains unknown. Nevertheless, daily colchicine is highly effective in preventing these attacks and hence may be important in understanding the pathogenesis of this disease. In a previous report, the present authors noted that patients with FMF often had premonitory symptoms of an attack, but while the patients were taking daily colchicine these early symptoms rarely progressed into a full attack (Dinarello et al., 1974). This finding suggested that the underlying excitatory process in FMF remained intact in these patients but that colchicine prophylaxis abated further development of the attack process. Dinarello et al. (1976) tried to correlate the therapeutic efficacy of colchicine in preventing these attacks with changes in host leukocyte function. There is ample evidence that colchicine rapidly binds to the protein subunits that form microtubules in cells (Wilson et al., 1974; Borisy and Taylor, 1967). Thus, leukocytes were obtained from patients taking therapeutic doses of the drug and then their cells were studied *in vitro*.

Changes in the ability of leukocytes from patients on colchicine either to release leukocytic pyrogen or to ingest staphylococci could not be demonstrated. In addition, there was no detectable change in numbers of circulating T and B lymphocytes. It is well known that *in vitro* colchicine interferes with cell division (Dustin, 1963). In the present colchicine-treated patients, the blastogenic responses of lymphocytes to three commonly used mitogens were normal. This result may be related to the fact that the presence of the drug is required *in vitro* to interfere with the process of division in cell cultures. Despite the fact that the impairment of cell locomotion by colchicine has been repeatedly demonstrated (Gallin and Rosenthal, 1974; Caner, 1964), both random and directed migration

of neutrophils obtained from patients on colchicine were normal in the study by Dinarello and coworkers (1976). In the previous studies (Gallin and Rosenthal, 1974; Caner, 1964) this impairment occurred only when there were high concentrations of the drug in the incubating medium.

Utilizing Boyden chambers, the effect of colchicine on the migration of human PMNL through Millipore filters with pores 3.0 microns in diameter toward suspensions of *S. albus* on the other side was investigated in a series of experiments (Caner, 1964). The Boyden chamber is a useful tool to study cell migration and cell invasion. It consists of a cylindrical cell culture insert nested inside the well of a cell culture plate. The directed migration of leukocytes towards a chemical compound has been referred to as chemotactic response, which presumably can be modified by COL. Migratory cells move through the pores toward the chemoattractant liberated by *S aureus* below and can be stained or quantified in a plate reader.

Schematic cross section of filter

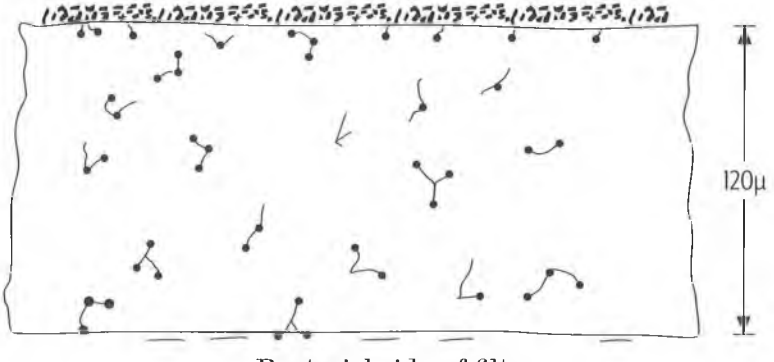

FIGURE 1: *Schematic cross section of filter. The filters after cleaning xylene allowed visualization of cells at all levels. Note the dense layer of leukocytes on the cell side and the ameboid forms actively migrating through the pores of the filter. Those that reached the far side spread out on the surface there forming an easily counted monolayer (CANER, Arthritis and Rheumatism, Vol 8 No 5, Oct 1965, Courtesy of The Rockefeller University Press)*

Evaluation of the results in Caner's (1965) study by comparison of average filter counts from individual experiments consistently showed a chemotactic response to *S. albus*, and dose-related inhibition of that response by colchicine. A semi-log plot of the combined data in the full range of colchicine concentrations employed showed that progressively fewer leukocytes crossed to the bacterial side from 0.025 to 2.5 micrograms per millilitre.

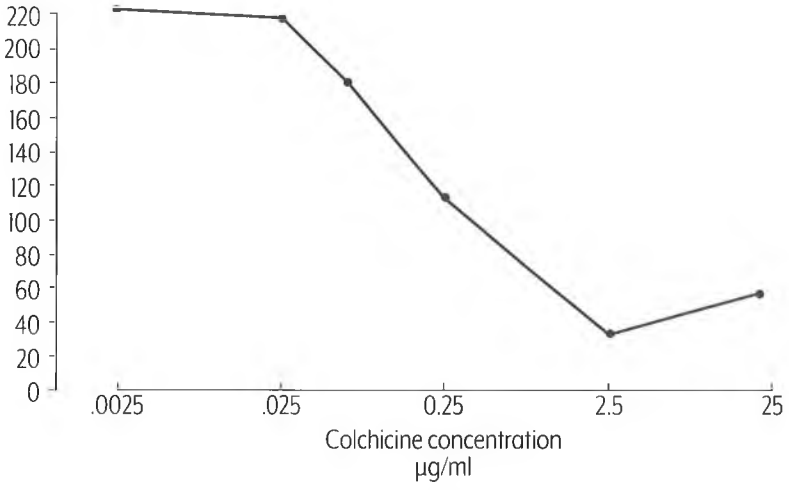

***FIGURE** 2: Mean count bacteria Boyden chamber with colchicine, vertical: mean count of leukocytes crossing the millipore filter. The higher the concentration the of colchicine, the less the number of leukocytes crossing the membrane towards chemoattractant of S aureus (CANER, Arthritis and Rheumatism, Vol 8 No 5, Oct 1965 Courtesy of The Rockefeller University Press).*

The reduction in the number of cells crossing at each concentration was analysed by *t* test and a significant reduction in the relative number of leukocytes crossing was apparent down to a colchicine concentration of 0.025 micrograms per millilitre Caner's (1965).

The study by Dinarello et al., (1974), using the skin-window test was able to demonstrate altered inflammatory responses in patients with FMF taking colchicine as compared to nor-

mal subjects or FMF patients taking a placebo. The abnormal responses were characterized by a reduction in the numbers of both neutrophils and monocytes accumulating at 9 to 24 hours after the initial stimulus. The authors believe that these observations are reliable because these tests were performed by an investigator who was blinded to the patients' therapy and because cell responses were counted by a technician who was unaware of the circumstances of the tests. Both neutrophils and monocytes migrated in normal numbers during the early phase of the skin-window responses, both on and off colchicine. This finding suggests that the inflammatory stimulus was similar in both groups. It also suggests that reduced leukocyte adherence to the glass coverslips was not an artifactual reason for the results observed, although other investigators have shown that colchicine does reduce leukocyte adherence to glass and nylon (MacGregor, 1974, Penny et al., 1966). The abnormal late phase of the skin-window responses with colchicine therapy may be related to an impairment of the generation of certain factors that amplify the acute inflammatory response. Neutrophil immobilizing factor (Goetzl and Austen, 1972), a complement-activating substance (Wright and Gallin, 1974; Goldstein and Weissmsnn, 1974), a neutrophil chemotactic factor (Wright and Gallin, (year unavailable); Zigmond and Hirsch, 1973), and a mononuclear cell chemotactic factor (Ward, 1968) have been demonstrated to exist preformed in neutrophils.

These and other factors are probably released as a consequence of phagocytosis and degranulation and may play a role in the amplification and localization of the inflammatory process. Because colchicine decreases neutrophil degranulation (Rajan, 1966), it may also modify release of cellular factors amplifying the acute inflammatory response. This failure to mobilize cellular factors associated with the amplification of the inflammatory response may account for both the diminished skinwindow response at 24 hours as well as the failure of progression of FMF attacks in patients taking colchicine.

10. Chapter: Chemotaxis of tumor cells and leukocytes

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Summary:
Although it has been known for many years that some motile plant and animal cells move towards or away from foci from which certain chemical substances are diffusing but the mechanism involved in these chemotactic responses are not understood. This applies particularly to chemotaxis among metazoan cells. Even in the case of mammalian leucocytes, there was a lack of information regarding the chemical nature of the stimuli cells respond to for a long time. While it was a relatively simple matter to show *in vitro* that leucocytes migrate towards a clump of bacilli, it was very hard to show whether a given soluble bacterial product is chemotactic (Boyden, 1962). Attempts were made to estimate the chemotactic effect of soluble substances by absorbing them onto various sorts of particles which were then tested for a capacity to attract leucocytes. This technique is very imprecise and it is impossible to use it for making quantitative comparisons between different substances for chemotactic activity. Earlier investigations showed that substances like human serum albumin (H.S.A.), which normally do not attract leucocytes, elicit a strong chemotactic response in the presence of the corresponding specific antiserum. This observation led to some experiments on the chemotactic activity of antibody-antigen complexes (Boyden, 1962).

Once it had been established that the migration of leukocytes and fibroblasts out of blood vessels is controlled by chemotactic signals, their sources were identified. It turned out that these signals are generated from a variety of sources, especially the complement system (Snyderman et al., 1971). In acute inflammatory reactions, such as those triggered by

tissue deposition of immune complexes, biologically active mediators (especially C5a from the fifth component of complement [C5]) seem to play a key role in directing the movement of neutrophils out of the bloodstream (Johnson and Ward, 1974). Lam et al., (1981) showed that neutral proteases (such as elastase), released either from inflammatory cells or from normal tissues, will act on C5, resulting in the production of a peptide that is chemotactic for tumor cells (Orr et al., 1979; Romualdez et al., 1976). The production of this signal *in vivo* could presumably cause the efflux of tumor cells from the bloodstream into the area in which the peptide has been generated. In their research, Lam et al., (1981) reported that the responses of tumor cells such as fibrosarcoma cells to chemotactic agents are similar to the responses of leukocytes to leukotactic factors. Just as in the case of neutrophils, tumor cells exhibited directed migration, volume changes, and increased adherence to nylon fibers following appropriate stimulation by chemotactic factor for tumor cells. *In vivo*, the same chemotactic agent induced diffuse local metastasis when injected into animals with circulating tumor cells. The data affirm the biologic activity of chemotactic factors for tumor cells and suggest that movement of tumor cells from the circulation may be under chemotactic influence in a manner similar to that *in vivo* responsiveness of neutrophils to leukotactic stimuli (Lam, et al., 1981).

It is generally accepted that tumor cells grown in tissue culture indefinitely retain their ability to produce tumors when injected into animals of the strain from which the tissue originated (Sanford, et al., 1955).. A clone (clone L 929) of mouse cells derived in 1948 from a single cell of strain L has been examined for tumor-producing capacity. Strain L originated in 1940 from an explant of subcutaneous connective tissue taken from a strain C3H (Andervont substrain) mouse, and had been cultured *in vitro* in a heterologous culture medium for over 10 years. The incidence of sarcomas produced by this strain had dropped from 68 per cent in 1943 to 1 per cent in 1946. It was found that clone L 929 cells were still capable of giving rise to a low percentage of sarcomas when injected into strain C3H mice. A second finding was that these cells induced an

immune reaction in the strain C3H mouse. A third finding of this study was that tumors derived from clone L 929 could be adapted by serial transfer *in vivo* to grow in all strain C3H mice injected (Sanord, et al., 1955).

The work by Martin (1981) described the direct establishment of a cell line that forms teratocarcinomas from normal pre-implantation mouse embryos when injected into mice. The pluripotency of these embryonic stem cells was demonstrated by the observation that subclonal cultures, derived from isolated single cells, can differentiate into a wide variety of cell types. This study suggested that a conditioned medium might contain a growth factor that stimulates the proliferation or inhibits the differentiation of normal pluripotent embryonic cells, or both. This method of obtaining embryonic stem cells makes the isolation of pluripotent cells lines from various types of noninbred embryo feasible, including those carrying mutant genes.

Main Body:
Many types of cell in the metazoan body are probably capable of characteristics such as adhesiveness, phagocytosis, ameboid movement and directional migration. The differences in the cells, from this behavioral standpoint, lie less in what they are capable of doing than in what particular stimuli induce them to do it. However, in very few instances indeed is information available on the precise nature of the factors which provoke the different sorts of behaviour in the various cell types. Even in the case of mammalian leucocytes, which are relatively easy to handle *in vitro* and which have received a great deal of experimental attention, we are very ignorant of the factors which control the characteristic activities of these cells. By what mechanism, for instance, does the polymorphonuclear leucocyte discern differences between healthy indigenous cells of the host on the one hand and dumps of bacteria on the other, so that it actively migrates towards, and ultimately engulfs the latter, while ignoring the former? The same question can be asked in respect of the ameboid cells of all metazoa. Although good evidence is available that the leucocytes respond by active migration towards chemical substances diffus-

ing from clumps of bacteria, starch grains, etc., (McCutcheon, 1946; McCutcheon, 1955; Harris, 1954), information on the actual nature of the chemotactic stimuli has almost completely been lacking. The general opinion, based on very little evidence, seems to be that the cells react to general classes of chemical substances, such as amino acids, or polysaccharides which are thought to be given off by the attractive particles. The possibility is worth considering, however, that the chemotactic response is a more selective process than this, and that the phagocytes, like the cells responsible for antibody production, respond to the presence in their environment of macromolecules which differ in structure from those normally present in the host. The simplest interpretation of the facts is that antibody and antigen combine to form a complex which interacts with a heat-labile substance (probably an enzyme, perhaps a component of complement), and that as a consequence of this interaction a heat-stable substance is produced which has a direct chemotactic influence on the leucocytes. It is reasonable to suppose that antibody-antigen complexes can exert a chemotactic effect *in vivo* as well as *in vitro*. This has obvious implications in relation to specific acquired resistance to natural microbial infections; it may also offer an explanation for the massive polymorphonuclear infiltration which follows intradermal injection of antigen in the Arthus reaction (Boyden, 1962).

Lam et al (1981) suggested that there is a remarkable parallelism between the response of tumor cells and leukocytes to chemotactic factors. *In vitro*, both cell types show chemotactic migration, cell volume expansion, and hyperadherence in response to chemotactic factors. Their study focused on the responses in tumor cells, as in leukocytes, to the chemotactic factor *in vitro* and found that they correlate with responses to the same factors *in vivo*. These analogous responses indicate that there are quite likely to be underlying similarities in the mechanisms of the responses of the different cell types to their respective chemotactic factors. For instance, it can be predicted that the large body of information concerning leukocyte-chemotactic factor interaction may be applicable to further study of the chemotaxis of tumor cells where considerably less is known. The particle counter assay may be a

useful rapid method of assaying tumor cell chemotactic factor activity, since this assay is much less time-consuming than the Boyden chamber assay. They preseneted the first direct evidence that the derivative of C5a, which is chemotactic for tumor cells *in vitro*, has the ability to bring about the accumulation of chemotactically responsive cells *in vivo*. These results support the earlier observation of Ozaki et al. (1971), who, working with hepatoma cells, described the ability of a chemotactic factor derived from tumor tissue to induced intradermal localization of circulating tumor cells. One hypothesis for the mechanism through which the *in vivo* accumulation of circulating tumor cells is induced by chemotactic factor was suggested that would take into account the observations to date. It was suggested that the cell swelling response and increased adherence of tumor cells, initiated by exposure to chemotactic factor, contribute to the mechanism whereby circulating tumor cells become sequestered at secondary sites *in vivo*. After the initial arrest of the cells, they could migrate through the endothelial junction to the extravascular locale where the chemotactic signal has originated.

The data presented by Lam et al., (1981) indicated that these steps are unlikely to be solely due to the action of vasopermeability agents, although it is difficult to imagine that widening the endothelial junction would strengthen the barrier between the intravascular circulating cells and the extravascular tissues. There is evidence that some tumors have a propensity to metastasize to sites of inflammation, (Ozaki et al., 1971; Fisher and Fisher, 1967) and our previous studies *in vitro* have shown that conditions necessary for the generation of the C5-related chemotactic factor could occur at such locations (Orr, et al., 1979). It may be that the slight enhancement of metastasis induced by the intraperitoneal injection of bovine serum albumin is the reflection of an inflammatory response to this foreign protein. There are several points of resemblance between the processes of cancer metastasis and inflammation. In both, cells circulate in the vasculature, stop, and cross vessel walls, thereby entering the extravascular tissues (Der Hagopian et al., 1978; Movat, 1979). In the case of neoplastic cells, their subsequent proliferation results in the formation of grossly visible met-

astatic tumors. Lam et al (1981) emphasized that tumor cells suc h as fibrosarcoma cells share with leukocytes functional characteristics that could contribute to their ability to leave the circulation in response to extravascular signals. These studies emphasize the possible broad biologic significance of tumor cell chemotactic responses in the process of metastasis.

Sanford et al., (1955) studied a clone (clone L 929) from mouse cells derived from a single cell of strain L with four objectives in mind. The first was to determine whether clone L 929 cells are capable of producing sarcomas in strain C3H mice. Second was to establish whether clone L 929 cells induce an immune reaction in the strain C3H mouse. Third was to determine whether clone L 929 cells can be adapted to grow progressively in 100 per cent of strain C3H mice inoculated. Fourth was to test whether clone L 929 cells can grow in other inbred strains of mice.

The first finding of their study was that the clone of cells derived from strain L 929 was still capable of giving rise to a low percentage of sarcomas when injected into strain C3H/He mice. This incidence (15 per cent) was higher than that obtained in 1947 with strain L cells (1 per cent), but in Sanford and coworkers's (1955) study approximately 6 times the number of cells were injected into each mouse; this increased number of cells may account for the higher percentage of sarcomas produced, since, with tumor strains known to induce resistance in the host, the dosage of injected cells appeared to be an important factor in tumor development (Foley, 1952; Gross, 1943). The high percentage of sarcomas (68 per cent) developing from clone L 929 cells in x-radiated mice suggested that cells of this clone induced a resistance in the nonirradiated host that could be partially abrogated by whole-body x-radiation before cell injection. Further tests confirmed this hypothesis. It was thus demonstrated that clone L 929 cells induce an immunity in the strain C3H mouse.

Although generally a tumor derived from a mouse of an adequately inbred strain will grow in all mice of the same strain, a few cases have been reported of immunity induced in inbred

strains of animals from tumors autogenous to the strain. Gross (1943) demonstrated that a methylcholanthrene-induced sarcoma of strain C3H mice that had been carried for 34 transfers in the strain of origin induced immunity in strain C3H mice. Lewis et al. (1951) found that sarcomas that had originated in two inbred strains of rats induced an immune state in the strain of origin when the tumors were caused to atrophy and regress by strangulation. MacDowell et al. (1934) immunized strain C58 mice, cellosaurus 58 mice, a cancerous cell line, with lymphatic leukemia cells that had originated spontaneously in this inbred strain many generations previously. Fink (1953) demonstrated that, by the 65th generation *in vivo*, a fibrosarcoma induced by methylcholanthrene in a strain BALB/c mouse could immunize mice of this strain. There was some evidence of mutation in the host strain, since the strain of mice in which the tumor originated could be less effectively immunized than a substrain separated about 32 generations prior to tumor origin. Goldfeder (1954) has recently reported that a spontaneous mammary adenocarcinoma of the DBA mouse under certain conditions induced demonstrable immunity in the DBA mouse after eight generations *in vivo*. Foley (1952) likewise demonstrated immunity of strain C3H mice to spontaneous lymphosarcoma 6-C3H-Ed, lymphoma cells of Gardner's original line, originating in this strain. In addition, six unselected, methylcholanthrene-induced sarcomas of strain C3H/He mice were found regularly to induce immunity in this strain when the sarcomas were caused to regress as a result of ligation (Foley, 1953); furthermore, immunity could be induced against all sarcomas at either the first or second transplant generations; thus, an incompatibility between tumor and host was demonstrated before repeated transplants had been made.

In the study of Sanford et al. (1955), the incompatibility between clone L 929 tissue culture cells and the strain C3H mouse has probably resulted from mutational differentiation either in the tissue culture cells or in the host strain C3H mouse during the 13-year period of tissue growth *in vitro*. Just when the initial change or changes took place is difficult to ascertain. When this strain of cells was first tested in 1942 after 2 years of growth *in vitro* and after exposure to the car-

cinogen 30-methylcholanthrene (Earle, 1943), only 46 per cent of the mice injected developed sarcomas. Other strains that originated from the same explant and that had been treated with varying doses of methylcholanthrene, when tested in the same groups of mice, gave varying percentages of sarcomas, ranging from 8 per cent in the strains subject to the highest dosages of carcinogen to 86 per cent in the control untreated strains. It is possible that the varying incidence of sarcomas produced by these six cell strains in 1942 already reflected antigenic variations among the tissue culture cell strains.

When in 1943 and 1944 the six cell strains were studied in transparent chambers, only 13 per cent of all 50 transplants grew progressively as tumors; the others either underwent early necrosis or they grew temporarily and later regressed or survived; however, larger implants of entire cultures of these six strains inoculated into the same source of strain C3H mice gave rise at this time to significantly higher percentages of sarcomas. It appeared that low numbers of cells were not able to grow so readily as larger numbers in the strain C3H mouse. Since, with strains of cells known to induce immunity in the host, the dosage of injected cells is a critical factor in the success of progressive cell growth (Foley, 1952; Gross, 1943; MacDowell, 1934), the failure of these small transplants to grow progressively may have reflected even at this time an incompatibility between strain L cells and the host strain of mouse. In 1948 the first definite indication of an immune reaction in the strain C3H mouse to strain L cells was reported (Algire, 1950).

An additional finding of the Sanford and coworkers' (1955) study was that clone L 929 cells could be adapted by serial transfer to grow progressively in all strain C3tI mice injected. It is not known at the present time whether this adaptation involved the selection of a more malignant or possibly less antigenic sarcoma cell type from a mixed cell population which arose among the progeny of a single cell, or whether further mutational changes occurred in the tumor cells during the serial transfers *in vivo*. From the tests of this study tumors derived from clone L 929 and transplanted from the strain C3H mouse grew progressively only in strain C3H mice; a

specificity of clone L 929 tumor cells for the strain C3H mouse was thus demonstrated.

The observations of Martin's (1981) studied the inner cell mass (ICM). ICM is the mass of cells inside the primordial embryo that will eventually give rise to the definitive structures of the fetus. This study demonstrated that ICM isolated from normal mouse blastocysts and cultured in medium conditioned by an established embryonal carcinoma cell line can give rise to cultures of cells with the characteristics of mouse teratocarcinoma stem cells. These properties include cell morphology, pluripotency, and the ability to form typical teratocarcinomas when injected into mice. In addition, it has been found that the embryonic stem cells described here express the Stage-specific embryonic antigen-1 (SSEA-I) (Solter and Knowles, 1978) common to teratocarcinoma stem cells and early embryos but not expressed by most differentiated cell types (data not shown). SSEA-1, also known as CD15, is a member of a cluster of differentiation antigens that have been identified in various normal tissues and in different types of cancers including papillary and medullary thyroid carcinoma. By using this culture method, it should now be possible to examine *in vitro* the way in which normal early embryonic cells give rise to teratocarcinoma stem cells. The results of such studies may ultimately serve to resolve the controversy surrounding the identity of teratocarcinoma stem cell progenitors in the early embryo and the question of whether the tumor stem cells have undergone a process of neoplastic transformation. These experiments were undertaken on the premise that medium conditioned by teratocarcinoma stem cells might contain a factor, perhaps identical to a normal endogenous embryonic growth factor, capable of stimulating the proliferation of a small population of pluripotent cells in the normal embryo. The success of the approach reported here suggests that this working hypothesis has some validity.

It seems unlikely that it is commonly produced by other cell types. Fibroblastic feeder layers are generally considered potent conditioning agents, but in these experiments ICMs cultured on STO feeder layers, a line established from mouse Sandos inbred mice (SIM) embryonic fibroblasts and frequent-

ly used for establishing embryonic stem cells and maintaining them in an undifferentiated state, did not give rise to embryonic stem cells (ESC) cells unless teratocarcinoma-conditioned medium was present in the early phases of the culture procedure. Obviously, many questions remain to be answered about the teratocarcinoma-derived factor. Its purification will be difficult because the biological assay for its activity involves the growth of cells from isolated mouse embryonic ICMs. It is nevertheless interesting to speculate on its possible relationship to other known tumor-derived growth factors (Todaro and De larco, 1980; Todaro et al., 1980). Ultimately the information that is obtained about this factor should help to elucidate the mechanism by which growth and differentiation are regulated during embryonice development. The culture method described here, as well as the one recently reported by Evans and Kaufman (1961), also has immediate practical value for the isolation of new pluripotent stem cell lines. It provides a means of circumventing the need for "converting" an embryo to a tumor *in vivo*. This makes feasible the isolation of pluripotent cells from embryos that cannot directly form teratocarcinomas when they are transplanted to an extra-uterine site. These include all noninbred embryos because, for reasons that are not yet understood, stem-cell-containing teratocarcinomas can be obtained only when an embryo is transplanted to a histocompatible host; the alternative approach of obtaining stem-cell-containing teratocarcinomas by transplanting embryos to immunodeficient athymic mice has not been generally successful (Solter et al., 1975; Solter and Damjanov, 1979). Many interesting mutations that affect early embryonic development are not maintained in inbred stocks of mice, and it has therefore not been feasible to obtain teratocarcinomas from embryos carrying these mutant genes. In this context it is noteworthy that the pluripotent ESC line described here was isolated from embryos of a noninbred genotype and thus is derived from an embryo that would not otherwise give rise to a teratocarcinoma. Given these results, it seems likely that there will soon be available pluripotent, embryo-derived cell lines with specific genetic alterations that should make possible a variety of new approaches to the study of early mammalian development.

11. Chapter: Chemotaxis of fibrosarcoma, fibroblasts, fibronectin, tropoelastin, collagene

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Chemotaxis in fibroblasts

Fibroblasts play a critical role in inflammatory reactions and wound healing by synthesizing new connective tissue substances (collagen and mucopolysaccharides) and constructing scar tissue. However, the mechanisms by which fibroblasts are attracted to sites of inflammation are unknown. Postlethwaite et al., (1976) used a quantitative technique that measures fibroblast chemotaxis *in vitro* in modified Boyden chambers. While other workers have demonstrated that fibroblasts are capable of migrating *in vitro* and *in vivo* (Abercrombie et al., 1971; Goldman, 1971; Baum, 1971); this was the first description of a quantitative assay that measures fibroblast chemotactic response. Using this assay, they found that human lymphocytes stimulated by specific antigen or nonspecific mitogen *in vitro* produce a heat stable proteinaceous substance that is chemotactic for fibroblasts. It was revealed that peripheral blood lymphocytes stimulated by antigen or mitogen *in vitro* produce a factor that is chemotactic for human dermal fibroblasts. This substance was called "lymphocyte-derived chemotactic factor for fibroblasts" (LDCF-F). LDCF-F is different from the lymphokine that is chemotactic for monocytes or macrophages. Macrophages are required for the generation of LDCF-F by T lymphocytes stimulated by phytohemagglutinin. The fibroblast chemotactic factor is heat stable (56 degrees centigrade for 30 min), trypsin sensitive, and neuraminidase resistant. LDCF-F could function to attract connective tissue

fibroblasts to sites at which cell-mediated immune reactions are occurring *in vivo*.

Chemotaxis in fibroblasts to collagen

Fibroblasts require both a collagen substratum and fibronectin for adherence and subsequent migration in the Boyden chamber. Both fibronectin and lymphokines are good chemoattractants, whereas the collagens are weaker. Fibroblasts appear to require cytoskeletal organization and methylation reactions for chemotaxis, properties shown previously for macrophages and neutrophils. Fibroblasts, however, migrate more slowly than phagocytic cells and appear to require protein synthesis (Gaus-Mueller et al., 1980).

The chemotactic response of human dermal fibroblasts to type I, II, and III human collagens and collagenderived peptides was quantitated by an *in vitro* assay. All three native human collagens and constituent α chains can serve as chemoattractants for fibroblasts *in vitro*. When type I, II, and III collagens were digested by bacterial collagenase, the resulting peptides were also chemotactic. In addition, synthetic di- and tripeptides containing hydroxyproline were also chemotactic for fibroblasts. Since collagen is degraded and remodelled at sites of tissue injury and inflammation, these findings suggest that collagen and collagen-degradation peptides might function as chemotactic stimuli for fibroblasts *in vivo* and attract these cells to effect repair of damaged tissue.

Chemotaxis in fibroblasts to fibronectin

Plasma and cell-derived fibronectin are potent chemoattractants for human dermal fibroblasts *in vitro*. The chemotactic property of fibronectin resides in a major 140,000 molecular weight non-gelatin-binding fragment of the native molecule. Human monocytes and neutrophils do not recognize fibronectin as a chemotactic stimulus. These findings suggest that fibronectin and perhaps certain fragments of fibronectin may function *in vivo* as a specific chemoattractant for fibroblasts and could, therefore, induce directional migration of fibroblasts to sites of tissue injury, remodeling or morphogenesis (Postlethwaite et al., 1981).

