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THE MECHANISM OF ACTION OF STEROIDAL COMPOUNDS

ON THE PROCESS OF MITOSIS

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THE MECHANISM OF ACTION OF STEROIDAL COMPOUNDS ON THE PROCESS OF MITOSIS

CHAPTER I

INTRODUCTION

During the past 30 years there has been an ever-increasing interest shown in the physiological activity of various compounds and their relationship to the process of mitosis.

Since 1929 when A. P. Dustin reported that certain chemical agents, which he termed "poisons caryoclasiques", were able to exert a toxic action on nuclei, an increasing number of factors, both physical and chemical, have been recognized as being able to produce this effect in varying degrees. These agents vary in nature from changes in temperature to X-rays and from distilled water to the most complex protein and steroidal compounds.

Contributions by numerous workers with training and research experience in widely separated fields now comprise an extensive literature.

Factors have been reported as "active" on different materials, by different criteria, and under very different experimental conditions. The purposes of many studies have also varied. Some workers have entered this field with cancer therapeutics in mind. Others, such as geneticists for example, are interested in these agents because of their mutagenic

capacity. There is even some disagreement as to what conclusive results should be accepted as being comparable under all circumstances. Nevertheless, our knowledge of the mitotic process has become greatly enriched by the wide interest exhibited in this mechanism and in factors that are advantageous or detrimental to it.

The importance of polycyclic hydrocarbon compounds and of steroids to this field was recognized by von Möllendorff (1939) and his co-workers (1942-1943).

A number of steroidal compounds both natural and synthetic are at present being employed to combat neoplastic diseases of rather specific classification. While many of these are very effective in their action against cancer cells, they have very undesirable side effects due to their hormonal nature. It can only be through continued effort that new compounds will be developed with more specific cytotoxic effects and with their side effects reduced to a minimum.

It is the object of these studies to determine, by a morphological study of cells in mitosis, the mechanism of action by which various steroidal compounds of the estrogen series, as well as their products of metabolism and synthesized epimers, may affect the process of mitosis of cells grown in tissue culture.

The Normal Mechanism of Mitosis

A sequence of definite stages has become recognizable and is accepted as the typical pattern of cellular appearance in the course of cell division (Darlington, 1937; DeRobertis, Nowinsky, and Saez, 1954). Beginning with the resting stage, or interphase, the cell passes through

prophase, metaphase, anaphase, and telophase, and once more enters the resting phase. The principal differences in appearance are due to the orderly variations of the organized nuclear and chromatin materials. In order to form a basis for observation and discussion in this problem, a brief resume of the principal characteristics seen in the several phases is given.

Resting Stage or Interphase

During this period the nucleus possesses a nuclear membrane. The chromosomes are not organized, but are dispersed as fine chromatin granules upon an extensive linin network throughout the entire nucleus. The centriole lies within the centrosome outside the nucleus. A prominent nucleolus is also visible and is the center of storage of chromatin.

As the process of mitosis begins, it is observed to be accomplished by the interaction of three interdependent and self-perpetuating cell organs:

(1) the chromosomes and the nucleus which they form,

(2) the cytoplasm with its contractile superficial gel layer and less viscous endoplasm, and

(3) the centrosome with its gel layer and spindle fibers.

Prophase

During early prophase the cytoplasmic pseudopodia retract indicating decreased viscosity of the cytoplasm and gel layer of the cell body and an increased contraction of the pseudopodial gel layer. The centrosome divides and moves to opposite poles of the nucleus to form a duplex body with two centrioles. Spindle fibers protrude as pseudopodia

from each centrosome over the surface of the nucleus to become attached to the kinetochores of the chromosomes. The chromosomes during this period have become free and visible by a process of shrinking and gelation.

In late prophase the nucleus becomes elongated and the chromosomes are pulled to the medial plane of the spindle by the action of the retractile spindle fibers. Several groups of chromosomes may be drawn in before the nucleolus divides into its nuclear units.

Metaphase

The outline of the nucleus disappears and the chromosomes lie in irregular groups halfway between the centrosomes. The area formerly outlined by the nuclear membrane is now occupied by a series of fibrils which form the spindle and which converge on the centrosome. The chromosomes oscillate individually due to slight variations of the contractile tension of the individual spindle fibers and form a flat plate across the equator of the cell.