Chemotaxis in fibroblast to tropoelastin

Fibroblasts are known to have chemotactic responses to two components of the extracellular matrix, collagen and fibronectin. To extend these observations to other extracellular connective tissue macromolecules and their proteolytic fragments, fibroblasts from adult human skin and from late-gestation (270 d), fetal bovine ligaments were studied for chemotactic responsiveness to tropoelastin and elastin-derived peptides. Bovine ligament tropoelastin and elastin-derived peptides, generated from either human aortic elastin with human neutrophil elastase or from bovine ligament elastin with pancreatic elastase, elicited chemotactic responses that were maximal at 0.2 microgram per millilitre (3×10^{-9} M) and 0.5-2.0 microgram protein per millilitre, respectively. Fractionation of the elastin-derived peptides by gel filtration indicated that comparable levels of chemotactic activity were present in all fractions, and amino acid analysis of the fractions showed no relationship between chemotactic activity and desmosine concentration. Taken in conjunction with the observations on tropoelastin, it appears that fibroblast chemotaxis to elastin components does not involve the cross-links of elastin. These results demonstrate that the influences of the connective tissue matrix upon fibroblast migration might include elastin precursors and fragments of elastin.

Contact inhibition in fibroblasts

The term contact inhibition was coined (Abercrombie, 1954) for a particular form of locomotory behaviour shown by fibroblast-like cells (hereafter called simply fibroblasts) in culture on a plane surface. It was defined as the prohibition of continued movement such as would carry one cell over the surface of another when contact between cells occurred, so that one cell does not use another as a substratum. Amongst fibroblasts and epithelial cells this inhibition seems to be brought about by a mechanism which it is suggested consists essentially of a spasm of contraction in the region of the contact, set off by some signal from the cell contacted. Many other kinds of cells show the general phenomenon of contact inhibition; but there is no certainty that they have the same contractile mechanism. The survey of the literature which this review has entailed

suggests that it might be useful to end with four somewhat negative points. First was that contact inhibition as originally defined is not concerned with mitosis. It may of course become so. Second, contact inhibition of movement is difficult to analyse reliably without quantitative estimations and deliberate experiments. Third, malignant cells are not properly described as being devoid of contact inhibition. It is suggested that they are defective as compared with their cells of origin. Fourth, from the point of view of invasion *in vivo*, the homologous contact inhibition of tumour cells by tumour cells is of little direct interest. It is the heterologous inhibition of tumor cells by normal cells that is relevant (Abercombie, 1970).

Phagocytosis by polymorphonuclear leukocytes

Phagocytosis of zymosan particles by human peripheral blood polymorphonuclear leukocytes (PMN) results in the release of lysosomal enzymes into the extracellular medium (Weissmann et al., 1971) and enhanced glucose oxidation and lactate production (Karnovsky et al., 1971). The antibiotic, cytochalasin B (CB), inhibits phagocytosis by PMN (Davis et al., 1971; Zigmong and Hirsch, 1972), presumably by interfering with the membrane-associated network of microfilaments (Wessels et al., 1971). CB is therefore useful in studies of the interrelationship between phagocytosis and metabolic changes in stimulated PMN. The study by Skosey et al. (1973) confirms earlier reports that CB inhibits phagocytosis by PMN (Davis et al., 1971; Zigmong and Hirsch, 1972; Malawista et al., 1971) and carbohydrate metabolism in phagocytosing PMN (Zigmong and Hirsch, 1972; Malawista et al., 1971; Skosey et al., 1972). The release of the lysosomal enzyme, β-glucuronidase, however, was moderately stimulated by CB under conditions which resulted in almost total inhibition of phagocytosis. Phagocytosis of zymosan by human peripheral blood PMN, measured by the uptake of [^{14}C] inulin from the incubation medium, was inhibited by CB. CB also decreased zymosan-stimulated oxidation of [1 ^{14}C] glucose to $^{14}CO_2$ and production of lactate, but moderately stimulated the release of β-glucuronidase. The results suggest that release of β-glucuronidase from cells exposed to zymosan does not require phagocytosis of the particles.

Chemotaxis in neutrophils

Orientation of nucleus, centriole, microtubules, and microfilaments within human neutrophils in a gradient of chemoattractant (5 percent *Escherichia coli* endotoxin activated serum) was evaluated by electron microscopy. Coordinated orientation of the entire neutrophil population did not occur in buffer (random migration) or in a uniform concentration of activated serum (activated random migration). Conditions of activated random migration resulted in increased numbers of cells with locomotory morphology, i.e., cellular asymmetry with linear alignment of nucleus, centriole, microtubule array, and pseudopods. Thus, activated serum increased the number of neutrophils exhibiting locomotory morphology, and a gradient of activated serum induced the alignment of neutrophils such that this locomotory morphology was uniform in the observed neutrophil population.

In related studies, cytochalasin B (3.0 microgram per milliliter) prevented migration and decreased the microfilaments seen but allowed normal orientation of neutrophil structures. In an activated serum gradient, colchicine, but not lumicolchicine, decreased the orientation of nuclei and centrioles, and caused a decrease in centriole-associated microtubules in concentrations as low as 10^{-8} to 10^{-7} M. Functional studies of migration showed that colchicine, but not lumicolchicine, minimally decreased activated random migration and markedly inhibited directed migration but had no effect on random migration. These studies show that, although functioning microfilaments are probably necessary for neutrophil migration, intact microtubules are essential for normal pseudopod formation and orientation, and maximal unidirectional migration during chemotaxis (Malech et al., 1977).

Chemotaxis in tumors

That malignant tumor cells in the circulation do not necessarily result in the development of metastatic foci is a reasonably well-established observation (Zeidman, 1961). Considerable work has centered on mechanisms that might account for the extravascular localization of tumor cells. Various mechanisms, including "invasiveness" of tumor cells, the role of coagula-

tion factors, and increased locomotive activity of tumor cells, to name only a few, have been postulated (Abercombie and Ambrose, 1958; O'Meara, 1958; Abercombie and Ambrose, 1958). In many respects the ability of malignant cells to pass from the blood stream into tissues resembles the behavior of leukocytes, which infiltrate tissues in response to inflammatory stimuli. Chemotactic mechanisms, which are defined as unidirectional migration induced by a concentration gradient of attractant, have been strongly implicated in cellular inflammatory responses, as demonstrated by the obligatory presence of chemotactic factors in developing reactions that become leukocyte-rich (Wilkinson, 1974; Ward, 1974; Sorkin et al., 1970), and by the recognition that inadequate cellular inflammatory responses result from conditions in which either leukocytes are unable to respond to chemotactic stimuli or there is defective generation of chemotactic factors (Ward, 1974). It seems possible that migration of tumor cells such as fibrosarcoma cells, or L 929, from the blood stream into extravascular locations might also be a response to chemotactic stimuli specific for tumor cells. Indeed, an extract from rat hepatoma AH 109A cells has been reported to be chemotactic for a variety of tumor cells, but this substance has no chemotactic activity for neutrophilic leukocytes (Yoshida et al., 1970). Injection of this extract into rat skin led to the development of metastatic nodules, emphasizing the potential biological importance of chemotactic factors in the localization of tumor cells from the blood stream (Ozaki et al., 1971). The studies reported in this communication confirm the ability of tumor cells to respond to chemotactic stimuli. They also indicate that a novel chemotactic factor for tumor cells is generated from the complement system. These findings suggest the possibility that complement products may significantly influence the migrational behavior of tumor cells *in vivo*.

Role of fibronectin in malignancy

Frozen sections of tumors induced by injecting virally transformed cells into animals were stained for fibronectin by immunofluorescence. Many tumor cell lines do not express fibronectin in tumors in situ even though some of them express fibronectin in culture. Cell shape and hormones appear to

influence the expression of fibronectin in culture; however, it is unclear how fibronectin expression is regulated *in vivo.*

Collagen is chemotactic for tumors

Organs that are rich in collagen such as liver, lungs, and bone are frequently sites of tumor cell metastasis. In an *in vitro* study, the cultured tumor cells of human and rat origin migrated unidirectionally in response to collagen. Synthetic di- and tri- peptides that contained amino acid sequences found frequently in the collagen helix caused similar effects. These results are consistent with the hypothesis that collagen or collagen fragments released during connective tissue remodeling may be important in tumor cell metastasis.

Vinca alkaloids inhibit tumor invasiveness

Inhibition of the invasiveness of MO_4 mouse fibrosarcoma cells by the vinca alkaloids, vinblastine (VLBS), vincristine (VCR) and vindesine (VDS), has been examined *in vitro*. MO_4 cells are virally transformed fibroblastic C3H mouse cells (Billiau et al., 1973) which produce invasive (Mareel et al., 1975; Meyvisch and Mareel, 1979) and metastasizing (Meyvisch et al., 1980) tumors after transplantation into syngeneic mice. At doses between 0.006 microgram per milliliter (minimal effect) and 0.1 microgram per milliliter (complete inhibition) these drugs interfered with the invasion of MO_4 cells from an aggregate confronting a fragment of embryonic chick heart in three-dimensional culture. The effect of these drugs was examined on the growth, directional migration and assembly of the cytoplasmic microtubule complex of MO_4 cells. Growth and directional migration were affected by the same doses of vinca alkaloids as invasion. In contrast with the vinca alkaloids, 5-fluorouracil at 1 microgram per milliliter inhibited growth but allowed directional migration and invasion. At a dose of 0.3 microgram per milliliter VLBS, VCR and VDS interfered with the assembly of cytoplasmic microtubules, as visible after immunocytochemical staining with tubulin antiserum. Ultrastructural analysis demonstrated that inhibition of invasion in three-dimensional culture corresponds with abolishment of the cytoplasmic microtubule complex. Anti-invasive concentrations of VLBS, VCR and VDS represent clinically achiev-

able plasma concentrations. It is concluded that the anti-invasive effect of the vinca alkaloids may contribute to their antitumor activity.

Main Body: Chemotaxis in fibroblasts

A quantitative *in vitro* assay that measures fibroblast chemotaxis in modified Boyden chambers has been described. Blind-well-modified Boyden chemotaxis chambers and polycarbonate filters, 13 millimetre in diameter, containing 8 micrometre pores were used to measure fibroblast chemotaxis. The polycarbonate filters as obtained from the manufacturer were not suitable for use in the assay because fibroblasts would not adhere to and spread-out on the upper surface of the filters or migrate through the filter pores in response to a chemotactic stimulus. We found that fibroblasts would adhere to and migrate through filters previously treated with a dilute gelatin solution to change the surface properties of the filters. Therefore, filters used in all experiments were placed in wire staining baskets, heated at 50 degrees centigrade in 0.5 percent acetic acid solution for 20 minutes, rinsed two times in glass-distilled water at 25 degrees centigrade placed in a beaker containing gelatin in glass-distilled water (5 milligram per litre) at 100 degrees centigrade for 1 hour, dried with a hair dryer, and heated in an oven (100 degrees centigrade) for 1 hour (Postlethwaite et al., (1976). Substances being assayed for fibroblast chemotactic activity were mixed with serum-free maintenance media (0.4 millilitre per 0.35 millilitre). Aliquots of this mixture were placed in the lower compartment of blind-well chemotaxis chambers. Prepared polycarbonate filters (dull side up) were placed in the chambers to cover the filled lower compartment, and chamber caps containing the upper compartment were screwed into the chambers to affect a watertight seal around the periphery of the filter. The upper chamber compartments were then loaded with the fibroblast suspension, prepared as described above (Postlethwaite et al., (1976). Loaded chambers were incubated at 37 millilitre for 150 minutes in a humidified atmosphere containing 5 percent CO_2. After the incubation period, chambers were disassembled, filters removed, placed in staining baskets, fixed for 15 seconds in absolute ethanol, stained with hematoxylin, and

mounted on glass cover slips. During incubation, fibroblasts responding to a chemotactic stimulus migrate from the upper filter surface through the pores and adhere to the lower filter surface. Fibroblast chemotactic activity was quantitated by counting nuclei of fibroblasts on the lower surface of the filters in 20 oil immersion fields (× 1,000). All samples were assayed in triplicate, and final activity was expressed as the mean ± SEM of the replicates (Postlethwaite et al., (1976). It has been found that human peripheral blood lymphocytes when stimulated by mitogen (phytohemagglutinin or PHA) or antigens (streptokinase-streptodornase or SKSD, purified protein derivative or PPD) produce a factor that is chemotactic for human dermal fibroblasts. This substance was called "lymphocyte-derived chemotactic factor for fibroblasts" or LDCF-F. Production of LDCF-F by lymphocytes obtained by isopyknic centrifugation of peripheral blood with Ficoll-Hypaque appears to be synchronous with mitogenesis induced by antigen or mitogen stimulation. In addition, production of LDCF-F in the presence of PPD correlates with delayed hypersensitivity to this antigen as measured by skin testing. Purified T lymphocytes stimulated by PHA require macrophages for production of LDCF-F. Osteoclast-activating factor and interferon are two other lymphokines that require macrophages for their production by lymphocytes (Epstein et al., 1971; Horton et al., 1974). LDCF-F from antigen- or mitogen-stimulated lymphocytes has a molecular weight of approximately 22,000 daltons. It is clearly distinct from LDCF-M ("lymphocyte-derived chemotactic factor for monocytes"). The findings that LDCF-F is not chemotactic for human monocytes, and that LDCF-M is not chemotactic for fibroblasts suggest that chemotactic receptors on monocytes and fibroblasts may be different. In contrast to this dichotomy between fibroblast and monocyte receptors for lymphocyte-derived chemotactic factors, we have found that native collagen, constituent α1- and α2-chains of collagen and peptides obtained by collagenase, cyanogen bromide, and pepsin degradation of collagen are chemotactic for monocytes (Postlethwaite and Kang, 1976) and fibroblasts (Goldman, 1971). Thus, it appears that similar receptors for collagen and collagen peptides are present on both fibroblasts and mono-

cytes. The fibroblast surface membrane receptor for LDCF-F has not been fully characterized, but our data suggest that it is readily accessible, being present on cells dispersed by EDTA or brief trypsinization. The finding that prolonged trypsinization renders the fibroblast chemotactically unresponsive to LDCF-F could be due to removal or alteration of the receptor or to an effect of trypsin on other membrane proteins involved in the chemotactic response. (Wang E, Goldman RD, 1978). The demonstration that trypsin can temporarily destroy a membrane receptor has been extensively documented for the adipose cell receptor for insulin (Cuatrecasas, 1971; Kono and Barham, 1971; Sakai et al., 1973; Solomon, et al., 1975). Definitive information regarding the nature of the fibroblast receptor for LDCF-F will have to await its isolation and purification. The finding that fibroblasts are able to respond chemotactically to a Lymphocyte-derived factor *in vitro* may have significance *in vivo*. For example, cell-mediated immune reactions produced by tubercle bacilli and *Schistosoma mansoni* result in formation of granulomas that are characterized by the presence of increased members of fibroblasts (Poole, 1970; Cheever, 1965). Perhaps the release of LDCF-F by sensitized, stimulated lymphocytes might function to attract neighboring connective tissue fibroblasts to these granulomas. The organized connective tissue capsule that characteristically surrounds "benign" tumors may in like manner result from release of LDCF-F by host lymphocytes as they react to neoplastic cells.

Recently, humans with pulmonary fibrosis (Ahmed et al., 1976) and progressive systemic sclerosis (Stuart et al., 1976) have been found to have evidence of cell-mediated immunity to collagen. Both of these conditions are characterized by lesions that contain increased numbers of fibroblasts and increased deposition of collagen. Perhaps T lymphocytes sensitized to collagen in these disease states release LDCF-F upon contact with collagen *in vivo*, and this leads to fibroblast accumulation. This study demonstrates that lymphocytes under appropriate stimulation by antigen or mitogen *in vitro* produce a chemotactic factor for fibroblasts. The function and role of such a chemotactic factor *in vivo* remain to be defined.

Chemotaxis in fibroblasts to collagen

Fibroblasts are found in inflammatory lesions resulting from tissue injury by a variety of different agents (Postlethwaite et al., 1978). They effect repair of damaged tissue by synthesizing and laying down extracellular components which constitute the scar. The mechanisms whereby these specialized effector cells are attracted to sites of tissue injury and inflammation are poorly understood. Collagen is the most abundant and ubiquitous connective tissue protein. So far, at least four distinct types of collagens have been found. Type I, II, and III collagens and their constituent α chains have been well characterized (Piez et al., 1963; Gallop et al., 1972; Miller, 1971; Trelstad et al., 1974; Epstein and Munderloh, 1975; Butler et al., 1975; Seyer et al., 1976; Gay et al., 1975; Remberger et al., 1975; Seyer et al., 1977). Type IV collagen, isolated initially from basement membranes, has been more difficult than the other three collagens to characterize biochemically and may be heterogeneous (Kefalides, 1971; Kefalides, 1972; Chung et al., 1976).

Fibroblasts require both a collagen substratum and fibronectin for adherence and subsequent migration in the Boyden chamber. Both fibronectin and lymphokines are good chemoattractants, whereas the collagens are weaker. Fibroblasts appear to require cytoskeletal organization and methylation reactions for chemotaxis, properties shown previously for macrophages and neutrophils. Fibroblasts, however, migrate more slowly than phagocytic cells and appear to require protein synthesis (Gaus-Mueller et al., 1980).

Postlethwaite et al., (1978) measured fibroblast chemotaxis using an *in vitro* assay. The assay uses blindwell modified Boyden chemotaxis chambers equipped with gelatin-treated polycarbonate filters having 8 micrometre pores. Normal human dermal fibroblasts maintained as continuous cell lines were used as indicator cells in the assay. Fibroblasts were harvested from monolayers by either trypsinization or EDTA treatment. Fibroblasts chemotactic activity was determined after chambers were incubated for 150 minutes by counting the fibroblasts migrating to the lower surface of the polycarbonate filters in 20 oil immersion fields. Substances

were assayed in triplicate, and the final chemotactic activity of a sample was expressed as the mean ± SEM. Lyophilized collagen and α chain preparations were solubilized for use in the chemotaxis studies by dissolving them in 0.5 M acetic acid, stirring them overnight at 4 degrees centigrade, and then dialyzing them against large volumes of 10 mM phosphate/0.14 M NaCl at pH 7.4 for 24 hours and subsequently against 10 mM glycylglycine/0.14 M NaCl at pH 7.2 for 24 hours at 4 degrees centigrade.

Human type I, II, and III collagens and isolated α chains from these collagens are chemotactic for human dermal fibroblasts *in vitro*. The reasons for the difference in chemotactic potency of native collagen and α chains are not apparent from the data presented by Postlethwaite et al., (1978). Perhaps the ordered helical configuration of the native collagen molecule in contrast to the random coil structure of α chains more readily exposes the essential amino acid sequence(s) so that it (they) can make contact more efficiently with the appropriate receptor(s) on fibroblast membranes. Postlethwaite et al., (1978) observed a similar difference between the chemotactic potency of native type I collagen and its constituent α chains for human peripheral blood monocytes (Postlethwaite and Kang, 1976). Perhaps of an even greater interest is the observation that synthetic tri- and dipeptides containing hydroxyproline, isolated α chains, and smaller peptides derived from degradation of collagen by digestion with cyanogen bromide (CNBr), pepsin, or bacterial collagenase are also chemotactic for fibroblasts. These findings suggest that many regions of the collagen chains contain amino acid sequences capable of inducing fibroblast chemotaxis. Several characteristics of the fibroblast membrane receptor involved in the chemotactic response to collagen are apparent from our studies. First, there appears to be a common receptor for type I, II, and III collagens and α1 (Piez et al., 1963); second, hydroxyproline is an important and perhaps essential constituent of the amino acid sequence(s) recognized by the receptor; and third, the receptor involved in the chemotactic response of fibroblasts to collagen is probably different from the receptor involved in the chemotactic response to LDCF-F (Postlethwaite et al., 1978).

Posttranslational modifications, such as phosphorylation of the hydroxy group might also be important. In normal connective tissues, collagen fibrils are tightly arranged into larger bundles or fibers. Glycosaminoglycans are closely associated with the packed collagen fibers. The glycosaminoglycans and the structural nature of the fibers may effectively "shield" collagen from fibroblasts so as not to provide a chemotactic stimulus. After tissue damage and during inflammatory reactions, collagen fibers may become "unshielded" by the concerted action of various lysosomal glycosidases and hydrolases and be degraded by specific collagenases present in neutrophils, macrophages, and other cells (Lazarus et al., 1968; Lazarus et al., 1968; Wahl et al., 1975; Harris et al., 1969; Harris and Krane, 1974). After collagen fibrils are cleaved by collagenase, the digestion products denature under the physiologic conditions of body temperature, pH, and ionic strength (Sakai and Gross, 1967). These products can be degraded further by the action of nonspecific proteases (Sakai and Gross, 1967). The *in vitro* findings suggest that peptides generated by the action of collagenase and nonspecific proteases on collagen could function as chemotactic stimuli to effect fibroblast migration to sites of inflammation *in vivo*.

Chemotaxis in fibroblast to fibronectin

Fibronectin, a class of adhesive, high-molecular-weight glycoproteins, is present in connective tissue, primative mesenchyme, basement membranes, amniotic fluid, cerebrospinal fluid, and plasma (Vaheri and Mosher, 1978; Yamada and Olden, 1978). Fibronectin of plasma or cellular origin is chemotactic for human dermal fibroblasts *in vitro*. After cleavage of fibronectin by cathepsin D, chemotactic activity is found in a non-gelatin-binding fragment having an estimated molecular weight of 140,000. Gelatin-binding fragments do not possess chemotactic activity. Although the mechanism by which fibronectin causes the chemotactic migration of fibroblasts is not apparent from this study, these data strongly suggest that fibronectin does not produce its effect by acting as a simple ligand between cell surface.receptor and the gelatin-coated polycarbonate fhers. The concentration of fibronectin in plasma is approximately 300 micrograms per mililitre (Yamada

and Olden, 1978). In a study, fibroblasts were responsive to lower concentrations (0.4-2 microgram per mililitre) of native fibronectin, however, higher concentrations of cathepsin D-generated non-gelatin-binding fragments were required to effect fibroblast migration. Higher concentrations (more than 2 microgram per mililitre) of native fibronectin elicited no migration. A similar bell-shaped dose-response curve has been observed in the chemotaxis of neutrophiis to formylmethionyl peptides (Schiffmann et al., 1975).

The diminished response to large amounts of chemoattractant may result from rapid diffusion of the chemoattractant to the upper cell compartment, with a concomitant saturation of available receptors on plasma membranes on the responding cells. It is also possible that fibroblasts in vivo respond optimally to higher concentrations of fibronectin than they do in vitro under the artificial conditions of the assay system employed in a study. Fibronectin and/or its biologically active fragments may play a significant role in effecting fibroblast migration *in vivo*. The role of fibronectin as a fibroblast chemoattractant should be considered in relation to other chemotactic factors for fibroblasts as has been previously described (Postlethwaite et al., 1976; Postlethwaite et al., 1980; Postlethwaite et al., 1979; Postlethwaite et al., 1977). Specific chemotactic lymphokines have been characterized for human and guinea pig fibroblasts (Postlethwaite et al., 1976; Postlethwaite et al., 1980;. These lymphokines could be released at sites of cell-mediated immune reactions *in vivo*. A fragment from human C5 is also chemotactic for fibroblasts, suggesting that the complement system when activated could provide a specific chemoattractant for fibroblasts (Postlethwaite et al., 1979). In addition, collagen and collagen-derived peptides are chemotactic for fibroblasts (Postlethwaite et al., 1977). Because collagen and fibronectin are both present in the connective tissue matrix, their degradation by specific proteinases during the course of inflammatory reactions of all types could generate additional chemotactic signals for fibroblasts. Thus, collagen-derived peptides and biologically active fibronectin peptides could serve as important amplifiers of fibroblast chemotaxis and could greatly facilitate the accumulation of fibroblasts at

sites of connective tissue injury and inflammation. During embryonic development and morphogenesis, the chemotactic property of fibronectin and derived peptides could function to direct migration of fibroblasts to effect specific organization of connective tissue.

Chemotaxis in fibroblast to tropoelastin

Recent evidence indicates that fibroblasts have the capacity to demonstrate chemotaxis. Thus far a variety of chemoattractants for fibroblasts have been identified including lymphokines, collagen, collagen-derived peptides, a complement-related component, fibronectin, and fibronectin-derived peptides (Postlethwaite et al., 1976; Postlethwaite et al., 1978; Chiang et al., 1978; Postlethwaite et al., 1979; Gauss-Muller et al., 1980; Postlethwaite et al., 1981; Tsukamoto et al., 1981; Seppa et al., 1981). The chemotactic responsiveness of fibroblasts to collagen and fibronectin suggests that the extracellular matrix may influence migratory responses of fibroblasts in vivo. The findings also raise the possibility that other constituents of the extracellular matrix have chemotactic activity for fibroblasts. Others reported that tropoelastin and elastin-derived peptides have the capacity to produce chemotactic responses in fibroblasts. Their results demonstrate that tropoelastin, the soluble precursor of elastin, and elastin-derived peptides have the capacity to induce fibroblast chemotaxis. Accordingly, as with two other extracellular connective tissue macromolecules, fibronectin and collagen, components of elastin may also influence the migratory activity of fibroblasts (Postlethwaite et al., 1978; Gauss-Muller et al., 1980; Postlethwaite et al., 1981; Tsukamoto et al., 1981; Seppa et al., 1981).

Researches carried out their studies with fibroblasts from two sources, fetal bovine ligament and adult human skin fibroblasts. Chemotactic responsiveness to tropoelastin and elastinderived peptides by fetal ligament fibroblasts might be expected because these cells are heavily committed to the production of elastin late in fetal life (Mecham et al., 1981). However, because adult human skin fibroblasts, cells that synthesize very little elastin, also showed directed migration to elastin and tropoelastin, it seems likely that fibroblasts gen-

erally have such responsiveness. Observations that connective tissue components such as fibronectin, collagen, tropoelastin, and elastinderived peptides have chemotactic activity for fibroblasts in vitro are of interest, but at present the meaning of such findings remains unclear. It will be important to develop a variety of data to give perspective to this new information. The need for detaching fibroblasts with trypsin or ethylenediaminetetraacetic acid (EDTA) before testing them for chemotaxis raises questions about the mechanisms involved in vivo in releasing fibroblasts from their attachments. Further, because several components of the matrix express chemotactic activity for fibroblasts in vitro, it is not evident which component(s) exerts a biologically relevant directive force in normal and pathologic conditions. The role of elastin-derived peptides as chemoattractants for fibroblasts, for example, might be minor because fibronectin, which is ubiquitous and highly susceptible to proteolytic degradation into fragments with chemotactic activity for fibroblasts (Seppa et al., 1981), would likely be degraded simultaneously with elastin. Recently, it has been observed that alveolar macrophages from patients with idiopathic pulmonary fibrosis produce and release much more fibronectin than alveolar macrophages from normal subjects, suggesting a possible pathogenetic role for fibronectin in this disease (Rennard et al., 1981). However, the finding that fibroblasts release chemotactic activity for neutrophils and monocytes (Sobel and Gallin, 1979), which in turn can release proteinases that degrade connective tissue, emphasizes the complexity of the mechanisms that may be involved in disorders such as idiopathic pulmonary fibrosis where pathologic changes consist of admixtures of inflammation and fibrosis (Fulmer and Crystal, 1979).

Contact inhibition in fibroblasts

Contact inhibition of movement is defined simply as the stopping of the continued locomotion of a cell in the direction which has produced a collision with another cell; so that one cell does not use another as a substratum. When a chick heart fibroblast on a plane substrate is approaching another on a collision course, time lapse filming of the leading end with an interval of 2 seconds, and high-resolution phase contrast, fail

to show any change until after visible contact has been made. The effect of contact is often delayed for 10 to 20 minutes after the cells have apparently met, which allows time for some degree of superimposition of one cell on the other (Amercrombie, 1958). When the collision is not between two lamellae but is between the front end of one cell and the side of the other, the leading lamella of the former cell may pass some distance between the latter cell and the substratum. When, however, the collision is head-on, if there is an overlap, one leading lamella necessarily extends over the dorsal side of the other. Whether a leading lamella passes dorsally or ventrally depends presumably on the thickness of the edge of the cell with which it collides, and on the closeness of attachment of the two colliding edges to the substratmn. The region of the leading lamella seems to be the most firmly adherent to the substratum of any part of the cell and may be regarded as the locomotory organ of the cell (Weiss, 1958; Abercrombie, 1961).