Anaphase

The thickened chromosomes split into two longitudinal halves and are drawn to opposite poles to the centrosomes. Cellular elongation indicates increased contraction of the broad equatorial band of the gel layer. The spindle sap is compressed and the centrosomes, with the attached chromosomes, are forced toward the poles. Increased contraction of a narrow equatorial band of the gel layer constricts the cell. It first bends the endoplasm inward and then divides the spindle sap. The endoplasm becomes a slender stalk which temporarily unites the two daughter cells. The daughter chromosomes are drawn to the centrosomes by the spindle fibers.

Telophase

Each group of daughter chromosomes forms an irregular mass of interfolding adhering chromosomes that swell and lose their affinity for stain. A new nuclear membrane forms about each reconstituted nucleus and upon the reappearance of the nucleolus the cell again enters the interphase or resting stage.

While the precise moment of initiation and termination of each phase may vary slightly with each tissue and species, and while the descriptions by investigators may also have shown variations based on personal interpretations, the foregoing account of mitosis appears to encompass the concept held by the majority of cytologists.

CHAPTER II

HISTORICAL BACKGROUND

The Spindle Mechanism in Mitosis

The word mitosis is used by most biologists as an inclusive term to cover any nuclear division that involves a spindle apparatus and the division of chromosomes, although geneticists restrict the term to non-meictic division.

Though the most obvious feature of the mitotic process lies in an orderly distribution of chromosomes to the new cells, it is clearly recognized that this distribution at anaphase is only the culmination of a complicated but orderly series of steps represented in the preceding prophase, metaphase, and in the later telophase and interphase or resting period.

The various elements which participate in the mitotic cycle frequently show considerable independence.

It was shown by Boveri in 1897 that division of the centers may proceed even when cytoplasmic cleavage is prevented experimentally. It has also long been known from the work of Wilson (1901) and Lillie (1906) that chromosomes may continue to divide under the above circumstances. Pease (1941) reported that even such things as changes in hydrostatic pressure may affect mitosis. Harvey (1936) showed that complete absence of nuclear material does not prevent cleavage of cytoplasm.

While cell division was observed in living cells early in the 19th century, the first more or less exact concept of the mitotic spindle was not formulated until about 1875 by Strasburger and in 1879 by Flemming, who compared the phenomena in fixed preparations. In the normal living cell the spindle is visible primarily because the condriosomes and other intracellular granular materials do not enter it, but roughly outline its extent. The spindle itself shows no internal structure; however, Hughes-Schrader and Ris (1941) showed that some tissues of certain species, for example the spermatocytes of coccidae, do present more favorable optical characteristics for the observation of the spindle.

Vital dyes would be a great aid, but they stain nuclear components with difficulty and not without detriment to the living cell (Becker, 1936; Ries, 1938).

There has been much debate on the nature of the spindle, since much of our knowledge of it was obtained from fixed and stained material; however, its demonstration by almost all fixation methods suffices to prove the existence of a specific apparatus. Wassermann in 1929 differentiated between two types of spindles, basing his classification upon the presence or absence of centers and on the origins of the spindle fibers. In the one, or "direct" type, the chromosomal spindle fibers connect the spindle directly with the pole, and in the second, or "indirect", type the chromosomes are connected with continuous fibers.

The scanty knowledge of mitotic processes made it preferable to present a classification without physiological connotations and this was done by Bleier in 1931. He considered the spindle fibers as artifacts and the "direct" type as one in which the chromosomes are connected

directly to the poles, while in the "indirect" type the chromosomes are connected with a continuous fiber which arises between the poles.

The most commonly used terms employed in the description of the spindle are:

(1) Continuous fiber which connects the two poles or centers,

(2) Chromosomal fibers which connect with the kinetochore of chromosomes and may or may not extend to the center,

(3) Interzonal connections, connecting the two separating chromosomes in anaphase or telophase, and

(4) The center, which is the body toward which the spindle elements are oriented and in animals is composed of a spherical centrosome containing the centriole and, according to Wilson (1925), astral rays may or may not be associated with it.

The chromosome consists of several identifiable parts:

(1) Chromonema, a thread-like structure on which genes are carried. It is enclosed by a

(2) Matrix, which is stainable with chromatin dyes at metaphase. This seems to be coated by a

(3) Pellicle, or sheath, and the

(4) Kinetochore, located at the junction of the two arms of the chromosome. This kinetic organ is in close structural and physiological relation to the center and contains spindle spherules.