If contact inhibition is to occur, the first visible reaction of a leading lamella after it has made contact, is a contraction. The effects of contact inhibition on cell displacement are of two quite different kinds. Firstly, the contraction may move the cells. The contraction may at times fail to draw the cells much together before it ruptures their mutual adhesion, so that the cells then jerk apart. Differences in adhesion of the cells to substratum and to each other doubtless explain the different results of the contraction. The second effect on displacement, which follows the first effect, is the cessation of locomotion of the cell in the direction which has produced the collision; this of course is the defining characteristic of contact inhibition (Amercrombie, 1970). There is no total inhibition of the cell's movement: if part of the leading lamella remains unaffected, it may lead the cell in a new direction, or a newly developed leading lamella may do the same. Where contraction has broken the contacting cells apart, the inhibited leading lamella may recover and re-extend, usually to suffer another inhibition.

Mechanism of contact inhibition in fibroblast

Amongst fibroblasts and epithelial cells this inhibition seems to be brought about by a mechanism which it is suggested con-

sists essentially of a spasm of contraction in the region of the contact, set off by some signal from the cell contacted. Many other kinds of cells show the general phenomenon of contact inhibition; but there is no certainty that they have the same contractile mechanism. There are a few possible hypotheses that can explain the mechanism of contact inhibition in fibroblasts. First, it may be that one cell acts simply as a mechanical obstacle to another. A leading lamella, required to turn suddenly dorsally to surmount another cell, may be unable to bend, or may when so kinked be unable to act as a locomotory organ. Secondly, it may be that the firmness of the adhesion between colliding fibroblasts stops the movement. Adhesion is a conspicuous feature of contact inhibition, and it has been invoked as a cause of diminution of speed of movement (Amercrombie and Heaysmann, 1953). It is conceivable that the mechanism of fibroblast movement is such that tight adhesion of the anterior end of the leading lamella stops it. This hypothesis does not account for the contraction in contact inhibition. Thirdly, it may be that the dorsal surface of a fibroblast is totally nonadhesive to another fibroblast (in contrast to the edges) so that movement on it is impossible. Placed entirely on such a surface a cell would (gravity apart) round up as it does when suspended in fluid. Its effect would be to make the progress of a leading lamella impossible. Fourth hypothesis was that the dorsal surface may be adhesive enough to another cell to permit movement on it; but it may be less adhesive than the tissue culture substratum. Faced with a choice, a cell may be unable to detach itself from the substratum by means of its adhesions to the less adhesive surface of another cell. A different kind of hypothesis would make the inhibition and its concomitants a response to some signal received as the result of the collision. The signal may involve a diffusible substance. The approach of two cells towards each other can hardly fail to alter the diffusion gradients around the cells of substances consumed or produced in common by both cells, and hence alter concentrations at the cell surfaces facing each other. Such a concentration change might be the signal. It would of course need to become effective only at separations of no more than a micron or so, if the reaction is to appear to depend so invariably on contact (Amercrombie, 1970).

***In vitro* consequences of contact inhibition.**
Many features of the behaviour of cell populations in culture can be ascribed to the effects of contact inhibition (Abercrombie, 1961). Monolayering, directional movement (Abercrombie and Heaysman, 1966) and the failure of fibroblast foci to scatter into discrete cells (Abercrombie and Heaysman, 1965) have been analysed in terms of contact inhibition. The circular form of fibroblast colonies still awaits full analysis, but it can probably be ascribed to the tendency of contact inhibition to direct fibroblast movement towards cell free space (Abercrombie and Heaysman, 1954; Abercrombie, 1961). The complete cessation of transposition of a cell may often be due to all round contact, and its resumption, as when a wound is made in a culture (Fischer, 1930; Todaro et al., 1965; Castor, 1968; Vasiliev et al., 1969) to release from the inhibition. In this connection, however, the concept of mobilisation, the awakening of a dormant locomotory mechanism, needs to be borne in mind (Abercrombie and Ambrose, 1962). The study of primary explants has suggested that when the replication cycle *in vivo* is in abeyance, the locomotory mechanism also tends to be sluggish, and the same correlation may perhaps hold in stationary monolayers *in vitro*. Abercrombie (1970) concluded that contact inhibition as originally defined is not concerned with mitosis. It may of course become so. Furthermore, contact inhibition of movement is difficult to analyse reliably without quantitative estimations and deliberate experiments. Anecdotes are not enough. Malignant cells are not properly described as being devoid of contact inhibition. It was suggested that they are defective as compared with their cells of origin. From the point of view of invasion *in vivo*, the homologous contact inhibition of tumour cells by tumour cells is of little direct interest. It is the heterologous inhibition of turnout cells by normal cells that is relevant.

Phagocytosis by human polymorphonuclear leukocytes
Cytochalasin B (CB) has been shown to inhibit phagocytosis by human polymorphonuclear leukocytes (PMN) as measured by the uptake and killing of bacteria (Davis et al., 1971; Zigmong and Hirsch, 1972; Malawista et al., 1971) or by the direct examination of cells incubated with particles (Zigmong and

Hirsch, 1972; Malawista et al., 1971). The difficulties inherent in these methods have been discussed in various studies (Zigmong and Hirsch, 1972; Berger and Karnovsky, 1966). Skoskey et al., (1973) used the uptake of [^{14}C] inulin into cells as an index of phagocytosis, as described previously (Berger and Karnovsky, 1966). This large molecular weight compound presumably enters cells in the water taken into the phagocytic vesicle. Water also enters cells during the process of pinocytosis. The uptake of [^{14}C] inulin, therefore, serves as an index of endocytosis, and is not specific for either phagocytosis or pinocytosis (Berger and Karnovsky, 1966). Since endocytosis of either type is associated with metabolic changes (Graham et al., 1967), it is reasonable to relate PMN metabolism and function to [^{14}C]-inulin uptake.

Three possible mechanisms have been proposed for the release of lysosomal enzymes from phagocytosing cells (Weissmann et al., 1971). The first, release of enzymes after cell death, cannot account for release of lysosomal enzymes from PMN phagocytosing zymosan, since lactic dehydrogenase, a non-lysosomal cytoplasmic enzyme, is not released from cells in response to zymosan (Weissmann et al., 1971). A second mechanism which has been proposed is that of "regurgitation during feeding." Electron microscope examination of rabbit neutrophils phagocytosing zymosan particles (Henson, 1971) has suggested that lysosomes fuse with the phagocytic vesicle as it forms during particle uptake. The lysosomes appear to discharge their content of enzymes into the vesicle. Enzymes are then extruded through the unclosed "mouth" of the phagocytic vesicle. Henson (1971) has suggested that neutrophils which have phagocytosed many particles "run out of energy" and become inefficient in closing off developing phagocytic vesicles. Lysosomal enzymes which have been extruded into the vesicle are allowed to leak out into the medium. Since CB inhibits glucose metabolism (Zigmong and Hirsch, 1972; Estensen and Plagemann, 1972), it is possible that the early phases of phagocytosis occur in the presence of CB, but that the antibiotic deprives the cells of sufficient energy to close off the mouth of the vesicle. However, the observation that complete deprivation of the cells of exogenous glucose does not mimic the effects of CB argues

against this possibility. We favor a third mechanism, "directed exocytosis," or "reverse endocytosis," proposed for the stimulation of release of enzyme by zymosan. Phagocytosis is apparently not required for the particles to stimulate enzyme release. It thus appears possible that zymosan, by coming into contact with cell membrane receptors, triggers lysosomal enzyme release without the requirement for the development of a phagocytic vesicle. Orci et al., (1972) observed that CB enhances glucose-induced secretion of insulin by isolated rat pancreatic islets. They suggested that the network of microfilaments of the beta cell might act as a barrier which denies the insulin-secretory granules access to the cell membrane. CB, by disrupting the function of this network, could cause enhancement of insulin secretion. A similar mechanism could explain the moderate enhancement of zymosan-stimulated β-glucuronidase release from PMN.

Chemotaxis in neutrophils

Orientation of nucleus, centriole, microtubules, and microfilaments within human neutrophils in a gradient of chemoattractant (5 percent *Escherichia coli* endotoxin activated serum) was evaluated by electron microscopy. Purified neutrophils (Hypaque-Ficoll) were placed in the upper compartment of chemotactic chambers. Use of small pore (0.45 micrometer) micropore filters permitted pseudopod penetration, but impeded migration. Under conditions of chemotaxis with activated serum beneath the filter, the neutrophil population oriented at the filter surface with nuclei located away from the stimulus, centrioles and associated radial array of microtubules beneath the nuclei, and microfilament-rich pseudopods penetrating the filter pores. Reversal of the direction of the gradient of the stimulus (activated serum above cells) resulted in a reorientation of internal structure which preceded pseudopod formation toward the activated serum and migration off the filter. Coordinated orientation of the entire neutrophil population did not occur in buffer (random migration) or in a uniform concentration of activated serum (activated random migration). Conditions of activated random migration resulted in increased numbers of cells with locomotory morphology, i.e., cellular asymmetry with linear alignment of nucleus, centriole,

microtubule array, and pseudopods. Thus, activated serum increased the number of neutrophils exhibiting locomotory morphology, and a gradient of activated serum induced the alignment of neutrophils such that this locomotory morphology was uniform in the observed neutrophil population.

In related studies, cytochalasin B and colchicine were used to explore the role of microfilaments and microtubules in the neutrophil orientation and migration response to activated serum. Cytochalasin B (3.0 microgram per milliliter) prevented migration and decreased the microfilaments seen but allowed normal orientation of neutrophil structures. In an activated serum gradient, colchicine, but not lumicolchicine, decreased the orientation of nuclei and centrioles, and caused a decrease in centriole-associated microtubules in concentrations as low as 10^{-8} to 10^{-7} M. These colchicine effects were associated with the rounding of cells and impairment of pseudopod formation. The impaired pseudopod formation was characterized by an inability to form pseudopods in the absence of a solid substrate, formation of narrow pseudopods within a substrate, and a defect in pseudopod orientation in an activated serum gradient. Functional studies of migration showed that colchicine, but not lumicolchicine, minimally decreased activated random migration and markedly inhibited directed migration but had no effect on random migration. These studies show that, although functioning microfilaments are probably necessary for neutrophil migration, intact microtubules are essential for normal pseudopod formation and orientation, and maximal unidirectional migration during chemotaxis (Malech et al., 1977).

Chemotaxis in tumors

The data in one paper confirmed the ability of tumor cells to respond to chemotactic stimuli, as first reported by Yoshida et al. (1970). However, some findings differ with respect to the nature of the chemotactic factor. Yoshida et al. (1970) did not find extracts from tumor cells to be chemotactic per se, but they demonstrated an activity appearing after incubation of serum with the extract. The subsequent experiments showed that the generation of chemotactic activity involved a direct interaction of the extract with C5. On the basis of the ultra-

centrifugal features of the chemotactic factor, it seems likely that a C5 fragment is the chemotactic factor and that this factor has been produced as a result of direct interaction of the extract with C5. It seems evident, then, that tumor cells contain a C5 cleaving enzyme that has the ability to cleave C5, producing a fragment that is uniquely chemotactically active, inasmuch as no leukotactic activity is found by this method of C5 cleavage. In this respect, the C5 cleaving enzyme from tumor cells acts in a manner different from another C5 cleaving enzyme, trypsin, since trypsinization of C5 results in the intermediate appearance of a leukotactic factor, prior to its disappearance and the subsequent appearance of a tumor cell chemotactic factor. These data do not permit any definitive conclusions regarding the possible origin of a smaller (tumor cell) chemotactic factor from a larger C5 fragment that contains leukotactic activity. The possibility, as yet unexplored, exists those normal tissues contain enzymes that will cleave C5, producing a chemotactic factor for tumor cells. Obviously, this might have considerable significance for the fate of tumor cells in the circulation, if a chemotactic factor for tumor cells was produced extravascularly. In support of this possibility are the experiments of Osaki et al., (1971) who injected the tumor cell extract intradermally into rats that had been given tumor cells intravenously. Sometime later small metastatic nodules of tumor were noted at the site of injection, but not at intradermal sites that were injected with vasopermeability agents. These studies suggest that chemotactic factors for tumor cells may have important biological influences *in vivo*. There are three reports indicating the presence of C5 cleaving enzymes in tissues. These enzymes have been defined by their ability to generate leukotactic fragments from C5. Neutrophilic leukocytes contain a lysosomal-associated, trypsin-like C5 cleaving enzyme that is released during phagocytosis and has optimal activity at neutral pH (Ward and Avaifler, 1971). Another C5 cleaving enzyme is present in macrophages and has a very low pH optimum. Mechanisms for its release have not been established (Snyderman et al., 1972). A third C5 cleaving enzyme has been described in fluids of virus-infected kidney cells cultured *in vitro*. This enzyme is active at neutral pH and is blocked by ethylenediamine tetraacetate but not

by trypsin inhibitor (Brier et al., 1970). The enzyme described in the current report might be similar (because of activity at neutral pH) to the neutrophil contained enzyme or to the one released from virally infected cells, but much more detailed information is necessary. It also remains to be demonstrated if any of these, or similar, enzymes derived from non-neoplastic tissues can generate a tumor cell chemotactic factor from C5. Remarkable about C5 is the fact that its fragmentation products, depending upon their size and structure, have the ability to induce chemotactic migration of leukocytes and tumor cells. Although C3 fragmentation products can similarly influence leukocytes, there is as yet no evidence that they have any influence on tumor cell migration. This is shown both by the effect of trypsin on C3 and C5, and by the complete suppression by antibody to C5 of the tumor cell chemotactic factor generated in human serum by the tumor cell extract. If tumor cells respond chemotactically to the C5 fragments, and if this factor plays a major role in the development of metastatic lesions *in vivo*, the absence of C5 might have an important influence on the frequency of metastatic lesions. The absence of C5 might be advantageous to the host since the source of the chemotactic factor would be missing. There is no definitive evidence for or against an influence of C5 on the behavior of tumor cells *in vivo*. However, caution is necessary in making any predictions about the outcome of complex biological reactions that seem to depend in part on the complement system. This is well demonstrated in the case of immunologic vasculitis. The acute inflammatory lesions in a normocomplementemic animal are mediated by C5 leukotactic products (Ward and Cochrane, 1965; Ward and Hill, 1972). Yet, in C5 deficiency states, the inflammatory vasculitis reaction still occurs, apparently mediated by a C5-independent system (Crisler and Frank, 1965). It is possible that if C5 fragments do have a determinant role in the extravascular movement of blood-borne tumor cells *in vivo*, manipulative techniques designed to inhibit the activity of these C5 fragments might significantly impair the tendency of the tumor cells to form metastatic foci. This possibility remains to be proven, but it does provide a new conceptual approach to modulating the behavior of tumor cells *in vivo*.

Role of fibronectin in malignancy

In regard to human cancer, the role of fibronectin is not too encouraging. Since the normal epithelium of adult tissues rarely has fibronectin associated with it, it is difficult to argue about the significance of the "loss" of fibronectin. However, during metastasis, transformed cells have to penetrate the various basement membranes, which often contain fibronectin; this interaction of tumor cells with fibronectin may be significant in the development of a malignancy. For experimental tumors, where fibroblasts are often the host for oncogenic transformation, fibronectin is clearly a good cell surface marker to follow. Regarding the role fibronectin in malignant development, it seems to depend on how the untransformed normal parent cells behave *in vivo*. At present, it is hard to say how cells such as L 929, 3T3, CEF, Nil or BHK, that produce fibronectin in culture behave with respect to fibronectin expression in a physiologic environment. Perhaps one can place 3T3 cells on glass beads, inject or implant them subcutaneously into animals, and then examine whether fibronectin is expressed. If that is true, then the fact that SV-3T3, Py-3T3,3T6, and 3T12 do not express fibronectin in their tumors is of the utmost significance from an oncogenic transformation standpoint. In *in vitro* studies of oncogenic transformation in the cell system, fibronectin is undoubtedly involved (Chen et al., 1979).

Collagen is chemotactic for tumors

The process of tumor cell metastasis has been difficult to study *in vitro*, since multiple mechanisms may be responsible for the cellular events that occur between the shedding of tumor cells from the primary site and the appearance of a metastasis in a selected distant site. Technical problems such as the tight adherence of tumor cells to each other and to the surfaces of cultured vessels also limit in vitro studies of tumor cell movement. The most widely used system for studying cell chemotaxis in vitro is the Boyden chamber technique (Ward et al., 1975). Organs rich in collagen such as liver, lung, and bone are frequently sites of tumor cell metastasis. The *in vitro* Boyden chamber system was used by Mundy et al., (1981) to show that cultured tumor cells are attracted unidirectionally along a concentration gradient of Types I

and III human and rat collagens as well as collagen-derived fragments and synthetic di- and tri- peptides. Whenever tumor cells lodge in distant sites and form a metastatic nidus, they must migrate out of capillaries or sinusoids into the surrounding tissue. This process of transvascular migration of tumor cells may be a directed rather than random event. These data are consistent with the notion that the collagen molecule contains a chemotactic signal for tumor cells, and this chemotactic factor is released during the process of collagen degradation. It is likely that this chemotactic signal contains the amino acid residues glycine and proline and is at least two amino acids in length. It is possible that a whole series of fragments of the collagen molecule containing glycine and proline residues are chemotactic for tumor cells. The molar concentration of the synthetic peptides required to produce a consistent chemotactic response was usually one order of magnitude greater than the molar concentration of intact collagen which produced a similar response. The reason for this is unclear, but similar results have been found with these peptides and fibroblast chemotaxis (Postlethwaite et al., 1978). Postlethwaite et al. (1978) have suggested that the ordered helical structure of native collagen may facilitate binding of the chemotactic factor to the cellular receptor. We did not show significant loss of activity as the synthetic di-peptide was diluted beyond the lowest effective concentrations of intact collagen. This may occur because these smaller molecules are freely and rapidly diffusible across the filter membrane. The maximal effects observed with Type 1 collagen were at 1 micromolar, which is near the limits of its solubility. Recently, we found that remodeling or resorbing bone produces a factor that is chemotactic for tumor cells (Orr et al., 1979; Orr et al., 1980). This bone-derived factor is nondialyzable and macromolecular and is released into the media bathing organ cultures of resorbing bones. It may provide the explanation for the selective metastasis of some tumor cells to bone. Its relationship to collagen or collagen fragments is unresolved, but since the bone matrix comprises approximately 95 percent Type I collagen, it is possible that collagen fragments that are released during the process of bone remodeling are chemotactic signals to tumor cells.

Different tumors metastasize selectively to different organs. Spleen, cartilage, and skeletal muscle are rarely the site of metastasis, whereas most tumors metastasize to the liver or lungs. Bone is a frequent site of metastasis of some tumors such as breast cancer but is an uncommon metastatic site for carcinomas of the gastrointestinal tract or female genitalia (Mundy et al., 1981). The reasons for these patterns of tumor metastasis are not clear. One possibility is that organs such as the lungs and liver contain a rich venous blood supply so that circulating tumor cells gain ready access to these organs. However, this cannot explain the observation that some tumors migrate selectively to bone, and some do not. It is possible that the local environment of organs such as bone is unfavorable to the formation of some tumor metastases, but not to others. The data presented here suggests that another reason that tumor cells collect selectively in some organs may be due to the connective tissue remodeling of the stroma of that organ, causing the generation of fragments of collagen that may be chemo-attractants for tumor cells.

Vinca alkaloids inhibit tumor invasiveness

The antitumor activity of the naturally occurring vinca alkaloids, vinblastine (VLBS) and vincristine (VCR) and the semi-synthetic VLBS-derivative vindesine (VDS), has been demonstrated in experimental and human cancer (Gerzon, 1980; De Brabander et al., 1976). Mareel et al., (1978) and Mareel et al., (1980) reported that microtubule inhibitors (including VLBS and VCR) interfere with the capacity of MO_1 mouse fibrosarcoma cells to invade normal tissues in three-dimensional culture, and that abolishment of the cytoplasmic microtubule complex is responsible for this anti-invasive effect (Mareel and De Brabander, 1978; Mareel et al., 1980; Storme and Mareel, 1981). MO_4 cells are virally transformed fibroblastic C3H mouse cells (Billiau et al., 1973) which produce invasive (Mareel et al., 1975; Meyvisch and Mareel, 1979) and metastasizing (Meyvisch et al., 1980) tumors after transplantation into syngeneic mice. Invasion is a characteristic of malignant cells and, next to unbalanced growth, responsible for the fatal outcome of many tumors (Mareel, 1980). The relevance of invasion in three-dimensional culture has been shown in as much

as all cell lines that were invasive in vitro produced invasive tumors in syngeneic animals (Mareel, 1979). Furthermore, the histopathology of invasion in vitro resembled that of invasive tumors in the animals (Mareel and Meyvisch, 1981). The wide use of VLBS, VCR and VDS in the clinic prompted us to investigate whether these drugs are anti-invasive in vitro at clinically achievable plasma concentrations. The effect of these compounds was examined on activities of MO_4 cells which might be involved in invasion: growth, directional migration and assembly of the cytoplasmic microtubule complex. Mareel and coworkers (1981) reported that at anti-invasive doses the vinca alkaloids inhibited the growth of MO_4 cell aggregates in individual shaker cultures. Absence of normal mitotic figures, accumulation of C-mitoses (Levan, 1954) and formation of multimicronucleated cells (De Brabander, 1976) strongly suggest that interference of the drugs with the assembly of the mitotic spindle is responsible for inhibition of growth. Anti-invasive agents that do not interfere with growth have not been found. Therefore, only indirect evidence is available that the anti-invasive effect of the vinca alkaloids is not due to inhibition of growth. Previous experiments with a number of growth inhibitors (Mareel and De Brabander, 1978; Mareel et al., 1980; Storme and Mareel, 1981) that do not affect microtubules make it unlikely that inhibition of growth is responsible for the anti-invasiveness of the vinca alkaloids. Increasing the number of cells in the confronting aggregate (diameter of 0.4 mm instead of 0.2 mm) did not alter the anti-invasive activity of the vinca alkaloids. In presence of 5-fluorouracil, invasion from a larger (0.4 mm) MO_4 cell aggregate occurred more rapidly than from a smaller (0.2 mm) aggregate. These data strengthen the idea that growth and invasion are basically unrelated activities of MO_4 cells. They indicate that growth might indirectly influence invasion by increasing the number of invasive cells. For one microtubule inhibitor (Nocodazole) we have presented direct evidence that the anti-invasive effect was not due to interference with spindle microtubules (Mareel and De Brabander, 1978). In this experiment MO_4 cells were treated with 5-fluorouracil, which arrested cells in S-phase but allowed invasion. The antiinvasive effect of Nocodazole on these cells could only be ascribed

to abolishment of the cytoplasmic microtubule complex because spindle microtubules were not formed. Abolishment of the cytoplasmic mictrotubule complex by vinca alkaloids might well explain their anti-invasive effect. Various authors have reported that directional migration depends on an intact cytoplasmic microtubule complex (Storme and Mareel, 1980; DeBrabander et al., 1976; Vasiliev et al., 1970; Spiro and Mundy, 1980). It is highly probable, as reviewed in (Mareel, 1980), that directional migration is a vital activity of invading cells.

12. Chapter: Chemotaxis and locomotion of activated neutrophils

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Summary
The ability of a cell or organism to direct its movement along a chemical gradient is called chemotaxis and this process of requires transformation of directional information from the environment into a series of cellular responses resulting in directional movement. the leukocytes are the only vertebrate cells in which this ability has been shown definitively. Studies have focused on the chemotaxis exhibited by the neutrophilic polymorphonuclear leukocytes (PMNs), whose chemotaxis presumably facilitates their accumulation at sites of injury or infection (Zigmond, 1978).

Chemotaxis of polymorphonuclear leukocytes
The Boyden chamber system or a modification thereof has been used to measure chemotaxis of polymorphonuclear leukocytes and monocytes (Boyden, 1962; Snydermann *et al.*, 1972) and, more recently, as a measure of random locomotion of the same cell types (Goetzl *et al*, 1974). Zigmond and Hirsch (1973) have demonstrated that these cells exhibit a "random walk" type of behavior, and that the distance moved by the leading edge of the cell population as designated by the two front-moving cells was a true indicator of the movement of the cell population at large.

Phagocytosis by polymorphonuclear leukocytes
Phagocytosis of zymosan particles by human peripheral blood polymorphonuclear leukocytes (PMN) results in the release of lysosomal enzymes into the extracellular medium (Weissmann *et al.*, 1971) and enhanced glucose oxidation and lactate production (Karnovsky *et al.*, 1971). The antibiotic, cytochalasin B (CB), inhibits phagocytosis by PMN (Davis *et al.*, 1971; Zigmong and Hirsch, 1972), presumably by interfering with the membrane-associated network of microfilaments (Wessels *et al.*, 1971). CB is therefore useful in studies of the interrelationship between phagocytosis and metabolic changes in stimulated PMN. The study by Skosey *et al.* (1973) confirms earlier reports that CB inhibits phagocytosis by PMN (Davis *et al.*, 1971; Zigmong and Hirsch, 1972; Malawista *et al.*, 1971) and carbohydrate metabolism in phagocytosing PMN (Zigmong and Hirsch, 1972; Malawista *et al.*, 1971; Skosey *et al.*, 1972). The release of the lysosomal enzyme, β-glucuronidase, however, was moderately stimulated by CB under conditions which resulted in almost total inhibition of phagocytosis. Phagocytosis of zymosan by human peripheral blood PMN, measured by the uptake of [^{14}C] inulin from the incubation medium, was inhibited by CB. CB also decreased zymosan-stimulated oxidation of [$^{1\text{-}14}$C] glucose to ^{14}CO2 and production of lactate, but moderately stimulated the release of β-glucuronidase. The results suggest that release of β-glucuronidase from cells exposed to zymosan does not require phagocytosis of the particles.

Multimicronucleation in MO$_1$ cells due to antitubulins
Continuous 'treatment of MO or MO cell cultures induces a multimicronucleation response that is completely reproducible, as evidenced by all cells that enter mitosis. MO is an epithelioid-type C3H mouse embryo cell line which in culture shows contact inhibition of movement and mitosis. The multimicronucleation of MO cells seems to be induced only by interference with microtubular structures or functions and can easily be distinguished from the multinucleation induced by interaction with cytokinesis. Indeed, only drugs that are known to interfere with microtubules produce micronucleation, and not the unrelated cytostatic or cytotoxic drugs such as alkylating agents or antimetabolites. The specificity

is also substantiated by the inactivity of lumicolchicine, the isomer of colchicine that does not bind to tubulin. Moreover, multinucleation induced by antitubulins is preceded by a sequence of easily observable events (morphologic alterations and lobulated mitoses) that may help to eliminate ambiguity (De Brabander *et al.*, 1976).

Effect of leukocyte migration inhibition drugs on fibroblast and fibrosarcoma

Drugs that have been shown to affect the motility of leukocytes were tested for their effects on the fibrosarcoma (FS) and mouse embryo fibroblast (MEF) cells in the agarose drop assay. It was possible to inhibit the migration of both FS and MEF cells with certain of these agents. However, none of these agents inhibited migration at concentrations far below those concentrations that induced cytotoxic alterations or inhibited the growth rates of the cells.

Inhibition of the invasiveness of MO_4 mouse fibrosarcoma cells by the vinca alkaloids, vinblastine (VLBS), vincristine (VCR) and vindesine (VDS), has been examined *in vitro*. MO_4 cells are virally transformed fibroblastic C3H mouse cells (Billiau *et al.*, 1973) which produce invasive (Mareel *et al.*, 1975; Meyvisch and Mareel, 1979) and metastasizing (Meyvisch *et al.*, 1980) tumors after transplantation into syngeneic mice. At doses between 0.006 microgram per milliliter (minimal effect) and 0.1 microgram per milliliter (complete inhibition) these drugs interfered with the invasion of MO_4 cells from an aggregate confronting a fragment of embryonic chick heart in three-dimensional culture. The effect of these drugs was examined on the growth, directional migration, and assembly of the cytoplasmic microtubule complex of MO_1 cells. Growth and directional migration were affected by the same doses of vinca alkaloids as invasion. In contrast with the vinca alkaloids, 5-fluorouracil at 1 microgram per milliliter inhibited growth but allowed directional migration and invasion. At a dose of 0.3 microgram per milliliter VLBS, VCR and VDS interfered with the assembly of cytoplasmic microtubules, as visible after immunocytochemical staining with tubulin antiserum. Ultrastructural analysis demonstrated that inhibi-

tion of invasion in three-dimensional culture corresponds with abolishment of the cytoplasmic microtubule complex. Anti-invasive concentrations of VLBS, VCR and VDS represent clinically achievable plasma concentrations. It is concluded that the anti-invasive effect of the vinca alkaloids may contribute to their antitumor activity.

Effect of COL on chemotaxis of neutrophils
The orientation of neutrophils toward a chemotactic source is only partly microtubule dependent, and the degree of impairment is affected by the strength of the chemoattractant gradient (Zakhireh and Malech, 1980).