Evidence against the existence of spindle fibers has been accumulated. It was regarded by Martens (1929) and Robyns (1929) that the fibers represented artifacts as a result of faulty fixation. Micro-dissection experiments done by Chambers (1924) showed no displacement of

chromosomes when the micro-dissection needle was passed between the chromosomes and the pole. However, no statement has ever been made as to what degree of resistance such fibers could offer.

Lewis (1923) considered them artifacts because she could see the fibers only after acidifying the medium of her cultures to a pH of 4.0. She also ignored the organization of the cytoplasm at a neutral pH when the refractive index is so uniform as to obscure any structure even if it were present.

On the other hand, there are numerous arguments in favor of spindle fibers. It was shown by Belar (1929) that the spindle body underwent shrinkage in hypertonic media and that the shrinkage was greater in transverse than in longitudinal direction. Belar (1929) also showed that the Brownian movement of particles within the spindle body is greater in a longitudinal than in a transverse direction.

According to Lillie (1909), ultracentrifugation may produce bends in the fibers, or even tear them, indicating resistance to such distortion. This was also noted by Schrader (1934) and has been demonstrated many times since.

With improvement in optical methods and the advent of the polarizing microscope and with phase contrast equipment, direct observation of the spindle has become possible.

Electron microscopic studies have been done by Porter (1955) and reveal the spindle fibers as condensations of the fibrous material around a pair of distinct filaments. In cross section these latter elements appear as fine tubules about 250 Å in diameter. The spindle fibers are continuous from the chromosome to the centriole.

Birefrigence of the spindle at metaphase in a longitudinal direction was demonstrated by Schmidt (1936) and it was concluded that it consisted of a structure of protein molecules oriented in a parallel pattern.

An element of importance in chromosome movement is the kinetochore which was described 60 years ago by Metzner (1894). The spindle fibers are believed to be attached to the spherule and during anaphase the kinetic region takes the lead on the way to the pole and the arms of the chromosomes appear to be dragged behind. This flexion indicates the presence of the kinetochore even when it is not always visible.

The tensile forces involved in anaphasic movement may pull the spherule out of the kinetochore, stretching the chromonema at the same time (Schrader, 1939).

When a chromosome is deprived of its kinetochore, it behaves erratically and soon is lost (Upcott, 1937; Koller, 1938; Darlington, 1939). Lagging chromosomes may be due to an unready kinetochore and this results in misdivision.

It was shown by Rhoades (1940) that when one arm of a chromosome is lost, the remaining chromosome arm with a terminal kinetochore may behave quite normal for a while, but eventually it is lost.

Some evidence has been produced (Hughes-Schrader and Ris, 1941) that the kinetochore is distributed along the whole length of the chromosome rather than at the constriction. Fragmentation of the chromosomes by X-rays produced many small pieces that continued to divide long after.

A relationship between kinetochore and centriole has been noted by Darlington (1936). He found that they stain identically and have functional relationships.

It has been indicated by Pollister (1939) that the kinetochores may become detached from degenerating chromosomes and duplicate the appearance and behavior of the centriole, thus becoming extra centrioles. This may in part account for the multiastered mitoses we can see in some cells exposed to mitotic poisons and in some tumors.

As to the actual mechanism involved in mitosis, our knowledge is also very tentative and a multitude of hypotheses have been advanced.

Certain contractile elements were observed by Klein in 1878 in the cell. This concept of contraction of spindle fibers was further formulated by van Beneden in 1883. This author described that the metaphase chromosomes are attached by chromosomal fibers to the centriole to which they pull the chromosome on contraction.

While this concept was debated, it still appears to be the most popular one. Some workers, like Watase (1891), believed that chromosomes are pushed into metaphase by the chromosome fibers. However, certain mechanical difficulties that this theory entails remain unexplained.

Drüner in 1895 presented a hypothesis which combined the push-pull concepts. It was postulated that the chromosomes respond to the pushing effect in the first stages of mitosis and to the pulling effect in anaphase and telophase.

In 1927 Belar modified this theory, and he believed that the chromosomes glide by traction on the continuous fiber toward the pole, while the portion between the chromosomes in the interzonal region expands and helps to push.