Effect of COL on collagen synthesis on fibroblasts
The effect of colchicine on collagen secretion was examined with 3T3 cells by Diegelmann and Peterkofsky (1972). This is an established line of mouse embryo fibroblasts that actively secrete collagen into the culture medium. Examination of photographs taken after 1 hr of incubation in the absence or presence of colchicine reveal that the long processes characteristic of fibroblasts had been retracted in the colchicine-treated cells (Diegelmann and Peterkofsky, 1972).

Main Body:
The ability of a cell or organism to direct its movement along a chemical gradient is called chemotaxis and this process of requires transformation of directional information from the environment into a series of cellular responses resulting in directional movement. Many lower organisms including bacteria, protozoa, and slime molds exhibit chemotaxis (Zigmond, 1978). This ability helps them find nutrients, avoid noxious stimuli and aggregate at critical times in their development. Reports on higher organisms indicate that primordial germ cells (Zakhireh and Dubois, 1974; Dubois, 1965), neurons (Roisen and Murphy, 1973; Romualdez and Ward, 1975), tumor cells (Romualdez and Ward, 1975), and fibroblasts (Postlethwaite *et al.*, 1976) exhibit chemotaxis. However, the leukocytes are the only vertebrate cells in which this ability has been shown definitively. Studies have focused on the chemotaxis exhibited by the neutrophilic polymorphonuclear leukocytes (PMNs), whose

chemotaxis presumably facilitates their accumulation at sites of injury or infection. The chemical signals involved in leukocyte chemotaxis have been studied before. These include serum factors (Keller and Sorkin, 1966; Ward *et al.*, 1965; Wilkinson *et al.*, 1969; Wissler *et al.*, 1972), particularly a fragment of the fifth component of complement (Boyden, 1962; Shin *et al.*, 1968; Snyderman *et al.*, 1969; Ward and Newman, 1969), the bacterial metabolites (Keller and Sorkin, 1967; Turner *et al.*, 1975), cell-derived materials from sensitized lymphocytes (Ward *et al.*, 1970) and from PMNs (Borel *et al.*, 1969; Cornely, 1966; Phelps, 1969; Zigmond and Hirsch, 1973); and denatured proteins (Wilkinson and McKay, 1972).

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of endocytosis, and is not specific for either phagocytosis or pinocytosis (Berger and Karnovsky, 1966). Since endocytosis of either type is associated with metabolic changes (Graham *et al.*, 1967), it is reasonable to relate PMN metabolism and function to [^{14}C]-inulin uptake.

Three possible mechanisms have been proposed for the release of lysosomal enzymes from phagocytosing cells (Weissmann *et al.*, 1971). The first, release of enzymes after cell death, cannot account for release of lysosomal enzymes from PMN phagocytosing zymosan, since lactic dehydrogenase, a non-lysosomal cytoplasmic enzyme, is not released from cells in response to zymosan (Weissmann *et al.*, 1971). A second mechanism which has been proposed is that of "regurgitation during feeding." Electron microscope examination of rabbit neutrophils phagocytosing zymosan particles (Henson, 1971) has suggested that lysosomes fuse with the phagocytic vesicle as it forms during particle uptake. The lysosomes appear to discharge their content of enzymes into the vesicle. Enzymes are then extruded through the unclosed "mouth" of the phagocytic vesicle.

Henson (1971) has suggested that neutrophils which have phagocytosed many particles "run out of energy" and become inefficient in closing off developing phagocytic vesicles. Lysosomal enzymes which have been extruded into the vesicle are allowed to leak out into the medium. Since CB inhibits glucose metabolism (Zigmong and Hirsch, 1972; Estensen and Plagemann, 1972), it is possible that the early phases of phagocytosis occur in the presence of CB, but that the antibiotic deprives the cells of sufficient energy to close off the mouth of the vesicle. However, the observation that complete deprivation of the cells of exogenous glucose does not mimic the effects of CB argues against this possibility. We favor a third mechanism, "directed exocytosis," or "reverse endocytosis," proposed for the stimulation of release of enzyme by zymosan. Phagocytosis is apparently not required for the particles to stimulate enzyme release. It thus appears possible that zymosan, by coming into contact with cell membrane receptors, triggers lysosomal enzyme release without the requirement for the development of a phagocytic vesicle.

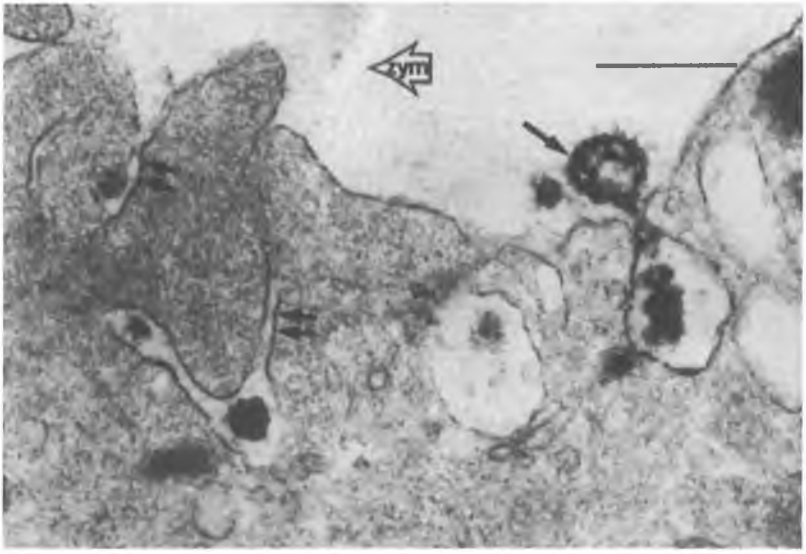

FIGURE 1: *a portion of a human blood neutrophil treated with cytochalasin B and then exposed to zymosan (zym). Peroxydase positive material is seen emerging from a lysosome that had fused with cell membrane (arrow). The double -arrow shown on the left side other deposits in lysosome open to the outer side of the plasma membrane (magnification 40.000, curtesy of Zurier, Hoffstein, & Weissann, 1978, The JOURNAL OF CELL BIOLOGY, VOL 58, pages 97-41. Courtesy of The Rockefeller University Press*

Orci *et al.*, (1972) observed that CB enhances glucose-induced secretion of insulin by isolated rat pancreatic islets. They suggested that the network of microfilaments of the beta cell might act as a barrier which denies the insulin-secretory granules access to the cell membrane. CB, by disrupting the function of this network, could cause enhancement of insulin secretion. A similar mechanism could explain the moderate enhancement of zymosan-stimulated β-glucuronidase release from PMN.

Multimicronucleation in MO_4 cells due to antitubulins
De Brabander *et al.*, (1976) observed the following sequence of events when the antitubulins (colchicine, vinblastine, vincristine) were added to cultures of MO or MO_4 cells. Within 10-80

minutes (depending on the drug and dosage used), the cells began to lose their characteristic stretched shape and assumed a rounded form, with loss of major cell processes. Ultrastructural observation showed that, with these drugs, this loss was accompanied by the gradual disappearance of the cytoplasmic microtubules. After a varying interval, depending on the culture conditions and the relative degree of synchronization, the cells rounded completely and assumed the bright character of mitotic cells. However, normal metaphase or anaphase figures were never observed. The cells remained rounded for approximately 5-6 hours, whereas normal mitoses are accomplished within 45 minutes. Then each rounded cell began to form progressively growing buds until the cells assumed the shape of a twisted string of sausages with multiple furrows. Time-lapse cinematographic observations showed that these cells displayed vigorous movements by peristaltic displacement of the furrows. After an additional 5-6 hours, each cell began to adhere onto the substrate and became multimicronucleated. By electron microscopy, it was noted that this type of cell was produced by separate enveloping of individual chromosomes or small clumps into a new nuclear membrane, as described by Starr (1963).

These multinucleated cells were viable for prolonged periods (more than 14 days), and, if allowed to recover in normal growth medium, could produce a progeny of normal-appearing mononuclear cells by complex multipolar mitoses and through the fusion of the micronuclei with each other. If the mitotic cells in treated cultures were shaken off and reseeded in the same medium, then all cells became multinucleated. This was confirmed by time-lapse observation of numerous individual cells (more than 300), which all became multinucleated after undergoing the abortive mitotic phase. Alternatively, the blocking of cells at a premitotic point of the cycle by 10 microgram 5-fluorouracil per millilitre resulted in a complete inhibition of multinucleation. A 99% inhibition was obtained by post-confluent inhibition of mitosis (De Brabander *et al.*, 1976).

Effect of leukocyte migration inhibition drugs on fibroblast and fibrosarcoma

Drugs that have been shown to affect the motility of leuko-

cytes were tested for their effects on the fibrosarcoma (FS) and mouse embryo fibroblast (MEF) cells in the agarose drop assay. It was possible to inhibit the migration of both FS and MEF cells with certain of these agents. However, none of these agents inhibited migration at concentrations far below those concentrations that induced cytotoxic alterations or inhibited the growth rates of the cells. Furthermore, even at drug concentrations that induced cytotoxic changes in the FS cells and inhibited the migration of the corona of cells by 90 percent, many individual cells were still observed to have migrated far from the edge of the agarose drop. The only drug tested which did effectively inhibit the migration of this population of cells was cytochalasin B, a drug which acts on the microfilament system (Zigmond and Hirsch, 1972). The inability to inhibit the migration of these cells with high concentrations of the various chemical agents may be of practical importance as well as of theoretic interest since it may be that these fast-moving cells are responsible for the invasive nature of this tumor. On the other hand, the number of cells that migrated beyond the leading edge of the corona could be reduced by 80 to 90 percent when normal human serum was used in place of fetal calf serum. This implies that this characteristic can be modified under the appropriate conditions.

Effect of vinca alkaloids on fibrosarcoma

The antitumor activity of the naturally occurring vinca alkaloids, vinblastine (VLBS) and vincristine (VCR) and the semi-synthetic VLBS-derivative vindesine (VDS), has been demonstrated in experimental and human cancer (Gerzon, 1980; De Brabander *et al.*, 1976). Mareel *et al.*, (1978) and Mareel *et al.*, (1980) reported that microtubule inhibitors (including VLBS and VCR) interfere with the capacity of MO_1 mouse fibrosarcoma cells to invade normal tissues in three-dimensional culture, and that abolishment of the cytoplasmic microtubule complex is responsible for this anti-invasive effect (Mareel and De Brabander, 1978; Mareel *et al.*, 1980; Storme and Mareel, 1981). MO_4 cells are virally transformed fibroblastic C3H mouse cells (Billiau *et al.*, 1973) which produce invasive (Mareel *et al.*, 1975; Meyvisch and Mareel, 1979) and metastasizing (Meyvisch *et al.*, 1980) tumors after transplantation

into syngeneic mice. Invasion is a characteristic of malignant cells and next to unbalanced growth, responsible for the fatal outcome of many tumors (Mareel, 1980). The relevance of invasion in three-dimensional culture has been shown in as much as all cell lines that were invasive in vitro produced invasive tumors in syngeneic animals (Mareel, 1979). Furthermore, the histopathology of invasion in vitro resembled that of invasive tumors in the animals (Mareel and Meyvisch, 1981). The wide use of VLBS, VCR and VDS in the clinic prompted us to investigate whether these drugs are anti-invasive in vitro at clinically achievable plasma concentrations. The effect of these compounds was examined on activities of MO_4 cells which might be involved in invasion: growth, directional migration and assembly of the cytoplasmic microtubule complex.

Mareel and coworkers (1982) reported that at anti-invasive doses the vinca alkaloids inhibited the growth of MO_1 cell aggregates in individual shaker cultures. Absence of normal mitotic figures, accumulation of C-mitoses (Levan, 1954) and formation of multimicronucleated cells (De Brabander, 1976) strongly suggest that interference of the drugs with the assembly of the mitotic spindle is responsible for inhibition of growth. Anti-invasive agents that do not interfere with growth have not been found. Therefore, only indirect evidence is available that the anti-invasive effect of the vinca alkaloids is not due to inhibition of growth. Previous experiments with a number of growth inhibitors (Mareel and De Brabander, 1978; Mareel *et al.*, 1980; Storme and Mareel, 1982) that do not affect microtubules make it unlikely that inhibition of growth is responsible for the anti-invasiveness of the vinca alkaloids. Increasing the number of cells in the confronting aggregate (diameter of 0.4 mm instead of 0.2 mm) did not alter the anti-invasive activity of the vinca alkaloids. In presence of 5-fluorouracil, invasion from a larger (0.4 mm) MO_4 cell aggregate occurred more rapidly than from a smaller (0.2 mm) aggregate. These data strengthen the idea that growth and invasion are basically unrelated activities of MO_4 cells. They indicate that growth might indirectly influence invasion by increasing the number of invasive cells. For one microtubule inhibitor (Nocodazole) it has been presented direct evidence that the anti-in-

vasive effect was not due to interference with spindle microtubules (Mareel and De Brabander, 1978). In this experiment MO_4 cells were treated with 5-fluorouracil, which arrested cells in S-phase but allowed invasion. The antiinvasive effect of Nocodazole on these cells could only be ascribed to abolishment of the cytoplasmic microtubule complex because spindle microtubules were not formed. Abolishment of the cytoplasmic microtubule complex by vinca alkaloids might well explain their anti-invasive effect. Various authors have reported that directional migration depends on an intact cytoplasmic microtubule complex (Storme and Mareel, 1980; DeBrabander *et al.*, 1976; Vasiliev *et al.*, 1970; Spiro and Mundy, 1980). It is highly probable, as reviewed in (Mareel, 1980), that directional migration is a vital activity of invading cells.

Effect of COL on chemotaxis of neutrophils

Zigmond (1977) used a novel assay of neutrophil orientation in a strong chemotactic gradient on glass coverslips and noted that only minor impairment of orientation occurred even in 10^{-3} M colchicine. Allan and Wilkinson (1978) showed in a cine-micrography study of neutrophils migrating toward a particulate chemotactic source that colchicine treatment resulted in a more tortuous path of migration than seen with controls when the cell was at a distance from the chemotactic source. But as the colchicine-treated neutrophil got closer to the source, the migratory path became quite straight. Thus, orientation of neutrophils toward a chemotactic source was only partly microtubule dependent, and the degree of impairment was affected by the strength of the chemoattractant gradient (Zakhireh and Malech, 1980).

Effect of COL on collagen synthesis on fibroblasts

The effect of colchicine on collagen secretion was examined with 3T3 cells by Diegelmann and Peterkofsky (1972). This is an established line of mouse embryo fibroblasts that actively secrete collagen into the culture medium. The control cells secreted up to 60 percent of the total collagen synthesized during a 2-hr incubation period. When the cells were first incubated in the presence of 10 micro molar colchicine, only about 12 percent of the collagen was secreted, an 80 percent

inhibition. Although the fraction of non-collagen protein secreted into the medium was much lower than collagen secretion, there was about the same extent of inhibition by colchicine. There was no concomitant inhibition of either collagen or non-collagen protein synthesis. Examination of photographs taken after 1 hr of incubation in the absence or presence of colchicine reveal that the long processes characteristic of fibroblasts had been retracted in the colchicine-treated cells (Diegelmann and Peterkofsky, 1972).

13. Chapter: Effect of anti-tubulins on the locomotion of chemotactic activated fibroblasts and fibrosarcoma cells in vitro

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1. Abstract
A new method for testing anti-tubulin agents COL and VBLS on chemotactically activated fibroblasts in a solution is presented in this study, which mimics cell movements in a solution and thus in 3-dimensional space. Different cell lines of fibroblasts from mouse and humans were examined *in vitro* for their locomotion and chemotactic response with migration rate under the influence of the anti-tubulin agents at different doses, respectively. Both anti-tubulin agents are known to be mitotic poisons that arrest human tumour cells, fibrocytes, higher plant cells, and green algal flagellate in the division phase of the nucleus by binding different cytoplasmic tubulin proteins which are responsible for different directions of locomotion. In a logarithmic growth phase, it was found that the highest rate of locomotion was on the day of maximum multiplication rate. On the following day, a significant decrease in both, rate of locomotion and multiplication, were observed.

For both anti-tubulin agents, a decrease in the locomotion was observed in all cell types. As the concentration increased, the decrease in the locomotion was observed with different characteristics for the individual cell types and anti-tubulin agents. The degree of locomotion of L-929 decreased by 50% at 5 mcg COL/ml CM and by 70% at 10 mcg VBLS/ml CM at 240 min, respectively. All cells had a reduction in migration frequency of up to 10% at 15 min. At longer contact time, the rate of the migration frequency remained constant (3T6;

L-929) or dropped (HT 1080, L6Y1, 3T3, C3H, HESF), similar to a plateau.

During random migration, at 15 minutes of incubation, there was an increase in activity in five cell lines (3T6, HT1080; 3T3, C3H; HESF), but the extent of this increase has differed; 0,05 mcg COL/ml caused an increase of almost 100% random migration in 3T6, whereas 10 mcg VBLS caused only a slight increase.

2. Introduction

Fibrosarcoma is a tumour of mesenchymal soft tissue affecting both children and adults. Fibrosarcoma also occurs in animals, particularly cats, horses, and dogs (Bostock *et al.*, 1979), which exhibit a tendency for metastasis. Fibrosarcoma arises and are often encapsulated in connective tissue as well as from subcutaneous tissue. The fibrosarcoma tissue masses are firm, poorly circumscribed, by the transformation of mesenchymal fibroblasts to form mitosis-rich, multimicronucleated cells interspersed with fibrous connective tissue strands.

The etiology of fibrosarcoma is unknown. The occurrence of fibrosarcoma in animals is associated with viral diseases, chronic inflammation, and implantation of foreign bodies (Boyland *et al.*,1968; Buoen *et al.*, 1975). Aromatic triphenyl dyes are also suspected of developing fibrosarcoma (Driessens *et al.*, 1963). Chronic localized inflammation and certain dietary supplements also witnessed to cause the frequent occurrence of fibrosarcoma (Grasso *et al.*, 1971).

The prognosis of fibrosarcoma to metastasis is unfavourable in the absence of effective antitumour therapy against it. The process of metastasis of fibrosarcoma could be guided by chemotaxis than a random seeding of tumour cells (Chen *et al.*, 1979; Lam *et al.*, 1981). Chemotaxis is a unidirectional process of migration, where eukaryotes actively migrate towards higher concentration gradient of a chemoattractant such as an antigen (Caner *et al.*,1965; Gaus *et al.*, 1980; Malech *et al.*, 1977; Mundy *et al.*,1981; Postlethwaite *et al.*, 1976; Romualdez *et al.*, 1975, 1976).

The *in vitro* migration behaviour of polymorphonuclear leukocytes under the influence of antigen/antibody complexes has been studied by using a special device, the Boyden chamber (Boyden *et al.*, 1961), later by Romualdez (Romualdez *et al.*, 1975, 1976). The leukocytes with "chemotactic activity" were separated from a solution by using a Millipore filter with 8 mcm pores and incubated in the special device to study their migration behaviour. Increased migration of leukocytes into the pores was observed in the presence of fresh serum containing antigen/antibody complexes (Lipton *et al.*, 1971). The Boyden chamber was also used for evaluating the targeted movements of fibroblasts, fibronectin (Gaus *et al.*, 1980), and fractions of type I collagen, and collagen peptides with amino acid sequences such as glycine-proline-hydroxyproline (Mundy *et al.*, 1981). The activated complement component C5 proved to be an effective chemoattractant for leukocytes (Romualdez *et al.*, 1976; Chen *et al.*, 1979; Postlethwaite *et al.*, 1976; 1978; 1981). The C5 component is a large natively folded molecule with pronounced antigenic properties that can be released from macromolecules by enzymatic reactions (Lam *et al.*, 1981). The presence of fragments of fibronectin, foetal calf serum (Lipton et al., 1971) in the growth medium as a chemoattractant might induce the directional migration of fibroblasts, which helps to study the migration behaviour of fibrosarcoma cells (Postlethwaite et al., 1976, 1981). Further, due to the presence of the various chemotactic substances, each of them exhibiting chemoattractive action by itself on fibroblasts, a summation effect can be taken into consideration on their action on directed cell migration. These molecules with induction of controlled migration behaviour, further to that a simple process of extraction and their purification, have been previously used to study tumour cells, especially fibrosarcoma cells (Chen *et al.*, 1979; Mundy *et al.*, 1981).

Random migration is the non-stimulated non-directed movement that occurs in addition to the directed movement. Several studies have enumerated the cellular causes of chemotactic directed movement and random migration (Zigmond and Hirsch, 1973).

The exogenously administered calcium ions and ATP influenced the directed movements of contractile proteins of fibroblasts. Thus the requirements for directed locomotion of fibroblasts *in vitro* is established (Hoffmann-Berling *et al.*,1964). Researchers from the National Institute of Cancer, USA, suggested that the migrating fibroblast could be lenticular and would generally move in the direction of its 'leading edge'(Gail 1973). Basis on this axial organization some recent studies asserted that 'the main locomotory organ of the fibroblast is its "ruffled membrane at the front end" (Abercrombie 1961). The migrating fibroblast thus resembles a shoe with its unattached central arch. In a study, it has been suggested that a 'cytoskeleton', composed of microtubules, is required to maintain such asymmetry, and indeed such distribution of microtubules have repeatedly been identified in moving fibroblasts (Buckley *et al.*,1967; Goldman et al., 1969; Goldman *et al.*, 1971).

Microtubules are also actively involved in the directed migration of fibroblastoma cells (Goldman *et al.*,1971; Tilney *et al.*,1971). Microtubules are non-branched helical protein chains with a molecular weight up to 60,000, building up quaternary structures, i.e. dimers, after ribosomal protein synthesis, in order to form large subunits with a diameter of about 220 - 250 angstrom. Microtubules are found in many different eukaryotic cells. During cell locomotion, microtubules align longitudinally with the direction of movement (Porter *et al.*, 1966; Stephens *et al.*, 1970). Thus, it is evident that the influence of microtubules of fibroblasts results in healing of wounds (Ehrlich *et al.*, 1977).

The locomotion of intact fibroblasts is a combination of cytoplasmic interactions of contractile proteins such as actin-myosin, tropomyosin, microtubules, and microfilaments, facilitated by cytoplasmic oxygen, ATP, GTP, calcium ions, and magnesium ions. In an early study on the locomotion of mouse embryo fibroblasts, (Freed et. al., 1968) it has been demonstrated that COL in a concentration of 10^{-5} M or higher can decrease directed migratory activity of cells by destruction or disassembly of quaternary structures of cytoplasmic microtubules, whereas the amoeboid motion of membrane and formation of microvilli

and microfilaments were not disturbed. Further, it has been identified (Gail 1973) that anti-tubulin agents such as COL at certain concentrations, are neither karyotoxic nor cytotoxic, but can modify locomotion of fibroblasts indirectly by incapacitating microtubules which are responsible for the ordered skeletal structure of fibroblasts. Thus, it is evident that microtubules are responsible for the 3-dimensional static structure of cells, whereas the microfibrils are serving for cell contraction and locomotion, respectively (Abercrombie, M, 1961; Carr *et al*, 1972). In insect *Notonecta glauca*, microtubules are responsible for the ordered structure and transport line of ribosomes. The transport line is full of ribosomes connected with microfilaments to the microtubules (MacGregor *et al.*, 1970). It disassembles if the concentration of COL is higher than 10^{5} M.

Intensive studies on the morphology of BHK21 fibroblasts under the influence of anti-tubulin agents, especially COL, have confirmed peculiarities of cell movements that depend on the presence and function of microtubules and microfilaments (Abercrombie *et al.*, 1958; Freed *et al.*, 1968; Goldmann *et al.*, 1971).

Microfilaments are intracellular structures consisting of helical protein chains. They are smaller than microtubules, assemble post-transnationally into larger subunits with diameters of about 50 angstrom, and were thought to be responsible for cell "spreading" (non-directed) and "rippled cell" membrane (Abercrombie *et al.*,1958 and Goldman *et al.*,1971).

Cell growth and locomotion are normally subject to contact inhibition in 3-dimensional space (Abercrombie *et al.*, 1967). The experimental evidence suggests that contact inhibition is abolished and cells form aggregates in 3-dimensional space for the locomotion of fibroblasts (Armstrong *et al.*, 1978; Abercrombie *et al.*, 1970).

The Boyden chamber, that has been used for evaluating the directed migration of leukocytes from the bloodstream into tissue, also has been used to study the migration behaviour of tumour cells such as fibrosarcoma cells under the directed influence of chemofactors and pharmaceuticals. Due to the fact,

that the process of directed migration would last only for a short period of time (i.e. no more than 240 min), the majority effects of pharmaceuticals on the metaphasic division rate and accumulation of arrested cells could have been excluded (Varani et al., 1978). The pharmaceuticals bind to microfilaments and exert their effect to slow down the locomotion. Basically, the undisturbed cell lines have an anterior flat extended pole with an anterior lamella, followed by the central cell body (Veseley *et al.* 1973). While moving, these cells appear elongated in the direction of movement and move consistently. (Abercrombie *et al.*, 1958; Postlethwaite *et al.*, 1981). Under the influence of anti tubulins, the cells changed their outer shape. Armstrong (1979) observed in fibroblasts treated with therapeutic concentrations of COL (10^{5} M) and VBLS (10^{6} M), the decrease of elongated shape and leading lamella.

Therefore as part of this work, the cells used were treated with VBLS and COL, two anti-tubulins with cytostatic effects that play an important role in today's chemotherapy of malignant tumours.

COL and VBLS have different chemical structures, that target tubulin proteins or receptor proteins, and show disorganization of the outer cell membrane of mammalian cells, respectively.

COL is an alkaloid extracted from the tree *Colchicum autumnale*, as reviewd in previous chapters. The COL alkaloid has a tricyclic chemical structure with a tropolone ring, which has been synthetized as an optical active compound (Schreiber *et al.*, 1959).

The IUPAC name of COL is (S)-N-(5,6,7,9-tetrahydrobenzo-1,2,3,10-tetramethoxy-9-oxobenzo(a)heptalene-7yl) (*) acetamide with a molecular weight 339 g/ mol. At position 7 (*) of the heptavalent ring there is an acetamido group that assigns chiral properties to COL. COL is soluble in cold aqueous solutions, e.g. Dulbecco's trypsin- EDTA bovine serum, which is used as a medium for growing fibrosarcoma cells (Martin *et al.*, 1981). Brues & Cohen (1936) carried out experiments with

various derivatives of COL, which they had obtained from the MERCK company, in order to gain clarification as to which molecular groups of COL might be responsible for its mitosis inhibition, but these did not yield any results.

COL is available in at least 2 stereoisomers, of which only the left-turning S (-) form binds to tubulin receptors. This optical active stereoisomer can be extracted from natural plant sources. It is assumed that the 4 methoxy groups in combination with the tricyclic spatial arrangement cause the binding to tubulin receptors. COL has been used as an experimental antineoplastic drug in human fibrocytes *in vitro* (Dominici *et al.*, 1932; Oughterson et *al.*,1937), sarcoma cells in experimental animals (Clearkin, 1937; Dustin *et al*, 1934), in the therapy of gout (Dustin *et al.*, 1978), and human cancer (Deckner 1938). Deckner has observed an unusual large proportion of nuclear divisions on the cytological examination of 30 different human carcinomas and among them an unusually large proportion of pathological forms, but no division FIGURE specific for the cancer cell or comparable to specific action of COL in non-cancerous cells. Similar findings were also obtained by MAUER (1938) with cancerized fibroblast cultures.

The chemical synthesis of COL with subsequent purification by recrystallization, chloroform can be bound up to 25% w/w, which influences the properties of COL, in particular on studies of the metaphase of mitosis (Brues *et al.*,1936; Schreiber *et al.*, 1959). Due to this fact for the following experiments, it must be ruled out that COL has been recrystallized with chloroform previously since chloroform itself influences both, the metaphase and migration of cells. In this study, it was used as an amorphous compound free of chloroform, which is in contrast to crystalline modification.

The medical use of COL has been reported as early as 457–475 A.D. by Jacques Psychriste, physician of Emperor Leon the Great (Lits *et al.*, 1938).

In *in vitro* studies on the cells of mice (strain C3H), COL has been observed to arrest the metaphase during the exponential

growth phase. The tumour growth in mice has been studied after injecting increasing dosages of COL (Amoroso 1935; Dustin *et al*, 1934; Lits *et al.*, 1933; Lits *et al.*, 1938). Ludford (1936) investigated the influence of COL concentration on cell division and metaphase arrests of tumour cells in mice. Further studies had been focused on evaluating the influence of COL on tumour growth in animals such as rabbits on Shope`tumour, a metastatic carcinoma in rabbits caused by Shope papilloma virus (Peyron *et al*, 1937), and of human fibrosarcoma cells (Oughterson *et al.*,1937), the growth of human fibroblasts, myolid leukemia (Paul *et al.* 1941), and Hodgkin disease (Broun *et al*, 1950). Quite surprisingly, experimental studies in mice have shown that repeated injections of 12 mcg COL per 24 hours increased the leukocyte count in the form of leukocytosis with up to 250,000 leukocytes per microlitre, after initial leukopenia. The number of cells arrested in metaphase also increased significantly. It was assumed that the leucocytosis was the result of central nervous stimulation of the bone marrow with predominance of granuloblasts (Widmann, 1947, 1948, 1949).

It has been observed that the influence of COL on the metaphasis of mitoses of cell division is through dose-dependent chromatin pyknoses. This results proposed the use of COL in human tumour therapy (*Lits et al.*, 1938; *Dustin* 1934). Administration of higher doses of COL with increasing concentrations caused damage to organelles of the cytoplasm and nucleus components (Haas *et al.*,1940). The histochemical and ultrastructural studies on HeLa cells incubated with increasing concentrations of COL (i.e. 10^{6} M to 10^{3} M) revealed numerous non-coordinated cytoplasmic fibrils with the decrease of microtubules (Robbins *et al.*, 1964).

At low concentration (0.01% to 0.5% COL), COL did not affect the cell division of green algae even after prolonged inoculation of up to 24 hours (Shyam *et al.*, 1976). However, when administered at a high concentration (above 1% COL) and exposure times of 4 hours to 10 hours, cells with polyploid nuclei were observed. Higher doses also affected the external cell shape, where COL treated cells changed from a spherical to epithelioid like, rounded oedematous swollen shape (Goldman *et al.*,

1971). But under much lower COL concentration, i.e. 10^{-6} M cells showed tetraploidy, known as c-mitosis (Eigsti *et al.* 1955). As demonstrated with green algae, eukaryote cells have not been affected visibly by COL concentration lower than 10^{-6} M.

The change from external cell shape to roundness and anterior position of the nucleus has been observed not only in cultures of green algae, fibrosarcoma cells such as L-929 (Armstrong *et al.*, 1957), but also in cultures of plant cells treated with COL. Phytopharmacological studies by *Macht & Livingstone* (1922) on the effect of COL on the roots of lupines, as lined out in other chapters of this book, showed pronounced clubbing with rounding of the cells. For the time being, the effect of different concentrations of colchicine on root growth was established. Colchicine cryst. in dilutions of more than 1 : 90.000 has a growth-inhibiting effect on lupine roots at room temperature. Under the influence of colchicine, the characteristic clubbing of the roots described by a number of researchers for various plants occurs on the lupins. *Havas* (1937 a) was the first to briefly mention the formation of clubs on the roots of wheat. *Eigsti* (1938) independently described the formation of clubs on roots of *Raphanus salivus*, *Triticum vulgare, Allium cepa* and *Zea mayz. Levan* (1938) observed the clubbing of *Allium cepa* tumours are formed by the root meristem, while the lenght growth ceases altogether. *O'Mara* (1939) emphasises that hypotrophy on roots does not occur in the meristematic zone but in the elongation zone. *Mangenot* (1939), *Garriques* (1939) and *Shimamura* (1939) also note hypertrophy of the growth zone. *Boas and Gistl* (1939) describe clubroots on *Zea, Vicia, Taba, Phaseolus, Lupinus, Hordeum and Linum usitatissimurn* and bring very clear illustrations of clubs on the roots of plants treated with colchicine. *Bhaduri* (1939) has described an early differentiation of vessels and sieve tubes in the root clubs. Whereas *Macht* always uses length growth as a criterion in his studies on the effects of other poisons, here the macroscopically visible symptom of the colchicine effect is the clubbing of both the main root and the lateral root.

Mairold (1943) incubated roots of Lupinus with increasing concentrations of COL at 20 degrees centigrade, i.e. 0.005 %

and 0.01 % COL cryst. in water. In 0.01 % COL solution the growth inhibition compared to the control is only 11 % , while after 48 hours 53 % , after 72 hours 69 % and after 96 hours already 77 % inhibition was observed. The inhibition becomes more and more obvious after 48 hours and later, because while the control roots continue their growth, the longer lasting poison influence becomes more and more noticeable, until finally the colchicinated roots stop their growth completely.

With increasing concentration, the inhibition of the length growth of Lupinus roots does not increase uniformly. Up to concentrations of approx. 0.005 % colchicine, there is a very marked increase in inhibition, while a further increase in concentration up to 0.01 % or 0.02 % colchicine shows a very insignificant increase in inhibition.

COL is taken up by cells by passive diffusion (Taylor *et al.*, 1965), and thus, uptake is not saturable. It has been shown, that approximately 90% of accumulated COL is bound to cytoplasmatic proteins (Borisy *et al.*, 1967), and further, to a COL-binding protein, in particular, tubulin (Krishan *et al.*, 1971; Wilson *et al.*, 1967). By binding to the cytoplasmic tubulin protein, COL inhibits polymerization of tubulin monomers to functioning microtubules and blocks cell functions caused by them, e.g. mitosis, leukocyte mobility, synthesis and secretion of collagen, antibodies, and polarization of cellular shape.

As early as 1881, Pernice demonstrated that COL blocks mitotic division in interphase with the formation of micormultinucleated cells (Benitz *et al.*, 1954; Deysson *et al.* 1944; Eigsti & Winn 1949).

The effect of COL depends on the number of tubulin-COL complexes. One dimer of tubulin can fix one molecule of COL. The rate of inhibition of polymerization of rat tubulin by 50% (ID 50) has been determined as 2.5×10^{-6} M (DeBrabander *et al.*, 1976). The inhibition threshold of c-mitosis, i.e. COL-mitosis of other eukaryotic cells has been determined (Eigsti *et al.*,1955; Meier *et al.*,1947) in chicken fibroblast cultures *in vitro* as being 1 nanogram COL/ mL. However, binding of COL to

cytoplasmatic proteins appears to be reversible (Borisy *et al.*, 1967) in human fibroblasts (strain K.B.), since COL reached an equilibrium in human fibroblasts for a given extra cellular concentration, and then lost gradually after suspension in a fresh medium (Minor *et al.*,1975) in a first-order decay with a half-life of approximately 4 hours. This decay depends on temperature, pH, ionic strength, and concentration of tubulin molecules (Wilson *et al.*, 1982). COL has not been fixed by cytoplasmatic molecules such as actin, myosin, or albumin (Borisy *et al.*, 1967), however might support synergistically it`s binding to tubulin proteins. The chemical quality and concentration of tubulin are of paramount importance for the colchicine-binding and thus activity on the mitotic division.

COL undergoes intensive metabolization in humans, i.e. demethoxylation, with the formation of inactive anti-tubular metabolites, such as octahydro-demethoxy-desoxy-desacetamido-colchicine (Rapoport *et al.*, 1951). There have been many reports on COL poisoning in the 19th century. After the discovery of COL influence on mitoses, a histological study had been performed on a fatal outcome of COL intoxication in a 41 year old female dosed with approximately 60 mg of COL (Dustin et al.,1941). The COL influence has arrested the mitotic metaphase, haemorrhagic destruction of the anterior lobe of the hypophysis has been observed (Dinarello et al., 1976). It had been reported on a fatal COL intoxication with hyponatraemic syndrome of the inappropriate release of anti-diuretic hormone (Gaultier *et al.*, 1975). In an early paper on the action of COL on tubulin *in vitro*, it has been shown that assembly could be completely prevented although less than 4 % of all the tubulin subunits were bound to COL (Olmsted *et al.*, 1973). It had been shown that 0.005% COL solution slowed down cell growth in interphase (Deysson *et al.*, 1944). The destroying effect of anti-tubulin agents on collagen secretion had been recently reported (Dieglemann *et al.*, 1972). In mouse fibroblasts both COL and VBLS (10^{-6} M) prevented the incorporation of radioactive C-14- proline, the major amino acid of collagen. It has been observed that COL at low concentrations (below 10^{-6} M), had blocked the secretion of collagen from 3T3 fibroblasts, however, did not prevent protein synthesis at the

ribosomes (Ehrlich *et al.*,1974). Golgi vesicles and lysosomes of isolated chondrocytes were scattered around the cytoplasm, presumably disturbed in their function (Moskalewski *et al.*, 1975), as observed with HeLa cells, exposed to the low dose of COL (Robins *et al.*,1964). Doses of COL and VBLS of 10^{-5} M completely removed the cytoplasmic microtubules, disbanded the ordered structures of the Golgi vesicles, and clustered lysosomes in isolated chondrocytes from foetal guinea pigs. In pathological conditions such as liver cirrhosis (after carbon tetrachloride intoxication), COL inhibits the formation and secretion of amyloid and collagen (Rojkind *et al.*,1975).

Degradation of microtubules has been described under COL concentrations of 5 to 40 mcg/ mL culture medium for BHK21 fibroblasts (Goldman *et al.*, 1971). An exposure of 6 to 8 hours at 10^{-7} M COL was sufficient to block essentially all the cells in metaphase of human K.B. cells, thus indicating that COL is bound to the majority of interphase cells (Bostock *et al.*, 1979; Taylor *et al.*, 1965). The temporal degradation of microtubules is dependent on COL concentration and temperature. At high concentrations of COL (10^{-3} M, 0.4 mcg / mL), the degradation rate of microtubules was approximately 3 min, compared with 30 min at 10^{-4} M (0,04 mcg/ mL) COL and 25 degrees Celsius (Inoue *et al.*, 1952). The time-dependent inhibition of locomotion/aggregation of both 3T3 and BHK21 cells by COL and VBLS in cell suspensions at 37°C had been attributed to destabilization of cytoplasmic microtubules (Waddell *et al.*, 1971). Inhibition had been more effective with lower concentrations of VBLS than with the same low concentration of COL. Both anti-tubulin agents exerted their inhibitory effect on the locomotion of fibroblasts within a few minutes after addition to the conditioned culture medium, which had been measured by Coulter Counter.

VBLS is an anti-tumour alkaloid isolated from *Vinca rosea Linn* (Johnson *et al.*, 1960), commonly used as the cationic sulphate salt (VBLS). The natural extract of VBLS consists of a mixture of stereoisomers, which not have been completely identified (Benvenuto 1981). The active compound has not yet been isolated so that always the mixtures of the extract have

been used. The structure of VBLS is completely different from COL. It consists of an indole part connected to a vindoline moiety. It would not be surprising if VBLS would be metabolized in the organism applied to, or to fibroblasts. This contact could affect the demethoxylation of the complex ring structures. However, to date, no such metabolites have been reported, to our knowledge. In the cell growth medium, it is usually present as a positively charged cation, after chemical derivatisation of the intact molecule with sulfuric acid (Merck Index 1976; Na *et al.*, 1982). VBLS is taken up by cells by passive diffusion and carrier-mediated transport and bound intracellularly to protein structures. It has been shown, that VBLS is extensively bound to cells in an exchangeable form, wherein the exchangeable part of VBLS is most likely not due to an active process (Beck *et al.*, 1981). Beck demonstrated that VBLS is accumulated intracellularly by human lymphoblasts relative to the culture medium, with more than 5-fold cell/medium ratio. The intracellular accumulation of VBLS appears to occur due to binding to cell organelles and is not due to active energy-dependent transport. The equilibrium of VBLS in cell/medium is temperature dependent and is established within less than 30 min.

Similar to COL, it inactivates tubulin and thus has corresponding effects.

There are two binding constants between tubulin and VBLS, the first one with 6.2×10^{6} /Mol being a tight binding and the second one with 8×10^{-4}/ Mol (Bhattacharyya *et al.*, 1976). The rate of half-maximum inhibition of tumour cells as being 0.83 nanograms VBLS/ mL had been determined (Johnson *et al.*,1960). Further, followed by the determination of the half-maximum inhibition of mouse fibrosarcoma cells as being 6 nanogram/ mL (Marceel *et al.*,1982). The rate of inhibition of polymerization of rat tubulin by 50%(ID 50) as $2,8 \times 10^{-7}$ M (i.e. 1 nanogram / mL), which was more effective than seen with the same concentration of COL, respectively (De Brabander *et al.*, 1976).

DNA synthesis, especially the new synthesis of purines was significantly reduced in suspensions of thymus cells by VBLS

at concentrations up to 50 mcg/mL solution within 30 min (Jones *et al.*, 1966; Van Lancker *et al.*, 1966).

There are differences in the sensitivity of cell suspensions to VBLS that have been attributed to individual cell characteristics (Waddell *et al.*, 1974). In particular, binding and inactivation by the formation of ordered paracrystals.

The formation of macro tubules and spiral assemblies of tubulin is closely related to the most apparent action of the Vinca alkaloids, i.e., the assembly of tubulin into crystalline birefringent structures. These were first described in L-strain fibroblasts with relatively low concentrations of VBLS (from 1 x 10^{-5} M to 4 x 10^{-4} M), the crystals appeared in less than 1 h, could reach a length of 8 mcm, and were formed of hexagonally packed tubules with a diameter of 27 - 28 nm. Some macro tubules of 28 nm were also observed by the authors who concluded that the crystals were made of tubulin (Bensch *et al.*, 1969). In particular, binding and inactivation by the formation of ordered paracrystals consisting of tubulin and VBLS described by electron microscopy, indicate intensive binding of the cationic charged VBLS to tubulins and anionic structures such as ribonucleic acid from ribosomes (Warnecke *et al.*, 1968). Isolated tubulins form protein dimers *in vitro* that form ordered tubulin aggregates under the influence of low magnesium ion concentration (less than 5 mM) and VBLS, which form paracrystals with VBLS (Na *et al.*, 1982). Others have found such paracrystals in micro-multinucleated cells, e.g., L-929 fibroblasts (Krishan *et al.*, 1971).

Both VBLS as well as COL, have demonstrated cytotoxic effects on target cells - *in vitro* (Chen *et al.*, 1976), in particular inhibition of the enzyme ornithine decarboxylase by VBLS. The inhibitory effect of VBLS is reversible by the addition of ornithine, arginine (Johnson *et al.*, 1960). The enzyme is responsible for the formation of polyamines on ribosomes. Polyamines stabilize ribosomes in protein synthesis, which is necessary for the formation of tubulins in the interphase of mitotic division and locomotion.

Surprisingly, under the influence of low concentrations of VBLS (10^{-5} to 10^{-6} M), blood monocytes show a maximal enhancement of migration into micropore filters without loss of viability (Crispe *et al.*,1976), which is in contrast to the impairment of locomotion of fibroblasts by VBLS and COL (Waddell *et al.*, 1974).

In this study, we focused on the influence of the 2 anti-tubulin agents, i.e. COL and VBLS on the locomotion of seven different fibrosarcoma cells and fibroblasts in a conditioned solution of a known chemoattractant by using the modified Boyden chamber (Boyden *et al.*, 1961; Postlethwaite *et al.*, 1976) from our laboratory. We have also evaluated the presence of different concentrations of the anti-tubulin agents and different times of contact between anti-tubulin agents and individual cell lines.

3. Results

The tests for functional activity of the cells at the end of the incubation showed an increase in LDH of approx. 4% compared to the beginning of the incubation with anti-tubulin. This was also shown in the decrease of cell movement within the first 15 min to 20 min after the start of incubation, which was true for all examined cell lines. Comparable was also the increase of pink stained cells with indication of metabolic disturbances and decrease of cell viability.

Logarithmic growth phase

In vitro behaviour of the cell lines was investigated without the influence of anti-tubulin agents. A maximum migration rate of cell lines was observed on the conditioned medium (CM) after the days of last trypsinization. These cell lines were used to obtain the optimal results.

The cell lines (L6Y1, HT1080, L-929, 3T6, 3T3, HESF, and C3H) were evaluated for the highest degree of locomotion on filters by using microscopic analysis. The maximum migration rate for L6Y1, HT1080 was observed at the end of the second day (i.e. 48 hours), and the maximum migration rate for 3T6, L-929, 3T3, HESF, and C3H, was observed at the end of the

third day (i.e.72 hours). In comparison with the relative multiplication rate of the cells in the flasks (see table 1 and Figure. 1), it was found that the highest rate of locomotion was on the day of maximum multiplication rate, i.e. maximum number of cells on a filter. It was observed that on the following day, a significant decrease in both rates of locomotion and multiplication was observed.

The results are listed in Table 1 and FIGURE 1 and correspond to the multiplication rate of cell lines.

Table-1: Relative multiplication rate of cell lines (one to five days after trypsinization)

CELL LINES	0 hr	24 hrs	48 hrs	72 hrs	96 hrs	120 hrs
L6Y1	100%	170%	160%	120%	130%	80%
3T3	100%	160%	160%	160%	70%	(--)
3T6	100%	160%	160%	160%	70%	(--)
L-929	100%	150%	170%	180%	80%	(--)
HT1080	100%	230%	180%	180%	(--)	(--)
C3H	100%	160%	160%	175%	75%	(--)
HSEF	100%	160%	165%	170%	70%	(--)

Table-1: *Relative multiplication rate of cell lines used, one to five days after trypsinization. In the periods marked with (--) the cells were already dead or detached. The measurement of cell density per microscopic visual field (x160) always took place at the end of the respective 24-hour period. It was checked in relation to the original initial concentration, which was given as 100%.*

FIGURE 1: Multiplication rate of cells after trypsinization

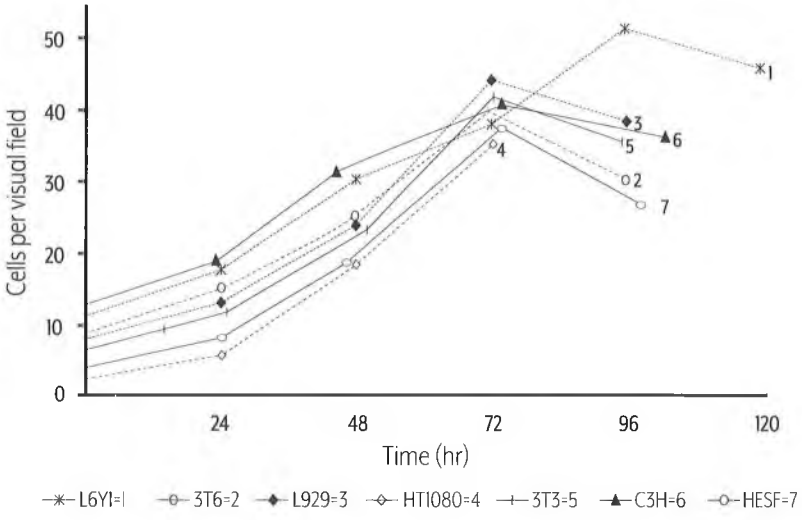


FIGURE 1: *Multiplication rate of cells after trypsinization, expressed as CPV. The highest rate of locomotion was on the day of maximum multiplication rate, i.e. the highest number of cells on filter: at 48 hours maximum multiplication rate of L6Y1, HT1080; at 72 hours maximum multiplication rate, of 3T6, 3T3, L 929, C3H, HESF.*

Anti-tubulin agents - conditioned medium
Cell lines change their migration behaviour when COL and VBLS were dissolved in the chemoattractant containing medium. The migration behaviour of the cell lines in the presence of increased concentrations of COL and VBLS (when COL and VBLS were dissolved in the chemoattractant) was evaluated, in order to learn to what extent it changes.

The anti-tubulin agents at four different concentrations (each lower by one power of ten) were evaluated. The maximum concentration used for VBLS was 10 mcg VBLS/mL (11 nmole/mL) and COL was 5 mcg COL/ mL (12.5 nmole/mL), as a highly effective anti-mitotic concentration (Armstrong *et. al* 1979).

For both anti-tubulin agents (COL and VBLS), a decrease in migratory rate was observed in all cell types, however with different expression concerning cell lines and anti tubulins, respectively. As the concentration increased, the decrease in the locomotion was observed with different characteristics for the individual cell types and anti-tubulin agents. The representative migration rate of cell lines is illustrated in Figure. 2 and Figure. 3.

The anti-tubulin agents are independent of their chemoattractive effects. For example, the degree of migration of L929 decreased by 50% at 5 mcg COL/ml CM and by 70% at 10 mcg VBLS/ml CM. Similar results were obtained with HT 1080, where COL caused 20% decrease at maximum concentration, whereas VBLS caused 75% decrease. On the other hand, at the same concentrations for 3T6, the decrease was 60% in each case, and for L6Y1, the decrease was 50% in each case. Under the influence of the highest concentration of COL, the migration of cell types of 3T3, C3H and HESF decreased by 50%. In the presence of the highest concentration of VBLS, C3H cells were most sensitive with a decrease of visible migration by almost 80%, followed by 3T3 with a 60% decrease, HESF with 50% decrease. The results strongly indicate that the migration rate varies for each of the cell lines and concentration of the anti-tubulin agent, respectively.

FIGURE 2: Migration rate of cells in the presence of colchicine and CM

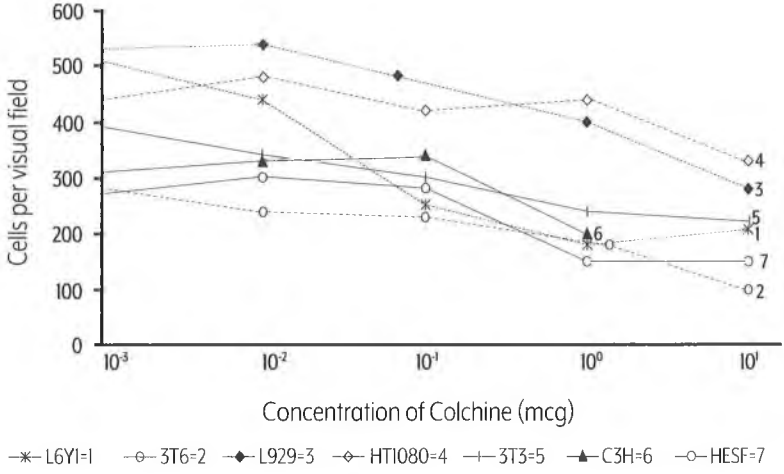


FIGURE 2: Migration rate of cell lines against chemoattractant together with increasing concentrations of COL. Increasing concentrations of COL from 10^{3} to 10^{0} microgram / ml were added to the chemoattractant, and poured into the lower part of the Boyden chamber. The upper part of the Boyden chamber was filled with the respective cells moving towards chemoattractant and anti-tubulin. The highest decrease of migration rate was observed with 3T6, followed by 3T3, C3H, HESF, L6Y1, L929 and HT1080.

FIGURE 3: Migration rate of cells lines against chemoattractant together with increasing concentartions of VBLS

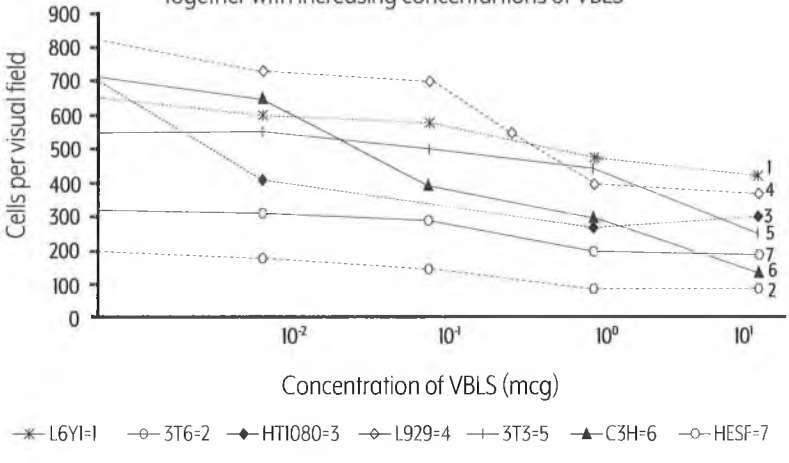


FIGURE 3: Migration rate of cell lines against chemoattractant together with increasing concentrations of VBLS. Increasing concentrations of VBLS from 10^{-3} to 10^{0} microgram / ml were added to the chemoattractant, and poured into the lower part of the Boyden chamber. The upper part of the Boyden chamber was filled with the respective cells moving towards chemoattractant and anti-tubulin. The highest decrease of migration rate was observed with C3H, followed by HT1080, L929, 3T3, 3T6, HESF, and L6Y1.

From the FIGURE 3, it is evident that increasing concentrations from 10^{-3} to 10^{0} microgram/ml of VBLS were added to the chemoattractant, and poured into the lower part of the Boyden chamber. The upper part of the Boyden chamber was filled with the respective cells moving towards chemoattractant and anti-tubulin agents. The highest decrease of mobility was observed with C3H, followed by HT1080, L929, 3T3, 3T6, HESF, and L6Y1.

Anti-tubulin agents - different incubation time - conditioned medium

It is known that the motility of the cell lines changes when cells were treated with COL and VBLS at different concentrations. In the present study, the motility of the cell lines treated with COL and VBLS at different concentrations for different times were evaluated. In this context, initially the DMEM containing 10% (v/v) FCS was poured into the culture flasks and later replaced with 10 ml of DMEM containing anti-tubulin agents in different concentrations. Two variables i.e. the concentration of COL/ VBLS, and the time of incubation of the anti-tubulin agents with cell lines tested were systematically changed. Considering the results of the migration behaviour experiments, wherein a decrease of locomotion with the increase of concentration were shown in the same direction for all cell lines. Accordingly, only two concentrations of each anti-tubulin agent were used for further experiments, i.e. 0.05 mcg COL/ml DMEM (C1),5 mcg COL/ml DMEM (C2), 0.1 mcg VBLS/ml DMEM (V1) and 10 mcg VBLS/ml DMEM (V2). The cell lines were undergone pre-incubation at various time intervals i.e., 15, 30, 60, 120,

180, and 240 minutes (Postlethwaite *et al.*, 1978). The incubation with anti-tubulin agents was followed by the chemotaxis experiment against the conditioned medium.

For each cell line, control experiments were also performed. The cell lines were pre-incubated according to the experimental time intervals with only DMEM (without anti-tubulin agents /additives) as a control. The results of the viability of the cells in the presence of DMEM are shown in Figure. 4. All cell lines had a reduction in migration frequency of up to 10% at 15 min, whereas three cell lines were recovered after 30 min and 60 min. At longer contact time, the rate remained constant (3T6, L-929) or dropped slightly (HT 1080, L6Y1, 3T3, C3H, HESF), similar to a plateau. This control experiment provided information about the effect of contact between the cell lines in the presence of incubation medium DMEM, without the addition of anti-tubulins.

FIGURE 4: Incubation of different cell lines in the presence of incubation medium DMEM, without the addition of antitubulins

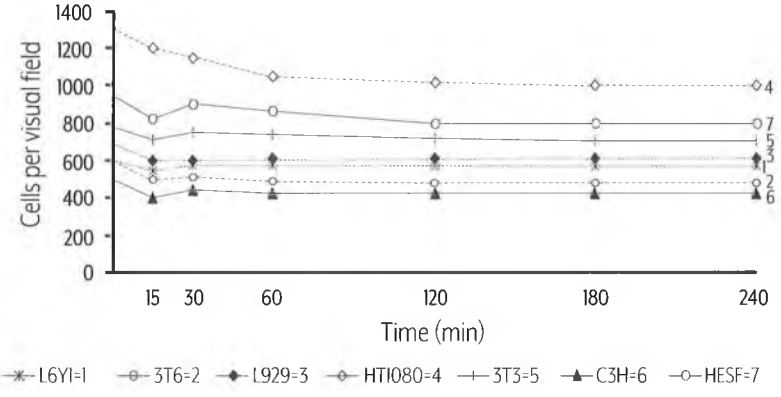


FIGURE 4 *Incubation of different cell lines in the presence of incubation medium DMEM, without the addition of anti-tubulins. At 15 min, there is a reduction of migration frequency by 10%, with recovery at 30 min to 60 min. Migration rate remained constant for 3T6, L929, and slightly dropped at 240 min of incubation for 3T3, C3H, HESF, HT1080, and L6Y1.*

From FIGURE 4, it is observed that the migration rate remains constant for 3T6, L929, and slightly dropped at 240 min of incubation for 3T3, C3H, HESF, HT1080, and L6Y1. The following data obtained from the migration rate of each cell line in the direction of the chemo attractant under the influence of different concentrations of each of the anti-tubulin agents and different times of preincubation of cells with an anti-tubulin agent are illustrated below.

c.1. 3T3: Mouse embryonic fibroblast cells

The anti-tubulin agents with various concentration i.e., 0.05 mcg COL/ml DMEM (C1),5 mcg COL/ml DMEM (C2), 0.1 mcg VBLS/ml DMEM (V1) and 10 mcg VBLS/ml DMEM (V2) and the control, were incubated separately, with 3T3 cells for different time intervals.