Some of the less popular theories attempting to explain the mechanism of mitosis include one proposed by Wassermann (1929) based on

an orderly sequence of viscosity changes in the cell which depend on variation in the hydration of various zones and structures. An earlier one by Lillie (1905) involved the action of chromosomes as electrified particles that move in an electrostatic field. A hypothesis of diffusion forces was promoted by Rashevsky (1938). Seventy years ago Berthold (1886) hypothesized a mechanism involving streaming currents in the substance of the spindle. Lamb (1907) proposed a theory involving hydrodynamic forces produced by phasic vibrations set up in a fluid medium by bodies. A theory was advanced by Pfeiffer (1939) involving positive and negative tactoids with polar orientation of micellar constituents. This theory was later further outlined by Bernal (1940), who took into account the various phases of mitosis.

Considering all hypotheses, the one still in greatest favor and still most acceptable is the original one propounded by van Beneden in 1883, with definite fibers which contract or shrink and draw the chromosomes toward the cell poles. However, the other theories should not be disregarded and no doubt most of them can be fitted into the picture as a whole to at least some degree. Their proponents, like the six men of Hindustan, are all partly in the right and partly in the wrong. Some of the more recent concepts attempt to incorporate some of these more peripheral interpretations. Based on the work of Meyer(1929) it is now believed that polypeptide chains exist in spindle fibers and these can shorten when they release their water of hydration and enter an isoelectric state.

The actual time involved in the completion of the mitosis varies greatly and has been found by Martens (1927) and Rabinowitz (1941) to be

anywhere from 10 minutes to several hours. The duration of the cycle varies with species, tissue involved, age, temperature, oxygen-carbon dioxide balance, and numerous other factors which may exercise their effects over a long period or perhaps only momentarily.

In view of the rather great sensitivity of the mitotic mechanism to induced changes in its environment, numerous experiments have been done affecting mitosis and numerous observations have been made relative to abnormal mitosis.

The principal target for anti-mitotic agents appears to be the spindle apparatus and its role in the organization and execution of mitosis. When this organ is disturbed, various things may happen. The normal chromosome number may be changed. While under normal conditions the somatic cell has a total complement of 2n chromosomes (diploid), it has been observed by Hsu and Pomerat (1953) that in mice the count may vary from n $\pm$ to 16n $\pm$. Liver cells showed the greatest range of deviation, while the skin exhibited a greater degree of aberrations quantitatively. This latter is perhaps due to the fact that the skin is more exposed and thus more liable to be subject to such alterations, due to the action of external factors.

While the normal diploid chromosome number in the mouse is 40, variations in number apart from multiples of the haploid count varied from 39 to 46. This indicates that not only deletions but also fragmentations occurred.

An even greater spread in the diploid number was noted in the guinea pig. Approximately 30% of the somatic cells demonstrated either more or less than normal diploid numbers.

Studying human endometrial cells, Timonen in 1950 found a spectrum of chromosome counts that ranged from 4 to approximately 100. He correlated the frequency of mitosis in tissues to cancer incidence and from this is indicated that the frequency of aneuploidy in cells may be influenced by the frequency of mitosis.

Mitotic Poisoning Action

The history of the study of mitotic inhibition goes back to the work of Fol in 1876, who experimentally induced multipolar mitosis and arrested the movement of the chromosomes by subjecting sea-urchin eggs to low temperatures. In 1887 the Hertwigs showed that this condition may be produced by various toxic agents, such as strychnine and chloral hydrate and by narcotics. Two years later, in 1889, Pernice discovered colchicine mitosis, or c-mitosis as it is commonly called.

A number of excellent reviews may be found in the literature dealing with the problem of mitotic poisons and their possible mechanism of action (Dustin, 1947; Loveless, 1949; Ludford, 1953; and Cowdry, 1955). The term "poisons caryoclasiques" was introduced by Dustin (1929) to designate the great variety of chemical substances which produce extensive nuclear disintegration.

The term "mitotic poison" falsely seems to imply that such substances are of a specific class with a precise function. The term "mitotic poisoning action" is therefore preferable (Ludford, 1953) when we consider the physiological characteristics of these substances.