After 15 min of pre-incubation, the moiety (control) decreased by about 15%, to reach the original mobility at 60 min. Further, the pre-incubations with the two lower concentrations C1 and V1 led to an increase in mobility after 120 min and remained at this value till 240 min. The two higher concentrations C2 and V2 also led to a significant reduction in mobility after an initial decrease of 15 min of preincubation and a short recovery phase after preincubation from 120 min to 240 min was observed. Recovery did not occur in the meantime. The mobility of 3T3 cells at different concentrations of anti-tubulin agents is shown in Figure. 5.

FIGURE 5: Incubation of 3T3 cells with different concentrations of antitubulin

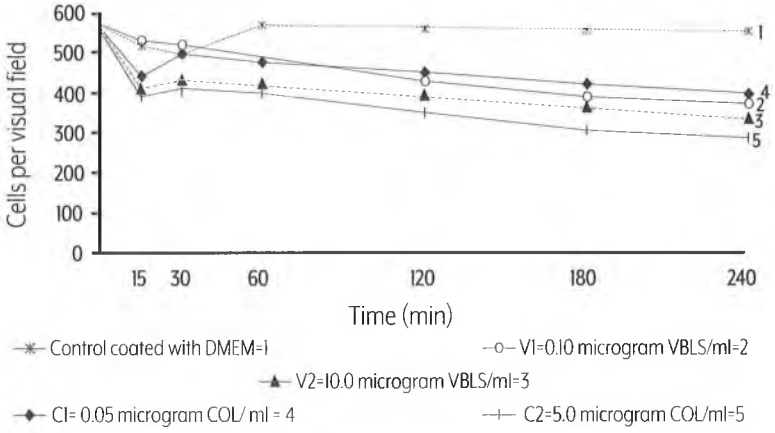


***FIGURE 5: 3T3 cells** were incubated in the presence of different concentrations of antitubulin, i.e. 1(controls without antitubulin); 2 (C1= 0.05 microgram COL/ ml), 3 (C2=5.0microgram COL/ml), 4(V1=0.10 microgram VBLS/ml) and 5 (V2=10.0microgram/ mL).*

It is evident from FIGURE 5 that the control recover quickly in mobility after a dent/decrease at 15 min, whereas higher concentrations of COL and VBLS decrease the migration under the influence of chemoattractants.

c.2. L6Y1

The anti-tubulin agents with various concentration i.e., 0.05 mcg COL/ml DMEM (C1),5 mcg COL/ml DMEM (C2), 0.1 mcg VBLS/ml DMEM (V1) and 10 mcg VBLS/ml DMEM (V2) and the control, were incubated separately, with L6Y1 cells for different time intervals.

Similar to the control experiment, there was a reduction in migration at 15 minutes at all concentrations, but much more pronounced decrease (50%) was observed in the presence of COL and VBLS, respectively. There was also less recovery, almost to baseline in the control group (60 minutes). After 30-minute of pre-incubation, a significant increase in migration was observed only at the low COL concentration (C1) whereas the other concentrations did not show an increase until af-

ter 60 min, like the control. In this experiment, the recovery phase of the control group remained constant on a plateau. After short drop-in locomotion between 0 min and 30 min, as also observed in the other cells, the locomotion rate remained in the range of the original locomotion rate up to 240 min of incubation time. The mobility of L6Y1 cells at different concentrations of anti-tubulin agents is shown in Figure. 6.

FIGURE 6: Incubation of L6Y1 cells with different concentrations of antitubulin

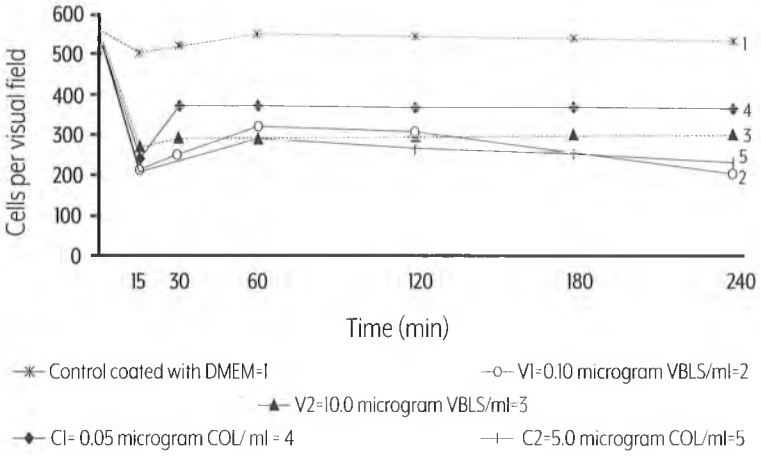


***FIGURE 6: L6Y1 cells** were incubated in the presence of different concentrations of antitubulin, i.e.1(controls without antitubulin);2 (C1= 0.05 microgram COL/ ml), 3 (C2=5.0microgram COL/ml); 4(V1=0.10 microgram VBLS/ml); and 5 (V2=10.0 microgram/ mL).*

It is observed from FIGURE 6 that the control recovered quickly in mobility after a dent/decrease at 15 min whereas the higher concentrations of COL and VBLS decrease the migration under the influence of chemoattractants.

c.3. L929

The anti-tubulin agents with various concentrations i.e., 0.05 mcg COL/ml DMEM (C1),5 mcg COL/ml DMEM (C2), 0.1 mcg VBLS/ml DMEM (V1), and 10 mcg VBLS/ml DMEM (V2) and the control, were incubated separately, with L929 cells for different time intervals.

In the control experiment, a slight recovery phase was reached after 60 minutes, followed by a constant level, as seen with L6Y1. At the same time (as shown in Figure. 7) at the beginning of the experiment, the cells in contact with COL / VBLS showed minimum locomotion. This was significantly less at the higher concentrations (C2/ V2) than at the lower concentrations (C1/V1), respectively. After four hours of incubation, the degree of locomotion slowly increased, with the curves of the two concentrations of COL on the one hand and those of the VBLS, on the other hand, converging, respectively. The mobility of L929 cells at different concentrations of anti-tubulin agents is shown in Figure. 7.

FIGURE 7: Incubation of L-929 cells with different concentrations of antitubulin

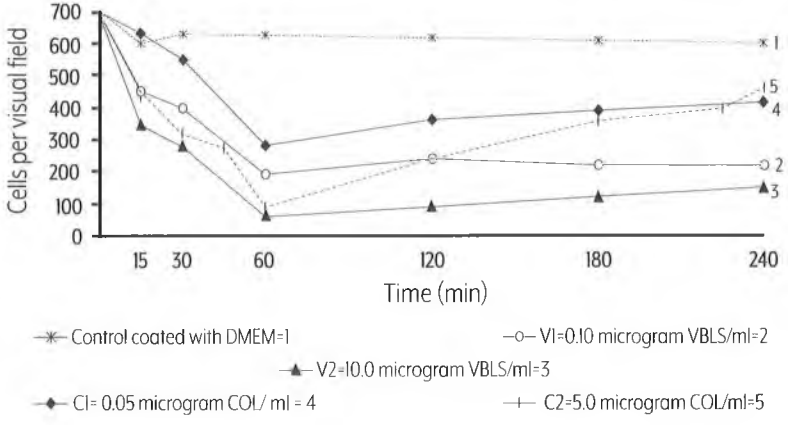
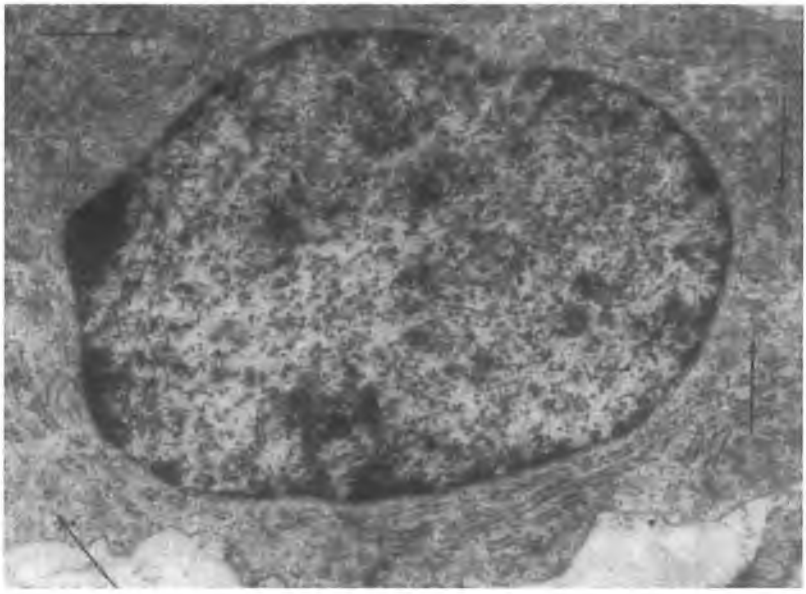


***FIGURE 7: L-929** cells were incubated in the presence of different concentrations of antitubulin, i.e.1(controls without antitubulin); 2 (C1= 0.05 microgram COL/ ml);3 (C2=5.0 microgram COL/ml); 4(V1=0.10 microgram VBLS/ml); and 5 (V2=10.0 microgram/ mL).*

It is observed from FIGURE 7 that the control recover quickly in mobility after a dent/decrease at 15 min whereas the higher concentrations of COL and VBLS decrease the migration under the influence of chemo attractants. However, C1, C2, and V2 recovered continuously from 60 min till 240 min.



***FUGURE 7.1.: L929** in the presence of Colchicine 10^{-5} M, 60 min contact time. The accumulation of Golgi apparatus and rough endoplasmic reticulum is found between the microtubules (arrows) close to the nuclear membrane. All other structures of the cytoplasm appear normal; x 14.000 (curtesy of Dr V.B. Bivali).*

c.4. C3H-mouse fibroblast cells

The anti-tubulin agents with various concentration i.e., 0.05 mcg COL/ml DMEM (C1),5 mcg COL/ml DMEM (C2), 0.1 mcg VBLS/ml DMEM (V1) and 10 mcg VBLS/ml DMEM (V2) and the control, were incubated separately, with C3H cells for different time intervals.

Similar to other cell lines, there was 10% decrease in mobility in the control at 15 min, which then returned to the original mobility at 60 min keeping that plateau till 240 min. The preincubation with low concentrations of anti-tubulin agents decreased to 35% at 15 min, and then briefly recovered to below 20% mobility in comparison to the control. At 240 min of preincubation, 40% mobility was reduced to 40% of the control. The two higher concentrations of anti-tubulins C2 and V2 also

resulted in a significant decrease in motility at 15 min. Further pre-incubations up to 240 min resulted in 60% decrease in motility. The mobility of C3H cells at different concentrations of anti-tubulin agents is shown in Figure. 8.

FIGURE 8: Incubation of C3H cells with different concentrations of antitubulin

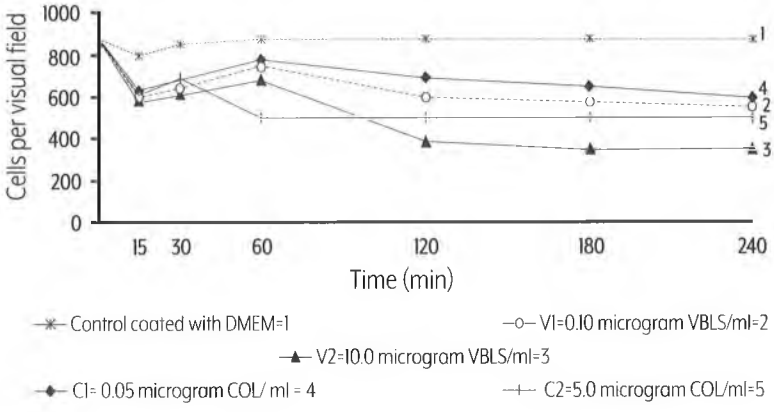


FIGURE 8: *C3H cells were incubated in the presence of different concentrations of antitubulin, i.e. 1 (controls without antitubulin); 2 (C1= 0.05 microgram COL/ ml); 3 (C2=5.0microgram COL/ml); 4(V1=0.10 microgram VBLS/ml); and 5 (V2=10.0 microgram/ mL).*

From FIGURE 8, it is observed that the control recover quickly in mobility after a dent/decrease at 15 min whereas the higher concentrations of COL and VBLS at a longer time of preincubation decrease the migration under the influence of chemoattractants.

c.5. HT 1080

The anti-tubulin agents with various concentration i.e., 0.05 mcg COL/ml DMEM (C1),5 mcg COL/ml DMEM (C2), 0.1 mcg VBLS/ml DMEM (V1) and 10 mcg VBLS/ml DMEM (V2) and the control, were incubated separately, with HT 1080 cells for different time intervals.

In contrast to the control, the HT 1080 cells also showed a recovery phase at 30 min of incubation (as shown in Figure. 9).

Only the high concentration of VBLS (V2) caused a significant decrease of migration at this time interval and remained at this low level for a longer period of incubation. With the other three concentrations, a significantly lower value was reached after 60 min of incubation, in comparison to 30 minutes. This tendency continued under COL influence even after 240 min of incubation, with the curves of C1 and C2 approaching each other. The attenuation of the migration rate was less under VBLS with longer incubation but was plateau-like only at V2. The mobility of HT1080 cells at different concentrations of anti-tubulin agents is shown in Figure. 9.

FIGURE 9: Incubation of HT1080 cells with different concentrations of antitubulin

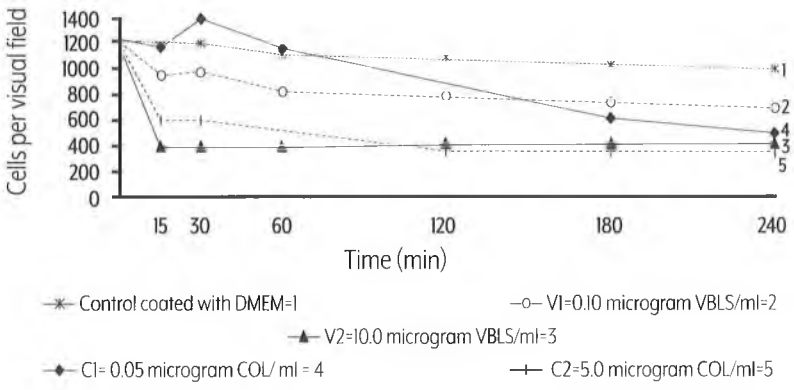


***FIGURE 9: HT1080** cells were incubated in the presence of different concentrations of antitubulin, i.e. 1(controls without antitubulin); 2 (C1= 0.05 microgram COL/ ml); 3 (C2=5.0microgram COL/ml); 4(V1=0.10 microgram VBLS/ml); and 5 (V2=10.0 microgram/ mL).*

From FIGURE 9, it is observed that the control decreased in mobility at 15 min and further 60 min of preincubation, and stabilised from 120 min to 240 min of preincubation. C1 caused an increase of mobility at 60 min of preincubation, then continuously decreased till 240 min of preincubation. C2 caused an initial decrease of motility at 30 min, similar to V2, whereas V1 reflected a loss of mobility at 60 min, however, which was not high as C1, C2, and V2 at 240 min.

c.6. HESF

The anti-tubulin agents with various concentrations i.e., 0.05 mcg COL/ml DMEM (C1),5 mcg COL/ml DMEM (C2), 0.1 mcg VBLS/ml DMEM (V1), and 10 mcg VBLS/ml DMEM (V2) and the control, were incubated separately, with HESF cells for different time intervals. At 15 min, the motility of the control decreased by about 10% and then returned to baseline at 60 min. This motility remained on the plateau until 240 min preincubation. Under C1, the motility decreased at 15 min of preincubation, as observed in the control. Thereafter, it increased to a level approximately 10% below the control at 60 min. The higher concentrations of COL and VBLS significantly reduced the motility at the beginning of incubation, from which they did not recover later. At 240 min, the cell line remained significantly below the mobility of the control, and the lower concentrations of the corresponding anti-tubulin. The mobility of HESF cells at different concentrations of anti-tubulin agents is shown in Figure. 10.

FIGURE 10: Incubation of HESF cells with different concentrations of antitubulin

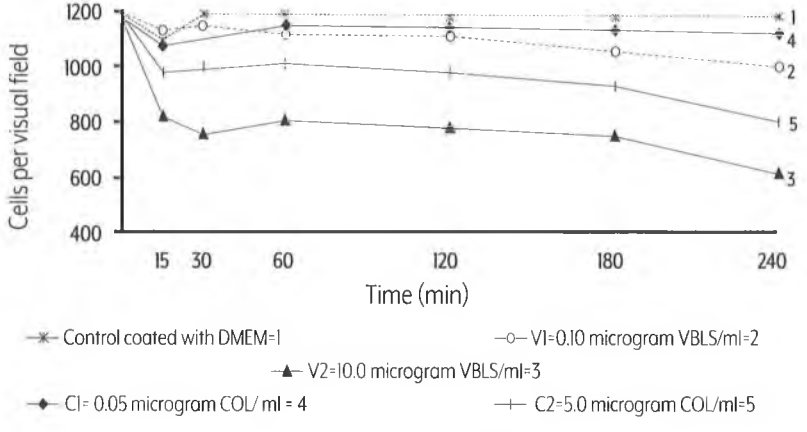


***FIGURE 10: HESF cells** were incubated in the presence of different concentrations of antitubulin, i.e.1(controls without antitubulin); 2 (C1= 0.05 microgram COL/ ml); 3 (C2=5.0microgram COL/ml); 4(V1=0.10 microgram VBLS/ml); and 5 (V2=10.0 microgram/ mL).*

From FIGURE 10, it is observed that the control recover quickly in mobility after a dent/decrease at 15 min whereas

the higher concentrations of COL and VBLS decrease the migration under the influence of chemoattractants.

c.7. 3T6

The anti-tubulin agents with various concentrations i.e., 0.05 mcg COL/ml DMEM (C1),5 mcg COL/ml DMEM (C2), 0.1 mcg VBLS/ml DMEM (V1), and 10 mcg VBLS/ml DMEM (V2) and the control, were incubated separately, with 3T6 cells for different time intervals. Again, a strong reduction in migration activity for the control was observed after 15 minutes, as observed with the other cell lines. A slight recovery was observed after 30 minutes, in line with the result from the control. It was striking, that both V1 and V2 produced similar results after 60 min of preincubation, comparable to C1, whereas C2 produced 60% reduction of migration within 30 min, and remain constant until 240 min. An increasing inhibition of migration towards chemoattractant occurred with the cells pre-incubated with anti-tubulin agents for more than 120 min. The mobility of 3T6 cells at different concentrations of anti-tubulin agents is shown in Figure. 11.

FIGURE 11: Incubation of 3T6 cells with different concentrations of antitubulin

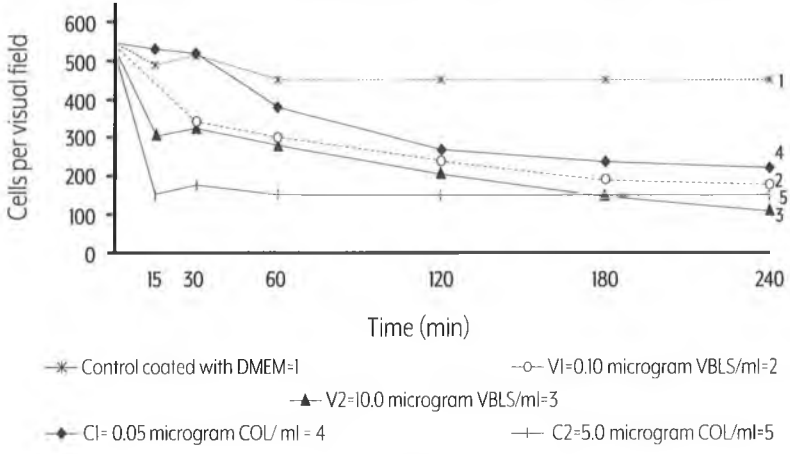


***FIGURE 11: 3T6 cells** were incubated in the presence of different concentrations of antitubulin, i.e. 1(controls without antitubulin); 2 (C1= 0.05microgram COL/ ml); 3 (C2=5.0microgram COL/ml); 4(V1=0.10 microgram VBLS/ml); and 5 (V2=10.0 microgram/ mL).*

From FIGURE 11, it is observed that the control recover quickly in mobility after a dent/decrease at 15 min whereas the higher concentrations of COL and VBLS decrease the migration under the influence of chemoattractants.

Random migration

It is known that anti-tubulin agents affect the random migration of cell lines. Random migration and attachment of the treated cells were also examined in this series of experiments. The attachment was undisturbed in all experiments, i.e. the filter side facing the upper chamber was densely occupied by the non-migrated cells after fixation.
Random migration almost reached the level determined with untreated cells for all cell lines and under all concentrations after four hours of incubation with anti-tubulin agents.

At 15 minutes of incubation, there was an increase in activity in five cell lines (3T6, HT1080, 3T3, C3H, HESF), but the extent of this increase differed. For example, C1 caused an increase of almost 100% in 3T6, whereas V2 caused only a slight increase. 3T3, C3H, and HESF reacted with an increase of only 15% to 25 % at 15 min of preincubation. However, at the longest time of preincubation, V2 caused a significant decrease in 3T3, C3H, and HESF, respectively.

FIGURE 12: 3T6 - Random Migration

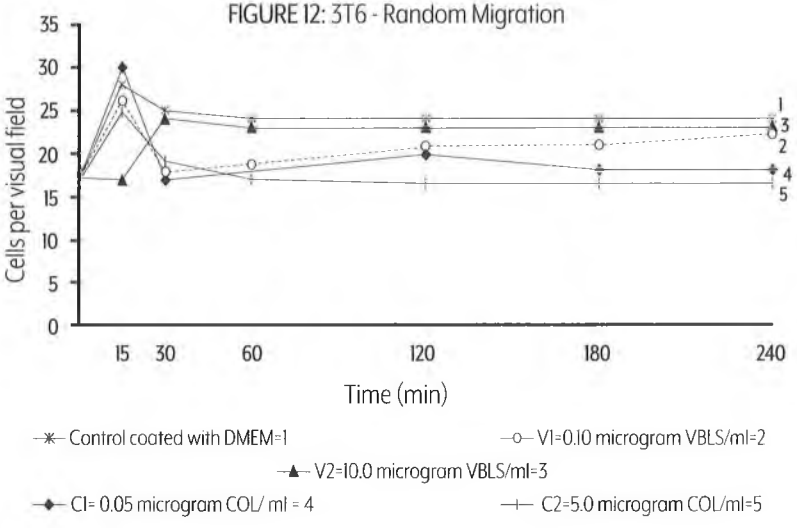


FIGURE 12: 3T6 random migration; cells preincubated under increasing time intervals, in presence of increasing concentrations of antitubulin: followed by migration against pure DMEM without additives 1 (controls incubation with DMEM); 2(C1=0.05 microgram COL/ ml); 3 (C2=5.0microgram COL/ mL); 4 (V1=0.1 microgram VBLS/ml); and 5 (V2=10.0 microgram VBLS/mL).

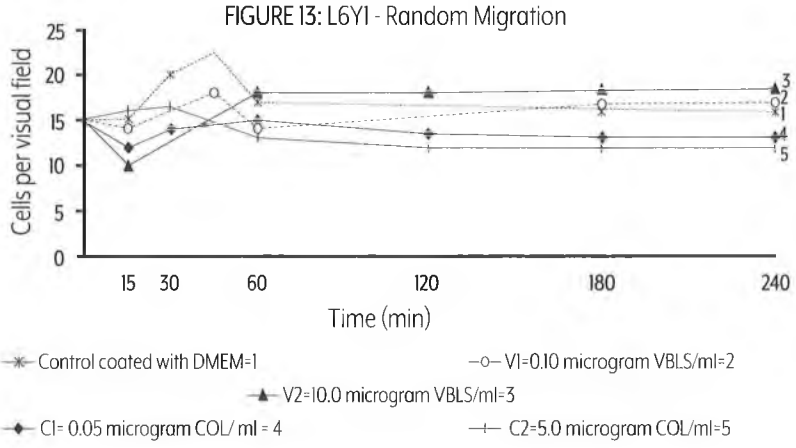


***FIGURE 13*: L6Y1** *random migration; cells preincubated under increasing time intervals, in presence of increasing concentrations of antitubulin, followed by migration against pure DMEM without additives 1 (controls incubation with DMEM); 2(C1=0.05 microgram COL/ ml); 3 (C2=5.0microgram COL/ mL); 4 (V1=0.1 microgram VBLS/ml); and 5 (V2=10.0 microgram VBLS/mL).*

L6Y1 responded for both anti-tubulin agents with a slight reduction in random migration after 15 minutes of incubation and a maximum at 30 minutes followed by a decrease to a plateau up to 240 min of incubation.

FIGURE 14: L929 - Random Migration

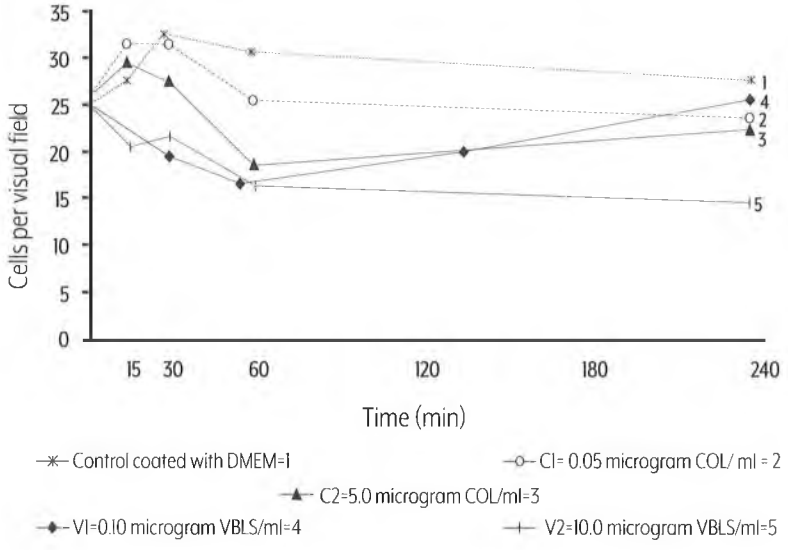


FIGURE 14: **L929** *-random migration; cells preincubated under increasing time intervals, in presence of increasing concentrations of antitubulin, followed by migration against pure DMEM without additives 1 (controls incubation with DMEM); 2(C10.05 microgram COL/ ml); 3 (C2=5.0 microgram COL/ mL); 4 (V1=0.1 microgram VBLS/ml); and 5 (V2=10.0 microgram VBLS/mL).*

L929 exhibited a decrease of random migration at a concentration of V2, which extended to a plateau up to 240 min of incubation/observation, whereas V1 dropped continuously until 60 min of incubation, after that gradually increasing until 240 min. C1 increased in random migration up to 30 min, then decreased until 60 min and extended into a plateau until 240 min. The random migration pattern of C2 was similar to V1, i.e. continuously increasing from 60 min to 240 min of incubation/observation.

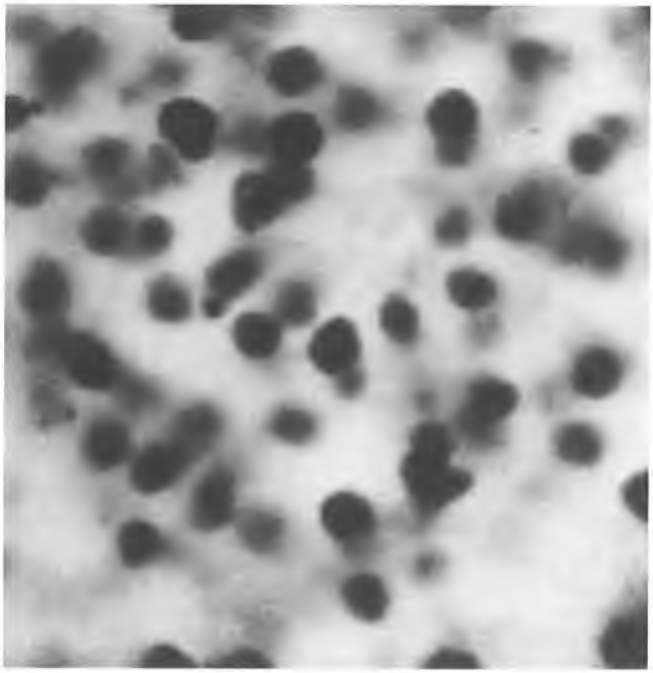


FIGURE: 14.1.
L929 cells that have migrated 25 mcm into the membrane of the Boyden chamber, plenty pyknosis, hematoxylin, x 400.

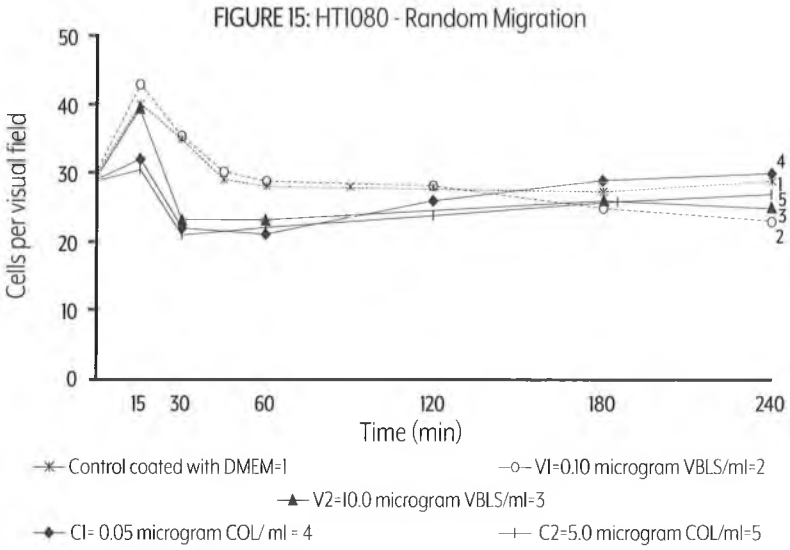


FIGURE 15: **HT1080** *-random migration; cells preincubated under increasing time intervals, in presence of increasing concentrations of anti-tubulin, followed by migration against pure DMEM without additives 1 (controls incubation with DMEM); 2(C1=0.05 microgram COL/ ml); 3 (C2=5.0microgram COL/ mL); 4 (V1=0.1 microgram VBLS/ml); and 5 (V2=10.0 microgram VBLS/mL).*

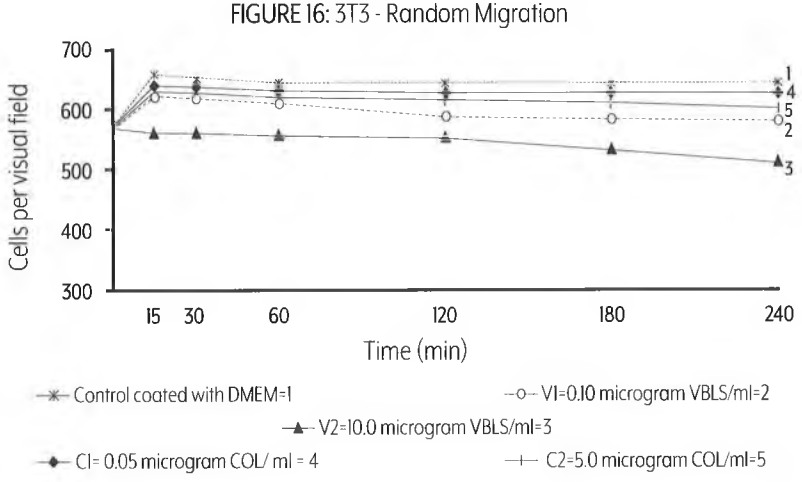


FIGURE 16: **3T3** *-random migration; cells preincubated under increasing time intervals, in presence of increasing concentrations of antitubulin, followed by migration against pure DMEM without additives 1 (controls incubation with DMEM); 2(C1=0.05 microgram COL/ ml); 3 (C2=5.0microgram COL/ mL); 4 (V1=0.1 microgram VBLS/ml); and 5 (V2=10.0 microgram VBLS/mL).*

FIGURE 17: C3H - Random Migration

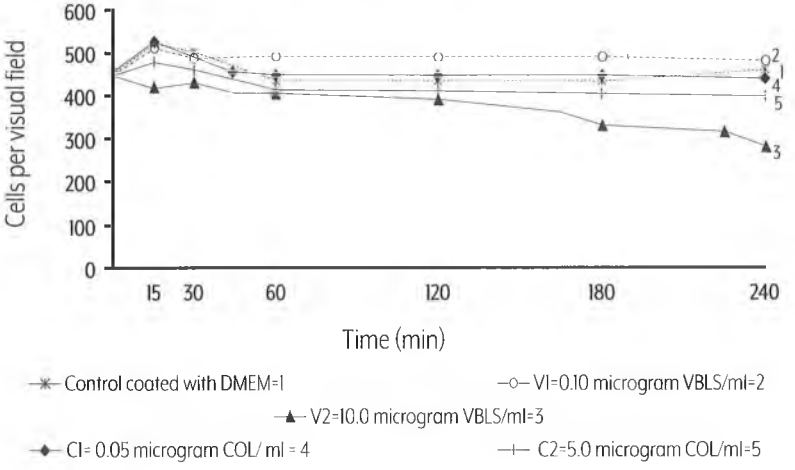


FIGURE 17: **C3H** *-random migration; cells preincubated under increasing time intervals, in presence of increasing concentrations of antitubulin, followed by migration against pure DMEM without additives 1 (controls incubation with DMEM); 2(C1=0.05 microgram COL/ ml); 3 (C2=5.0 microgram COL/ mL); 4 (V1=0.1 microgram VBLS/ml); and 5 (V2=10.0 microgram VBLS/mL).*

FIGURE 18: HESF - Random Migration

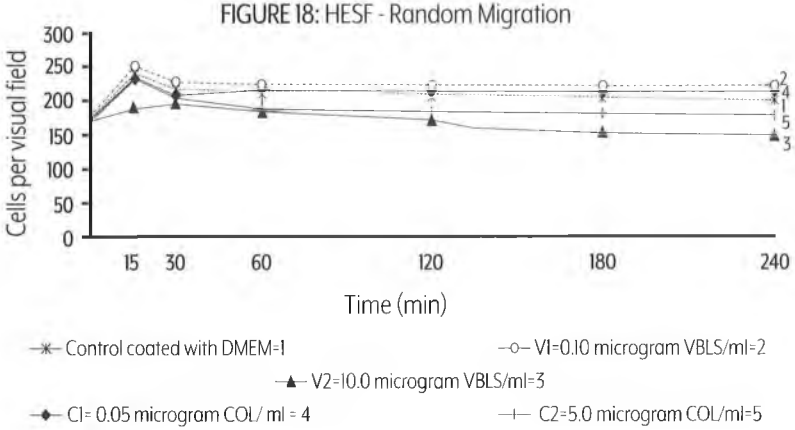


FIGURE 18: HESF *random migration; cells preincubated under increasing time intervals, in presence of increasing concentrations of antitubulin, followed by migration against pure DMEM without additives 1 (controls incubation with DMEM); 2(C1=0.05 microgram COL/ ml); 3 (C2=5.0microgram COL/ mL); 4 (V1=0.1 microgram VBLS/ml); and 5 (V2=10.0 microgram VBLS/mL).*

It should be emphasized again that among the 7 cell lines studied, overall random migration was affected only in a minor way by COL / VBLS treatment at short intervals of preincubation. The longer the preincubation, the more the metabolic effects of the anti-tubulins were expressed by reduction of the random migration, without abolishing the random migration completely.

These results are striking different from CM activated migration.

4. Discussion

In this article, the study of the cell migration in the direction of a chemotactic gradient have been attempted, which is similar to blood leukocytes. Based on extensive research on the chemotactic behaviour of leukocytes (Zakhireh *et al.*, 1980) and the influence of COL and VBLS on the individual steps of the mitotic division of cells ranging from plant cells (Macht and Livingston 1922; Eigsti *et al.*, 1955), green algae (Shyam *et al.*, 1974), tumour cells and fibroblasts (Oughterson *et al.*, 1937; Varani *et al.*, 1978), certain assumptions can be made about the movement behaviour of tumour cells in a solution containing chemoattractant. The results show that the fibroblasts also change their outer shape, in particular the boundary of the cellular outer membrane becomes rounded, and epithelioid-like forms under the influence of COL and VBLS. Lam (1981) hypothesized that the rounded tumour cells which had been described by Abercrombie (1958, 1961), Brues (1936), can migrate from the bloodstream into the tissue to the origin of the chemotactic stimulus and proliferate into a solid tumour, through the endothelial junction, abandoning the normal contact initiation of the healthy cells.

It has been known since 1889 (Eigsti *et al.*, 1949) that COL can arrest eukaryotic cells in the metaphase of mitotic division by binding to intracellular tubulin proteins and thereby disturbing the directed cytoplasmic movement of such cells (Eigsti *et al.*, 1955). Microtubule proteins have been associated with the structured, target-directed movement of fibroblasts (Margulis *et al.*, 1973; Tilney *et al.*, 1971), whereas the filaments and microfilaments presumably are associated with random migration of cells (Abercrombie *et al.*, 1958; Freed *et al.*, 1968; Goldman *et al.*, 1971), as they are not inhibited by anti-tubulin agents, but inhibited by cytochalasin B (Zigmond *et al.*, 1972) in particular by binding to microfilaments of leukocytes (Skosey *et al.*, 1973). There are various types of microtubules, considered from the primary protein structure, of which only one embodiment supports directional cell movement. This tubulin structure has receptors for independently binding COL and VBLS, which can interact with the function of microtubules. The biogenesis, biochemistry, the physiological function of microtubules of fibroblasts, and their interaction with COL and VBLS (Lam *et al.*, 1981; Margulis *et al.*, 1973; Wilson *et al.*, 1967) are known.

Immobile, multi-micro nucleated cells are formed above a critical concentration of COL (Eigsti *et al.*, 1949). The same applies to VBLS (Bruchovsky *et al.*, 1965; Lam *et al.*, 1981; Wilson *et al.* 1975), i.e. above 10^{-6} M, where cytotoxic effects have been observed (Armstrong *et al.*, 1979). Meier (1947) has shown that the mitotic division of fibrosarcoma cells is inhibited by 1 nanogram COL/ml, which is comparable to the concentrations of the present study. Marceel (1982) has shown that the mitotic division of fibrosarcoma cells has been completely inhibited between 1 nanogram VBLS / ml and 6 nanogram VBLS/ml, comparable to the results of Johnson (1960) and Palmer (1963).

Palmer (1963) has shown that minute concentrations of VBLS (1 nanogram/ml) can increase the amount of EHE cells in the metaphase arrest of up to 452%. Higher VBLS concentrations (10 nanogram/ ml) achieved 200%, metaphase arrest of EHE cells, and with 100 nanogram/ ml of VBLS a metaphase arrest of 129%. The higher the VBLS concentra-

tion the more pronounced was the cytotoxic effect on EHE cells. Varani (1978) has shown by using the Boyden chamber that directed migration of activated fibrosarcoma and mouse embryo fibroblasts, has been inhibited at concentrations of COL by 10 mcg / mL and VBLS by 1 to 10 mcg / mL. From the Boyden chamber migration experiment, the cells migrated on the filters having 8 mcm diameter were evaluated by microscopic study wherein it is concluded that there must be a different migration pattern between mouse embryo fibroblasts and fibrosarcoma cells, because the mouse embryo fibroblasts migrated 10 times more than fibrosarcoma cells. Higher concentrations of COL, i.e. 10^{+2} mcg/mL, and VBLS , i.e. 10^{-1} mcg/ mL caused toxic effects. The toxic concentrations of both, VBLS and COL can be regarded as high, compared to the concentrations used by Johnson (1960). Under higher concentrations of VBLS (above 1 microgram/ mL), the accumulation of metaphase cells ranging from 60% to 80% was observed (Johnson *et al* 1960). Furthermore, EHE cells have been arrested in metaphase much longer by VBLS, than comparable concentrations of COL. Similar results have also been reported by Cutts (1961), in particular, metaphase arrests of the "ball type", which Abercrombie & Ambrose (1958) described as "rounded cells".

Similar results have been published by Nebel (1938) on the influence of COL on mitotic division of higher plants. Wilson (1975) has shown that the dissociation and binding between COL and tubulin depend on a first order decay. All these known results show, that the influence of COL on tubulin of fibroblasts is reversible. Longer contact times between anti-tubulin agents and tubulin receptors might keep cells in prolonged metaphase arrest with the incapacitation of forming mRNA (Dustin *et al.*, 1963), and in addition, the functional proteins like sodium-potassium ATPase, which are necessary for the stability of the outer cell membrane. On the other hand, the cationic VBLS can bind to the anionic rRNA of ribosomes and obstruct protein synthesis further to the direct binding to cytoplasmic tubulin receptors with the formation of para crystals (Na *et al.*1982), which we observed for L929 in FIGURE 19.

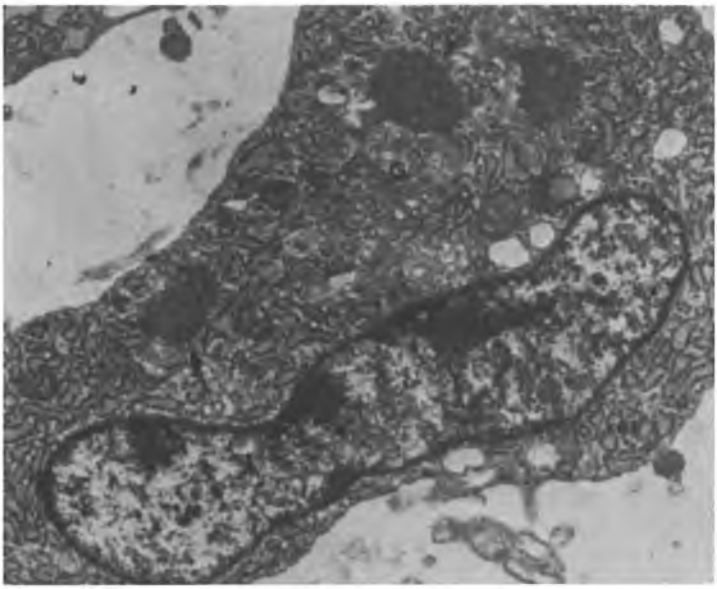


FIGURE 19: *The electron micrograph represents L929 after treatment with VBLS 10^{-5}M. Several dark coloured membranous aggregates, similar to para-crystals, can be seen indicated by arrows. In addition the cisternae of the rough endoplasmic reticulum appear to be vacuolar and to have lost their usual three-dimensional canalicular appearance (rer); the GOLGI complex (G) in the middle of the micrograph appears unaltered, x 14.000 (curtesy of Dr V.B. Bivali).*

This might result in rounded cells due to increased intracellular osmotic pressure, caused by the release of amino acids from destabilizing tubulin molecules, and in addition to the loss of a large number of cells. Abercrombie & Ambrose (1958, 1967) have described the same external characteristics of mouse sarcoma cells and mouse fibroblasts with rounded external cells with very active and mobile cell boundaries, which did not give up their mobility even in the presence of other fibroblasts and sarcoma cells.

Certainly, EHE cells cannot be compared for their antigenicity to the mouse fibroblasts or HESF cells of the present study. The outer membrane boundaries and functionalities are different

for each type of cell line. Since the blueprint of eukaryotic cells is similar, the effects of anti-tubulin agents on motility for each type of cell line should be comparable in the present study.

On this basis, it was of interest to test whether the structured formation of microtubule proteins of fibroblasts can be inhibited by "anti-tubulin agents" selectively, in such a way that the metabolism of fibroblasts will not be incapacitated cytotoxically. However, the microtubule proteins will become non-functional and could no longer support target-directed movement under the influence of a chemoattractant, which was added to the test system of the Boyden chamber.
The results of chemotaxis of the fibroblasts treated with COL and VBLS show that the fibroblasts were exposed to each of the anti-tubulin agents independently, or together with a known chemoattractant, responded with a decrease in their directed migratory activity.

However, this reduction did not occur to the same extent to all the cell lines. The cell lines responded to the anti-tubulin agents (COL and VBLS) were tested, at different concentrations, and the exposure duration. The effect of the mechanical manipulation of the pre-treatment was expressed in all experiments wherein after 15-minute exposure to anti-tubulin agents, i.e., the relatively rapid sequence of exposure and trypsinization as damaging factors, the cells showed a partly reversible reduction in migration rate, which was much more pronounced in the cells exposed to anti-tubulin agents than those treated with pure DMEM. Von Moellendorff (1937) had already pointed out that initially after addition of diluted colchicine solution (dilution up to 1:20 million) to cultures of fibrocytes in vitro there is an inhibition of the growth rhythm and thus also of the migration activity. He attributed this to nonspecific physical influence, although the initial colchicine effect could not be excluded. After initial decrease of mobility, we can assume from the further course under the influence of anti tubulins and CM that the initial inhibition of fibrocyte movement is a characteristic consequence of colchicine action. However, this primary damage, especially in metaphase, is not specific for the effect of COL, as Von Moellendorf (1937) has shown. As

demonstrated by von Moellendorf (1937), the inhibitory effect of anti-tubulins on motility of cells, having been preincuated up to 120 min, is clearly pronounced, which has been due to the inhibition of mitotic division in metaphase. From 180 min preincubation time onwards, damage to mitotic division and thus also to motility occurs more frequently, the so-called colchicine effect. The cells then show very strong amoeboid movements, imperfect lacing attempts and formation of cytoplasmic processes; very severely damaged cells behave inactively. Von Moellendorf was able to show that the strong accumulation of figures of cell division in the metaphase of mitosis, as observed by our experiments, was not due to an acceleration of the cell division rhythm, but due to the "mitotic arrest" in metaphase. In very severely damaged mitoses, chromosomes (including chromosome fragments, as well as pyknotic chromosome groups) may be scattered throughout the cell.

FIGURE 20 FIGURE 21 FIGURE 22

***FIGURE** 20: L929 pyknosis of the two chromosome packages in anaphase. Colchicine 1 mcg/mL 240 min preincubation; staining: carnoy-iron hematoxylin.*

***FIGURE** 21: L929 two chromosome packages show the density of telophase, but cell body constriction has failed. Colchicine 1 mcg/ mL; 240 min preincubation, stain/ fix.: carony, cardozo-pappenheim.*

***FIGURE** 22: L929 scattering of chromosomes throughout the cell. Colchicine 5 mcg/ mL; 240 min preincubation. stain/ fix: Krallinoer; Feulgen nuclear reaction.*

The experiments were all performed at a time period when the cells were in a stage of higher mitotic activity: At high rates of proliferation and migration, the cells were certainly more vulnerable than at resting times, so the results of this stage were more pronounced but qualitatively same.
In all cell lines tested, it was seen that COL and VBLS did not have a chemo-attractive effect in the presence of CM, but - in contrast - attenuated the migration rate with increasing concentrations of COL and VBLS. This is understandable under the results of Palmer (1961) and Cutts (1961), who had observed a decrease in metaphase arrests of EHE cells with increasing VBLS concentrations in EHE cells, which may be associated with a cytotoxic and karyotoxic effect of VBLS.
The attachment of cells to the filter of the Boyden chamber was still very pronounced in CM, even at the maximum concentration of the anti-tubulin agents, cell attachment to the top of the filter occurred at the usual rate, but the stimulus or ability of the cells to squeeze through the narrow pores was diminished. Thus, if in the main experiments, the cells exposed to anti-tubulin agents exhibited lower migratory activity, this had to be due to the direct effect of the anti-tubulin agents on the cells, rather than to a relatively reduced chemoattractivity of the CM, as disclosed by Inoue (1952), Johnson (1960) and Wilson (1975).

The two anti-tubulin agents, which - as already discussed - have different binding sites, had different effects on the individual cell types, and are not interchangeable in terms of their results. This could be attributed to their different chemical structure, solubility in DMEM, crystal forming, and precipitating action on microtubules, as has been extensively discussed in prior studies (Bensch *et al.*, 1969; Eigsti *et al.*,1955). As pointed out by James D Loudon and Manske (1952) it cannot be excluded that, COL might form pharmacologically non-active crystals in water based buffer solutions, such as DMEM, and thus escaping from the site of action or dissociation from binding sites within the cells were tested. COL is readily soluble in cold water (4 degrees centigrade), however, the solubility of COL decreases with the increase of water temperature. The active concentration of COL, and VBLS in our test solutions with fibroblasts has not been checked in the present study.

This was particularly evident in L-929 by long-term incubation (240 minutes, 37 degrees centigrade), where the effect of both VBLS concentrations (V1 and V2) was stronger than C1 and C2, which would be in agreement with the presumed metabolic effect of VBLS on nucleotides and protein synthesis (Armstrong *et al.*,1979; Krishan *et al.*, 1971; Warneoke *et al.* 1968). Wilson (1975) has shown that, the binding between VBLS and tubulin is complete within 5 min after first contact. Therefore, the longer incubation time of up to 240 min might have secondary effects on the metabolism of the cells, that is responsible for stability, function, and locomotion.

In L-929 cells, on a longer time of preincubation, the anti-tubulin influence of VBLS was less when compared to COL. This observation was not made with the other cells. The question arises here whether the L-929 cells have a possibility to abolish the effect of COL and VBLS - in case of continued incubation with the formation of cytoplasmic paracrystals consisting of VBLS/tubulin (Waddell *et al.*,1974; Na *et al.*, 1982; Krishan *et al.*, 1971), which might modify mobility.

However, it cannot be excluded that the fibroblasts we studied, in particular L929 cells, lead to a release of lysosomal enzymes by contact with low concentrations of spindle poisons, i.e. 10^{-5} to 10^{-6} M. Thus, smaller molecules could be released from DMEM that might have affected the migratory behavior of the cells studied. These smaller molecules may have a stimulatory effect on cell migration. Phelps (1969) reported such releases of stimulatory molecules from leukocytes in the presence of urate crystals. Mizel et al (1980) attributed this stimulatory effect to a factor named as interleukin-1, which can be released from tumor cells of mouse fibroblasts by stimulation. Furthermore, it cannot be excluded that the different cell lines might react differently to trypsinisation. From fibroblast it is a well known fact, that trypsinisation can lead to the degradation and inactivation of membranous receptors. This can lead to the blockade of chemoattractant receptors, with the result that the cell lines are not stimulated by the added chemoattractant. It has been extensively demonstrated, that trypsin can destroy membrane receptors of adipose cells for

insulin (Kono and Barham, 1971; Sakai et al., 1973; Solomon, et al., 1975). Therefor such modifying impact on the outer cell membrane of our cells cannot be excluded, and be responsible for the different migration pattern under the influence of the same anti tubulin agent condition.

The effect of the different concentrations of anti-tubulin agents was consistent with the expectation that a larger amount of drug would also have a stronger effect on the target cells (Wilson *et al.*, 1975). The only exception was L6Y1, wherein the lower VBLS concentration caused up to 20% greater inhibition. Since the tendency of both concentrations is very similar, V1 and V2 gave partly the same results. It must be assumed that in this cell line the effect of VBLS is less dependent on the concentration than its mechanism of action. The same cell line showed high sensitivity to the concentration of anti tubulin when COL was applied, the results of C1 and C2 differed in part by 30%. This could be an indication of differential reaction kinetics of COL and VBLS. Further, an influence of trypzination cannot be ruled out, as descried previously.

The activated HESF cells showed only slight decreases in motility under the influence of C1 and V1. Over the period of pre-incubation from 60 min to 240 min, motility remained slightly below that of control, which Zakhireh (1980) had observed with blood monocytes. However, high concentrations of C2 and V2 led to a strong decrease in motility, which can be attributed to possible metabolic disturbances. At these concentrations, Palmer (1961) and Cutts (1961) had found that there was an increase in arrested metaphase cells characterized by decreased motility, as Postlethwaite (1978) found for fibroblasts.

In contrast to the expected results, variation in pre-incubation times had produced two opposite results for HT1080 and 3T6. With longer incubation time, migration rates decreased more or less steadily, in some cases with an asymptotic course. The result was similar for L6Y1, except that here the minimum was reached very early (30 min) and was approximately maintained in the further course.

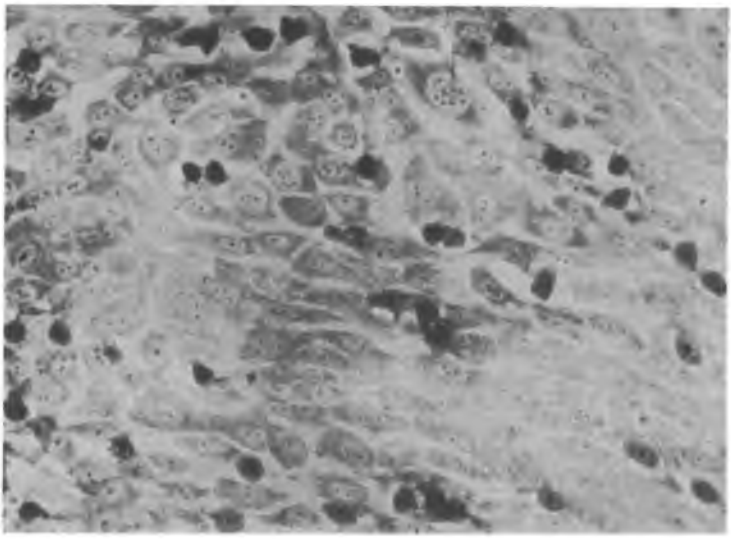


FIGURE 23: Accumulation of blocked metaphases in 3T6 culture after exposure to colchicine 5 mcg/mL for 240 min preincubation; multinucleated cells, with scattered chromosomes, x 160.

The opposite reaction was observed with L929 at 240 minutes of incubation, compared to 60 minutes, there was a rebound of the chemotactic response, more pronounced for COL than for VBLS. This phenomenon was already mentioned and discussed in connection with the observations on the different responses of COL and VBLS.

HT1080 cells showed the most differentiated response to the modifications of concentration and time course of pre-incubation with anti-tubulin agents, with a priori migration propensity.

Similar to L6Y1, activated C3H cells responded to increasing concentrations of COL and VBLS at pre-incubation of 120 min with a marked decrease in motility, which remained comparably low at 180 min and 240 min, respectively. It cannot be excluded that cytotoxic and karyotoxic effects of the two anti-tubulins are responsible for this pattern of mobility, as Palmer (1961) had shown for EHE cells.

As shown in the present study, for the other cells a prolonged incubation for 60 min in the presence of VBLS, and to a lesser degree with COL, led to a significant decrease in the mobility of activated 3T3 cells. This is consistent with the very low dissociation constants of VBLS and tubulin, which lead to a rapid establishment of the binding equilibrium. Complexes of VBLS and tubulin could both, disrupt ribosomal protein synthesis of tubulins and prevent their post-translational modification, which may be necessary for functional tubulin proteins. Future studies on the thermodynamic formation of tubulin dimers and quaternary structures could provide information on this issue.

It is important to note that even at the maximum concentration applied and at the longest time course of pre-incubation, chemotactic migration was only reduced but not abolished. Some cells migrated even under these conditions, at a rate still well above random migration. This has also been described by Armstrong (1978) in his morphological study on fibroblasts with respect to the migration in three-dimensional tissue, that could only partially inhibit but not completely suppress by the concentrations of COL and VBLS, respectively as mentioned in the present study.

COL in low dose, i.e. 1.000 microgram per day has been used successfully in the treatment of familial Mediterranean fever, which presumably is caused by fragile leucocytes. The number of leucocytes had been reduced under low dose therapy with COL, but chemotaxis and random migration were normal. The authors concluded that COL might have blocked the liberation of inflammatory proteins, but not basic locomotion (Dinarello *et al*, 1976).

Despite these differences in results, it can be said that chemotactic activity, i.e., directed migration, was reduced by VBLS and COL in higher concentrations (below 10^{-1} M) when compared with the control group.

When random migration is considered, it can be seen that the activity level in the non-directed migration was maintained over the entire time course of exposure. This result differs from

observations made by Zakhiren & Maleck (1980) in their work on blood monocytes. Random migration at higher COL and VBLS concentrations, i.e. both above 10^{-6} M or 10^{-5} M, had been also qualitatively reduced similar to the chemotactic response.

As mentioned already in previous articles, Macht and Livingston (1922) had observed both the inhibition of root growth and cell division in plants which had been in contact with COL in concentrations above 0.001 %, similar to the COL concentrations which Zakhiren & Maleck (1980) had used for their blood monocytes and which we used in our experiments with fibroblasts. At dilutions lower than 0.001 %, however, the roots of the colchicinated seedlings had shown a striking increase in both cell division and structural length of roots compared to the control. As with growth inhibition, a temperature dependence of the COL effect had also been observed for growth promotion and cell division in such plant cells. At concentrations of 0.000125 % to 0.001 % COL amorphous, a clear stimulatory effect on cell division has been evident at 16 degrees centigrade, while at 31 degrees centigrade growth inhibition predominated due to the increased toxicity of COL at higher temperature. It is understood, that plant cells are different from blood monocytes, or our fibroblasts. However it cannot be excluded that the basic reaction of both cytoplasmic and nuclear functional molecules to COL might be similar.