The name "radiomimetic drugs", so named because of their similarity of action to ionizing radiations, was applied by Politzer (1931) to

certain basic dyes that acted as chromosome poisons. The poisonous action of arsenical derivatives was demonstrated on mitosis in mice by Piton (1929) and on tumors by Dustin and Gregoire (1933). Ammonia can arrest cells in metaphase (Rosenfeld, 1933). In fact, the spindle can be inactivated by heat alone (Lewis, 1933).

Colchicine slows spindle contractions and dissolves the spindle fibers (Inoue, 1952). It changes the fibrous polarized spindle into an amorphous "pseudo-spindle" or "hyaline globule" which is incapable of moving the chromosomes (Gaulden and Carlson, 1951). The prolonged metaphase leads to degenerative nuclear changes, such as short thickened chromosomes.

Phenyl mercuric hydroxide was shown by Macfarlane and Katzberg (1951a) to shorten the distance between the poles of dividing cells due to an increased curvature of the spindle fibers. They also observed (1951b) that tilted spindles indicated the first step in spindle inactivation and that the metaphase-prophase ratio at first decreased and then increased as c-mitosis (spindle inhibition) followed the slowing of prophase. It was further reported by Macfarlane, Katzberg, and Albers (1951) that degrees of mitotic poisoning could be detected in corneal epithelium by variations in concentration and length of time of exposure to mercurial compounds.

Macfarlane and Messing (1953) reported that mercurial compounds may produce chimeras in plants and by producing chromosome breaks may have a mutagenic effect. The source of water used in preparing mercurial solutions was also shown to be a factor on the effectiveness of mercurial toxicity. The loss of resistance by cells to poisoning effect was greater

when Cincinnati water, whose source is the Ohio River, was employed than when Palm Beach, Florida, water was used (Macfarlane, Messing, and Ryan, 1951). Thus a synergistic effect was evident.

Spleen extracts have been shown to produce antimitotic effects in living cells. Diller and Watson (1949) reported that pyknosis, polyploidy, multipolar spindles, and coagulated chromatin were frequent occurrences in cells treated with these extracts. Other bizarre effects were reported by Katzberg, in 1952.

The property of disturbing cell division in some way or other is one which can be exhibited by almost any substance under appropriate conditions.

The wide range of classification of these substances and conditions becomes apparent when review lists are consulted (Murray and Kopech, 1953; Ludford, 1953). Distilled water also exhibits the capacity to affect cell division (Hughes, 1952). Cells in the process of cell division are more sensitive to such poisoning effects than resting cells (Ludford, 1936). This, however, may be due to the fact that internal disturbances are more readily observed in mitosis and are less apparent in resting cells (Ludford, 1953). By inhibition of spindle formation, cells are permitted to begin mitosis, but completion is delayed or arrested in metaphase.

Colchicine was employed to reveal foci of rapid cell division in tissues in which mitoses are otherwise a rare occurrence (Cowdry and Thompson, 1944). There was first a rapid rise of the ratio of mitotic cells to resting cells and then a gradual decline as recovery occurred. There is an agglutination of the chromosomes and vacuolization of the

cytoplasm.

Comparative in vivo and in vitro experiments with toxic substances suggested that the action came as a result of changes in the colloidal state of the cell in mitosis (Ludford, 1936). The frequent occurrence of deranged mitosis in tumors has been attributed to a disorganization of the ultra structure of the protein framework which is directly related to viscosity changes (Powell, 1946).

Cells are adaptable to physical and chemical changes in their environment to a certain degree and so may in part control the action of agents that tend to change their viscosity (von Möllendorff, 1938a). This regulation of viscosity is particularly operative during mitosis when the cell passes from a condition in which water is uniformly distributed through a series of phases where variations occur. The balance between hydration and syneresis is rather delicate and any variations in the rate of one may produce changes in the time required for the various mitotic phases. Since syneresis is involved in spindle formation, any substances such as halides and acetate which encourage hydration processes will prolong metaphase and at the same time hasten anaphase and reconstruction of the nuclei (von Möllendorff, 1938b). When the viscosity regulation mechanism is disturbed, mitosis will result in various derangements, such as aberrant chromosomes, clumping in metaphase, anaphase with incomplete chromosome separation, and telophase without cytoplasmic cleavage.