As has been observed previously with blood monocytes, some of our cells migrated more, as if they had been put into a state of higher activity by low concentrations of COL and VBLS, respectively. These phenomena could be explained with the observations of Dustin (1934), that COL can cause in a first phase of incubation a lively stimulation of the metaphase division activity and thus mobility of fibrocytes, in a second phase then a blockade of the mitoses in the metaphase ("stathmokineses"). Also Ludford (1936), who carried out investigations on tissue cultures in vitro and in vivo, was of the opinion that the accumulation of the cell division seen in FIGURE 20 / FIGURE 21 / FIGURE 22 would be rather caused by the mitotic arrest in the metaphase, without, however, being able to exclude a certain stimulation of mobility during mitoses.

The results/findings of the present study support the thesis of R. Goldmann (1971) on chemotaxis. Together with others (Creasey *et al.*, 1975; Crispe *et al.*, 1976; Ehrlich *et al.*, 1977; Freed *et al.*, 1968), he attributed "chemotaxis" to the directed migration of cells, which basically is facilitated by structured microtubules. In contrast to this, he assumed that the "random migration" of cells, i.e. spreading, is caused by the microfilaments, which are not affected by the anti-tubulin agents. This type of chemotaxis, attributed to random migration, he designated as "spreading", which he thought to be based on the microfilaments not being disturbed by the anti-tubulin agents.

In addition, the observations of altered morphology of cells treated with COL, i.e. undulation of the surface membrane at several sites of cells, rounded outer borderline of cells have been observed under the microscope.

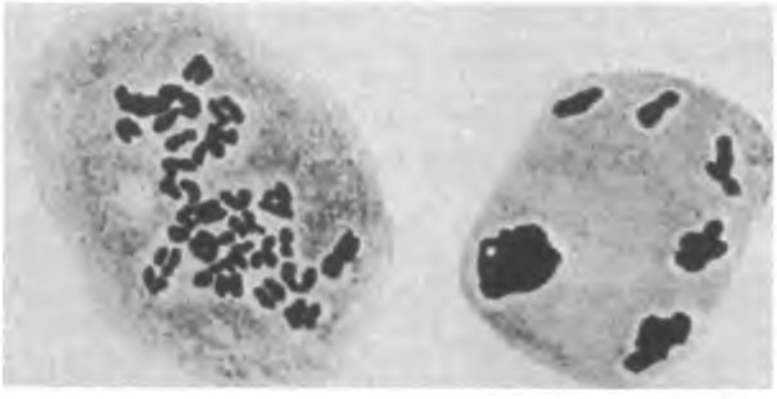


FIGURE 24 FIGURE 25

*The microscopic evaluation (**FIGURE 24, FIGURE 25**) of L929 revealed the disappearance of cytoplasmatic microtubules and nuclear membrane, and appearance of micro-multinucleated cells of the arrested metaphase. Scattering of chromosomes showing longitudinal cleavage; colchicine, 5 mcg/ mL; 240 min preincubation; staining carnoy- iron-hematoxylin, x 400.*

They used regenerating liver cells of rats in order to investigate the influence of COL on dividing metaphase cells. The

highest rate of metaphase arrested mitoses had been achieved with a sub-cutaneous colchicine dose (saline) of 100 mcg to 200 mcg per 100 g body weight. After parenteral application of COL into rats, they had observed the disappearance of the nuclear membrane, scattered chromosomes, and mitotic arrested cells with rounded outer cell border. COL at a dosage of 20 mcg to 50 mcg per 100 g body weight, significantly fewer metaphase arrested liver cells were observed. A higher COL dosage of 500 mcg which is equivalent to 10,000 mcg per 100 g body weight, i.e. lethal dose, caused much less arrested mitoses than the lowest administered COL dose. The authors concluded from their observation, similar to Palmer (1961) that, the high dose of COL prevented the liver cells from mitosis due to toxic effects with the result of fewer cells arrested in metaphase. In contrast to Dustin (1934), Brues & Cohen (1936) did not find a stimulation of division activity by colchicine on regenerating rat livers in their experiments. Abercrombie (1958) described the same features in fibrosarcoma cells (Eigsti *et al.*, 1955; Deysson *et al.*, 1944), and also observed the "retracted" processes where the whole cell becomes round. Furthermore, he could show that the determinacy of the preceding cell part had been removed. Similar aspects of leukocytes and fibroblasts have been extensively described (DeBrabander *et al.*, 1976; Goldman *et al.*, 1971; Spiro *et al.*, 1980; Zakhireh *et al.*,1980).

5. Conclusions

In this article, the study of the cell migration in the direction of a chemotactic gradient has been attempted which is similar to blood leukocytes. The main result is that a new method for testing anti-tubulin agents on activated fibroblasts in solution is presented, which mimics cell movements in solution and thus in 3-dimensional space.

The results of the experiments carried out in this work have shown that the cells at the time of the highest proliferation rate also exhibit the highest motility and mitotic activity. The experiments were all performed at a time point when the cells were in a stage of higher mitotic activity: At high rates of proliferation and migration, the cells were certainly more vulnerable than at resting times, so the results of this stage were more

pronounced, but probably qualitatively the same. In this state of increased vulnerability and mitotic activity, the anti tubulins COL and VBLS were able to inhibit the directed cell migration in varying degrees with all the modifications of preincubation time and concentration, respectively. However, this reduction did not occur to the same extent across all the cell lines. The cell lines responded to the anti-tubulin agents tested, their concentration, and the exposure duration, individually. Further to that, motility could not be brought to a complete cessation. The rate of chemotactically activated and migrated cells was significantly higher than the random migration, which was not negatively influenced by the two substances. Thus, if in the main experiments the cells exposed to anti-tubulin agents exhibited lower migratory activity, this has to be due to the direct effect of the anti-tubulin agents on the cells. These observations add new molecular insights into the knowledge of the role of two anti-tubulin agents, i.e. COL and VBLS on the motility of 7 different fibrosarcoma cell lines and fibroblasts in a conditioned solution of a known chemo attractant by using the modified Boyden chamber. Our results have shown that the Boyden chamber is a useful tool for studying the influence of both, COL and VBLS, on the directed and non directed movement of fibroblasts in the presence of a chemoattractant in solution.

6. Material & Methods

Cell lines

The following cell lines were obtained from the clinical laboratory of our hospital for studying the chemotactic response to anti-tubulins:

C3H	mouse fibroblast embryo cell line
HT 1080	cells of an epitheloid human fibrosarcoma
3T6	cells of a mouse embryo fibroblastoma
3T3	mouse fibroblast cell line
L929	cell line established by Earl 1948, cells of a mouse fibroblastoma from an explant of subcutaneous connective tissue taken from strain C3H mouse; doubling time: 28 hours, from cell counting
L6Y1	mouse tumour cells
HESF	humane embryo skin fibroblasts

Logarithmic growth phase

The first investigation was focused on when the cells showed the greatest possible migratory activity after trypsinization and distribution to the new bottles. 4 flasks were filled with 10 ml of the same cell suspension for each of the 7 cell types; on the first to fourth day after their introduction into the flasks, always one flask of each cell type was taken, the DMEM was decanted while removing the dead or detached cells as well. After rinsing with PBS and trypsinization, the cell number was counted. Subsequently, chemotaxis against conditioned medium and random migration was determined. This resulted in the respective information for the following experiments, which were set at the maximum of the chemotaxis rates thus tested. The restriction to the fourth day after trypsinization was made because at this point at the latest the cells had proliferated to such an extent that some of them died, detached, and showed a significantly reduced migration frequency.

Anti tubulin agents - conditioned medium

The next step was to make a statement about the chemo attractivity of COL and VBLS or their direct influence on the effect of CM.

Therefor the chemo attractivity of COL and VBLS or their direct influence on the effect of CM was evaluated.
For this purpose, five sets were prepared per cell line, investigated. The chemoattractant served unchanged as a counter sample in the first place. This was followed by four solutions, each with different concentrations of COL (0.005 mcg/ml, 0.05 , mcg/ml, 0.5 mcg/ml, 5 mcg/ml, respectively) dissolved in CM, respectively. Further to that VBLS (0.01 mcg/ml, 0.1 mcg/ml, 1.0 mcg/ml, 10 mcg/ml, respectively) dissolved in CM. The concentrations of anti-tubulin agents were used according to the experiments of Johnson (1960) and Wilson (1975) on fibrosarcoma cells from mouse and Malech (1977) and Zakhire (1980) on neutrophil cells, respectively.

Anti tubulin agents – different incubation time - conditioned medium

After these preparatory steps, the direct effect of the anti-tubulins on the cells was investigated. The extent with which the migration rate of the cells is reduced by pre-treatment with these anti-tubulin agents was determined. The investigation of the migration was studied in the medium free of anti-tubulin agents, with the exception of the amount of anti-tubulin liberated from the cells by passive diffusion into the medium during the experimental time (Beck *et al.*, 1983; Borisy *et al.*, 1967; Minor *et al.*,1975). For this purpose, two concentrations of COL (0.05 mcg = C1 and 5 mcg = C2) and VBLS (0.1 mcg = V1 and 10 mcg = V2) each per ml DMEM, were added to the cells in the culture flasks - after PBS rinsing and incubated at different times (15 min, 30 min, 60 min, 120 min, 240 min). The lowest concentration of VBLS, i.e. 0.1 mcg/ mL, was twice as high as that published by Johnson (1960) for inhibiting the half-maximum cell division of fibrosarcoma cells, i.e. sarcoma 180, in the metaphase.

At the end of the time interval, decantation of the anti-tubulin containing DMEM and careful rinsing with PBS was followed by the usual steps to obtain the cell suspension. The cells thus treated were then subjected to the Boyden chamber for their directed migration against the chemoattractant CM. For comparison, control cells were tested for their chemotaxis, that treated with DMEM free of anti-tubulin, for the times indicated.

Anti tubulin agents – random migration

In addition to directed migration, non-directed migration is also important for the evaluation of cell mobility. The random migration was generally determined on DMEM. In particular, the influence of COL and VBLS on non-directed migration was evaluated by using the experimental set-up similar to earlier experiments. Cell lines were preincubated with different concentrations of spindle poisons at different time intervals, collected, and transferred to the upper part of the Boyden chamber. Further, the cell lines were incubated against the pure DMEM without a chemoattractant in the lower compartment of the Boyden chamber.

Panel Discussion

Question Dr MILLER:
Both anti tubulins have completely different chemical structures. Colchicine is a tropolone derivative while vinblastine is a vindoline derivative. In addition, there are stereospecific differences, as Dr SCHREIBER had explained. They also have different binding receptors on the cytoplasmic tubulin proteins of the cells studied, as Wilson and Taylor had shown. Although the fibroblasts are of different origin, they lead to qualitatively comparable reactions in the movement pattern under the influence of the two different anti-tubulins. Similar to neutrophil granulocytes, random migration and directional movement are slowed down under the additional influence of chemoattractant. This effect was more pronounced for vinblastine sulphate than for colchicine at comparable concentrations. Are there any explanations for this?

Answer Dr MANULESCU:
We have done further studies on cell division under the influence of colchicine for L6Y1, L929 and 3T3 cells. We found that colchicine in low concentrations of 10^{-5} M and less (10^{-8} M) leads to a metaphase arrest in the fibroblasts studied, as Eigsti had found in his cell lines. Not all pharmaceuticals are producing such arrest of cell division. It can be regarded as a typical reaction for colchicine and vinblastine. This disruption of the tubulin chain may have led to the slowing down of the movement pattern in the Boyden chamber that we observed. Although the cells are different from three dimensional structure, the blueprint of the individual cell lines for their individual enzymes, structural proteins and secretory molecules appears to be highly similar. Their pharmacological properties are comparable despite their chemical differences. Both drugs have a narrow therapeutic range in humans. They lead to gastrointestinal disorders with nausea, vomiting and severe diarrhoea with loss of minerals in case of overdose. Hyponatremia can be observed in case of accidental poison. Polyneuropathies are frequently observed, as well as myoneuropathies. Metaphase arrest has been descried for both, bone marrow cells and gastrointestinal mucosa, but with quantitative differences between the two drugs, also depending on

the dosage and duration of use. This type of specific chemical binding to charged cytoplasmic molecules may be one explanation for the improvement of symptoms in Gout that we observe with colchicine or vinblastine treatment in patients.

Thus, it could well be, that different cell lines of fibroblasts might have similar or identical receptors for the two anti-tubulins, with the result of similar movement pattern of the cell. However, there may be differences in the transport of the pharmaceuticals through the cytoplasmic membrane into the cell interior, as the cell lines have different antigenic properties that may influence transmembrane transport. This would explain why the different cell lines react in the same way in terms of quality, but show considerable differences in terms of quantity, which may be due to the different transmembrane penetration behaviour and cytoplasmatic metabolization or binding to chemically charged molecules, such as nucleic acids and their metabolites such as urate.

Question: Dr Scanner
In your experiments on fibrosarcoma cells, you found that the cell lines studied showed a markedly increased migration rate when they were fixed on the filters of the Boyden chamber during the exponential growth phase. Can you tell us something about the effect of the anti tubulins on the growth rate and movement in this phase of the cell cycle? In prokaryote growth cycles, increased metabolic activities have also been observed during exponential growth phases, comprising increased formation of rough endoplasmic reticulum and granules with lysosomal enzymes.

Answer: Dr Gaitan
Similar to the migration of leukocytes in the tissue, fibrosarcoma cells also have to go through various movement processes during their metastasis in order to reach their target tissue. First, they have to bind to endothelial cells, then penetrate the cell wall or their interjunctions to enter the bloodstream and from there to the target tissue, either by random migration or mediated by chemoattractant.

The protocol for culturing, growing fibroblasts, tumour cells, and forming the chemoattractant used, followed the protocol as described by Boyden (1961), Varani (1978), and were further modified by Armstrong (1979) and Postlethwaite (1976), with minor adaptations to the conditions in our laboratory. Fibroblasts were cultured according to standard techniques (Sharma 1980, page 359), in Falcon plastic bottles with 75 cm^2 bottom surface and 250 ml volume. Dulbecco's Minimal Essential Medium (DMEM) was mixed with 10% (v/v) FCS, served as nutrient, with 40,000 U/mL penicillin, and 50 mcg/mL streptomycin added. Cells were maintained in a humified atmosphere at 37°C with 5% $C0_2$ added, and the medium was changed every two days until the bottom of the flask was confluent.

Trypsinization and Viability testing of Cells

Trypsinization was performed according to Gori's protocol (Gori *et al.*, 1964) adapted by Goldman, Postlethwaite, and Martin (Goldman *et al.*,1971; Postlethwaite *et al.*, 1976; Martin *et al.*, 1981). The medium was poured off, the cell culture was rinsed with 20 ml PBS (Na^+/K^+ - phosphate buffer, pH 7.2), and then trypsinized with 5 ml 0.20 % trypsin, 0.1 % EDTA in PBS. After 30 sec, the trypsin-containing PBS was poured off, and the cells were placed in the incubator for another minute. The monolayer was then detached by tapping the bottom of the flask. For culture, 30 ml of DMEM containing 10% FCS was added to the cells and they were distributed to two new flasks where they continued to grow as described above (Gori et al. 1964), adapted by Goldman (1971), Postlethwaite (1976), and Martin (1981). The structural and biophysical integrity of the cells examined was subjected to various standard laboratory tests. At the end of each anti tubulin experiment, aliquots were taken from the centrifuged supernatant for the quantitative determination of lactate dehydrogenase (LDH) in the incubation medium. LDH is a cytoplasmic enzyme that is not released from intact cells into the culture medium. One can assume, that the gradual increase in LDH activity would be an indication for physical disruption of the plasma membrane accompanied by a decrease in fibroblast biochemical viability. Furthermore, we stained the cells with 0.5 % eosin.

Viable cells actively pump the eosin dye out of the cell into the medium, while metabolically inactive cells are stained pink by the dye.

Preparation for chemotaxis experiment

The cells intended for the experiment were suspended in 15 ml DMEM containing 10% FCS to ensure cell recovery. The medium was centrifuged off and decanted. The cell sediment was resuspended in 5 ml DMEM without FCS, counted, and diluted to a cell count of up to 10^6/ml.

The fibroblast chemotaxis was measured with a blind well modified Boyden chamber manufactured in our clinical laboratory. The lower compartment was filled with 200 microliters of the solution to be assayed or the control medium.

Then the chamber was covered with millipore filters of 10 mcm thickness and 8 mcm in pore diameter. The filters were coated with 0.1 % gelatine to provide a substrate for the cells to adhere (Postlethwaite *et al.*, 1976). The 8 mcm narrow pores ensure that the cells with a nucleus diameter of 12 – 15 mcm can only pass thru the other side of the filter by active deformation.

In the upper compartment, 800 microlitres of cell suspension were placed containing up to 1 x 10^6 cells/ml, based on the protocol of Zigmond & Hirsch (1972) used for the chemotaxis experiments on leukocytes.

The chemoattractant used was the CM mentioned earlier, which is a mixture of various substances, consisting of nutrients and minerals. These substances are synthesized by cultured human embryofibroblasts (HEF) within 24 hours, and released into their culture medium.

CM was prepared following the known protocol of Martin *et al.*, 1981. Fibroblasts were cultivated as described, the medium (DMEM with 10 % v/v FCS) changed after one and after three days, respectively, and poured off again after four days. Further, the cells were washed with PBS to completely re-

move the FCS and then covered with 10 ml of normal DMEM. After 24 hrs., the now "conditioned" medium was decanted, centrifuged to clean cell debris, and frozen at -20 degrees centigrade until use.

Both, COL (clinical pharmacy, amorphous, melting point 142 -144 °C and VBLS, clinical pharmacy, melting point 282-285°C) were dissolved in DMEM (Armstrong *et al.*, 1979) in required concentrations as indicated i.e. 0.005 mcg/ mL DMEM to 10 mcg anti-tubulin agent/ mL DMEM, and then used directly for the experiments. COL at a concentration of above 0.02 mcg COL/mL and VBLS at a concentration of above 0.002 mcg VBLS/mL, respectively, have previously been used successfully for inhibition of locomotion of fibroblasts. Anti-tubulin agents when administered at 0.01 mcg anti-tubulin agent/mL, do not produce multinucleation, failed to exert any effect on the cell growth of the culture, as has been described by DeBranbander *et al.*, 1976,.

Cultures and solutions containing anti-tubulin agents were protected from sunlight in order to prevent degradation.

Methods

The chambers for the study of the migration behavior of the different cell lines were made of polyethylene in the workshop of our pharmacy, according to a model the original chamber disclosed by Boyden (1961). All the experimental sets used had the same basic principle.

Each individual chemotactic experiment was preceded by a control group for DMEM to test the chemotactic unaffected attachment. This basic principle of experimental setup was modified in different series of experiments to test chemotaxis under the influence of anti tubulin. As to the preparation phase, cells were suspended at a counted concentration as described, which was assigned as being 100%. The filters were labelled, placed flat on the lower 200 microlitre compartment filled with a solution of attractant and fixed, followed by the cell suspension (corresponding to a maximum of $8 \times 10^{+5}$ cells to $1 \times 10^{+6}$ cells) into the upper 800 microlitre compartment,

both compartments separated by a filter with pores as described. Each individual batch was performed in triplicate. Each Boyden chamber was incubated in an incubator for four hours at 37°C with humified air, 5% CO_2 added.

At the end of the incubation period, the cell suspension was removed, the top of the chamber was taken out, and the filter was carefully removed with forceps. In the experiments used to evaluate the attachment, the filters were not treated further mechanically. Cells attached to the filters on the upper side facing the cell suspension were removed with moist filter paper to facilitate the later microscopic evaluation of the cells that had migrated to the lower side. After mechanical treatment, cells on the filters were fixed in ethanol (90%) for 15 sec using a metal basket, rinsed with distilled water, stained with hematoxylin, dried, mounted on glass coverslips with immersion oil, as described by Postlethwaite (1976). Fibroblast chemotaxis was quantified by counting nuclei of fibroblasts on the lower surface of the filters under the microscope according to Mundy 1981 (magnification x 160 or x 400, cells per view, cells per vision - CPV -, as reflected by the corresponding graphs).

The cells adhering to the upper side of the filter had already been removed in preparation for fixation, which facilitated the counting. All the visible cells are on the lower side of the filter or in the pores, which indicates they must have migrated. Individual cells still adhering to the top of the filter could be detected during counting by focusing at a filter thickness of 10 micrometres. In order for the experiments to be scored, macroscopic uniform staining of the filters was required in both the sample and the control attachment. Five fields of view were counted from each filter; since three filters were used per experiment, this resulted in 15 values that were arithmetically averaged.

Modified methods

By varying this basic principle, the subdivision into several series of experiments was achieved.

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Colchicine in concentrations of 1×10^{-6} M to 5×10^{-5} M or higher interferes with biochemical cellular functions of leukocytes which are important for phagocytosis. However other studies have shown that the interaction of colchicine would not be with phagocytosis but with the directed and non directed movement of cells and the liberation of lysosomal granules across the outer membrane of cells.

The histological findings were confirmed by studies of digestive enzymes incorporated into cytoplasmic granules of leukocytes. In such experiments, Dr MALAWISTA (1975) treated leukocytes with colchicine in concentration of 2.5×10^{-5} M in DMEM with the result of more activity and less degranulation of digestive enzymes than controls.

Similar to the migration of leukocytes in the tissue, fibrosarcoma cells also have to go through various movement processes during their metastasis in order to reach their target tissue. First, they must bind to endothelial cells, then penetrate the cell wall to enter the bloodstream and from there to the target tissue, either by random migration or mediated by chemoattractant.

Question: Dr Fingal
In 1979, Bell suggested that messenger substances released from cells, e.g. fibroblasts, in response to external stimuli should be referred to as lymphokines. Is it possible that, in addition to the already known chemoattractants such as fibronectin or collagen, other messenger substances could also be responsible for the migration behaviour of fibroblasts?

Answer: Dr Brighton
To date, such substances have neither been isolated from fibroblasts nor characterised. However, it is quite possible that external chemical or physical stimuli can lead to the synthesis and release of "lymphokines" which influence the migration behaviour of cells. The future will show where this leads.

Question: Dr Bell
We know from studies of patients with uric acid arthritis

when treated with colchicine (i.e. 1 x 10^{-6} M in vitro) , that leukocytes show less adherence to endothelial cells than untreated patients (*Penny R, Galton DAG, Scott JT et al, 1966, Studies on neutrophil function, I. Physiological and pharmacological aspects, Br J Haematol, Vol 12, pp 623*). It may be possible to conclude that endothelial cells and fibroblasts exposed to colchicine treatment also exhibit changes in their outer antigenic plasma membrane, like migrating leukocytes. How do you comment this under view of your experimental results on isolated fibrosarcoma cells?

Answer: Dr Bondoc

Colchicine 1 x 10^{-3} M blocked the random migration of PMN. This finding was confirmed in cine-micrographic studies in which the individual speeds of many cells were measured, but lower concentrations of the drug (2.5 x 10^{-6} M and 1 x10^{-4} M) had no effect on random migration, whereas directed migration was slowed down. In further support of the involvement of microtubules, we found that the effects of vinblastine (2.5 x 10^{-5} M) on human leukocytes during phagocytosis were similar in all respects to those of colchicine and, on a molar basis, at least as strong. Thus there was inhibition of the increased oxygen consumption and glucose-1-^{14}C oxidation that normally accompany phagocytosis; there was inhibition of lysosomal degranulation and the formation of digestive vacuoles, indicated both in morphologic studies and in measurements of granule-associated acid phosphatase activity after phagocytosis; and there was no demonstrable effect either on the ingestion process itself, or, in the usual experimental system, on the intracellular killing of Staphylococcus aureus. Ultrastructual studies with vinblastine revealed the dissolution of normal microtubules in human leukocytes and led to the discovery of vinblastine-induced paracrystalline aggregates of microtubule protein. With regard to this, vinblastine has been shown to depolymerize tubulin polymers and precipitate colchicine-binding protein (*Bensch KG, Marantz R, Wisniewski H, et al, 1969, Induction invitro of microtubular crystals by Vinca alkaloids. Science Vol 165; pp 495).* Furthermore, Gillespie (*Gillespie E, Levine RJ, Malawista SE, 1968, Histamine release from rat peritoneal mast cells: inhibition by colchicine and potentiation by deuterium oxide. J Pharmacol Exp Ther Vol, pp 158*) has shown that the preincubation of secretory cells such as mast cells with

the secretion of histamine, in the presence of varying concentrations of colchicine and increasing incubation time, lead to a decrease in their cytoplasmatic secretory performance. Obviously there is an interaction of secretory vacuoles of cells with colchicine. Mast cells are not fibrosarcoma cells we studied, but they have basically the same blueprint as other eukaryotic cells. From this assumption, one could conclude that other cell functions of different cell lines, such as fibrosarcoma cells, also behave similarly, exhibiting their secretory or mobile properties or also cell stability functions, such as sodium-potassium ATPase.

A molecular target as microtubule protein, one would expect the potential effects of colchicine in man to be limited only by the number of cell systems to which the drug has significant access in the dose range of 10^{8} M to 10^{0} M, and by the lability (i.e. turnover time) of the microtubules that supports a given function. With regard to that, it can be assumed that colchicine binds to the disorganized microtubule protein but probably not to the polymerized microtubule itself. This state of intracytoplasmic disorganisation with depolymerisation of polytubulin proteins has been observed during the exponential growth phase with countless interphases, similar to that in prokaryotes and other cell systems. Obviously, in this state the receptors on the tubulin proteins are free for the binding of colchicine with the formation of tubulin-colchicine complexes that prevent the formation of tubulin polymers.

Supporting this view are the variety of systems involved in colchicine overdose in man, as well as the variety of functions affected in various cellular models such as fibrosarcoma cells (*Conference on the Biology of Cytoplasmic Microtubules, 1975, NY Acad Sci pp 233*).

This could explain the increase in three dimensional epitheloid shape of cells treated with anti-tubulin, which has also been observed by others, since it is possible that altered biochemical metabolism leads to an increase in intraosmotic pressure, with subsequent diffusional entrance of water from the culture medium into the cytoplasmatic space.

Others *(Phelps P, 1969: Polymorphonuclear leukocyte motility invitro. II. Stimulatory effect of monosodium urate crystals and*

urate in solution; partial inhibition by colchicine and indomethacin. Arthritis Rheum Vol 12: pp 189) have shown that very low concentrations of colchicine, i.e. 1 x 10^{-5} M to 1 x 10^{-8} M could decrease the locomotion of PMN in direction of a chemoattractant such as urate crystals and bacteria, but not completely stop it, as shown by Dr MALAWISTA for phagocytosis of bacteria by prokaryotes (1975).

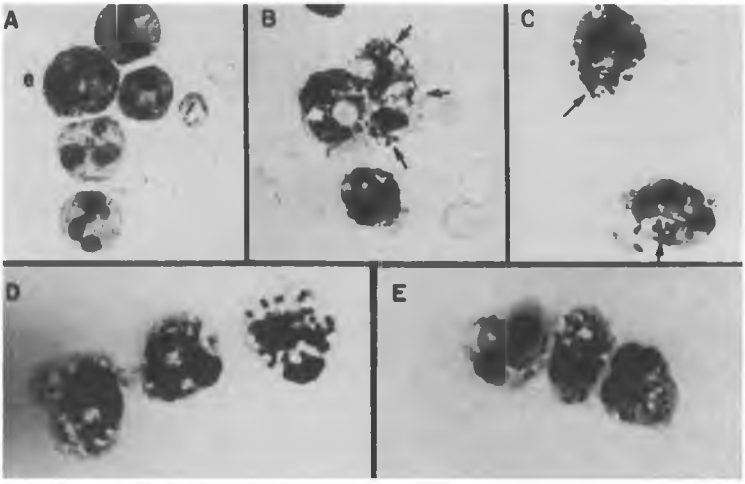

FIGURE 1:: *The effects of colchicine and ff vinblastine sulphate on the shape of granulocytes after phagocytosis of heat inactivated bacteria , i.e. staphylococci, The cells were incubated for 1 hour with or without colchicine (2.5 x 10^{5} M), and for an additional hour with or without bacteria.* ***A)*** *No COL; no bacteria: The granular cytoplasm is without vacuoles. Cells incubated with VBLS or COL for 1 hour had a similar appearance (not shown).* ***B)*** *No colchicine; bacteria: Phagocytosis is associated with the presence of prominent digestive vacuoles. Bacteria (arrows) are seen.* ***C)*** *COL; bacteria: neutrophils contain numerous staphylococci (arrows), but are without prominent cytoplasmic vacuoles. Cells with this degree of cytoplasmic integrity after phagocytosis were not seen in preparations without colchicine.* ***D)*** *No VBLS; bacteria, and* ***E)*** *VBLS; bacteria: In experiments with VBLS the results were similar to those seen with COL (Malawista SE, 1975, Microtubules and the mobilization of lysosomes in phagocytizing human leukocytes, Conference on the Biology of Cytoplasmic Microtubules, NY Acad Sci, Vol 253, pp 738).*

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