Another theory was propounded (Levan and Östergren, 1943) in which aberrations due to spindle inhibition are believed to be indicative of a type of narcosis, and arrested mechanisms may recover following the removal of the narcotizing agent. The activity of a compound varies in-

versely with its water solubility and so the decisive concentrations are those in the lipoids of the cell. It has been suggested that compounds associate themselves with the lipophilic side chains of the protein polypeptide chains and alter their capacity to fold (Östergren, 1944b). In this way fibrous protein molecules become more corpuscular and spindles cannot be formed. Furthermore, substances which inhibit spindle formation bring about the contraction of chromosomes (Inoué, 1952).

It has also been theorized that these compounds interfere at some significant point with the normal oxidation reduction systems (Levan and Tjio, 1948) or that this action is brought about by some product arising from their metabolism.

Chromosome fragmentation and stickiness with bridge formations were believed by Goldacre, Loveless, and Ross (1949) to be the result of a cross-linkage of protein fibers brought about by two reactive groups of a molecule combining two adjacent fibers.

Another theory of mitotic disturbances, based on disturbances of the nucleic acid metabolism and their deficiencies incorporated in a chromosome at prophase, was introduced by Darlington and Koller (1947). Trypaflavin, which prevents prophase initiation, is believed by Lettré (1948) to block nucleic acid metabolism.

Among the more unusual factors that have been found to be toxic to cells grown in tissue culture is one which was reported by Fedoroff (1955 and 1956), who found that serum obtained from blood of schizophrenic patients produced certain cytoxic effects on test cells. The toxicity was greater in serum obtained from women that that of serum obtained from men.

It has been reported by Lettré (1954) that the level of activity of a relatively mild antimitotic agent may be elevated by the addition of a small dose of a second agent that acts as a synergist. On the other hand, an agent may depress the action of a strong poison, that is, in its capacity as an antagonist. Thus near-threshold dosages of either compound may greatly augment the total antimitotic activity, when a synergist is employed while an antagonist may offset the effect of a rather large dose of a more active compound.

It has been suggested by Brachet (1946) that the breakdown of adenosine-triphosphate to adenosine diphosphate may produce the energy required in spindle activity. It has also been shown by Lettré (1950) that addition of ATP to a culture medium may inhibit colchicine poisoning. Other compounds that inhibit colchicine poisoning are meso-inositol (Chargaff, Stewart, and Magasenik, 1948) and ascorbic acid (Lettré, 1942).

Lettré and Lettré (1946) demonstrated that trypaflavin and proflavin, which may act as mitotic poisons at concentrations of 2.7 gamma per cc., may be counteracted by the addition of yeast nucleic acid of 1.6 mg. per cc. This, however, is ineffective against colchicine. von Möllendorff (1944) showed that vitamin C may neutralize antimitotic action and that vitamins B_1 and B_6 also are capable of making cells more resistant to this poisoning effect by steroids. Biesele (1955) has recently shown that Coenzyme A can counteract the effect of fragmentation and beading that is induced by 6-mercaptopurine on mitochondria of cells in tissue culture.

Intervention of sulphydryl groups in arsenical mitotic poisoning has been discussed by Hughes (1949). Dimercaptopropanol (Brit. Anti-

Lewisite, which is itself a mitotic poison, has been shown to be capable of reversing mitotic arrest and preventing the pyknotic action of arsenicals (Dustin, 1947).

In considering the action of chemotherapeutic agents in microorganisms, Work and Work (1948) concluded that the disturbance of any one enzymatic reaction by the introduction of a drug or a poison will extend far beyond that single reaction. If the cell survives, an overall adjustment will occur which will be evident in the alteration of metabolic processes, in the growth rate, or in the response to further contact with drugs, and these alterations may be the inductors of mitotic aberrations rather than the direct damage to intracellular mechanism.

Review of Literature on the Effect of Steroidal Compounds on Growth and Mitosis

Numerous studies have been made upon the effect which various naturally occurring steroidal compounds have upon growth of cells in tissue culture and upon the mitotic process.

Sterols

Cholesterol is the most common of steroids and probably forms the basic molecular skeleton for all steroid compounds. It has been shown by von Möllendorff (1942a) and Thomann (1942) to be incapable of affecting the mitotic process. The same holds true for cholestenone, its product of oxidation. Likewise 7-aminocholesterol is inactive as a mitotic poison (Lettré, 1943b).

Sex Hormones

Early studies by Yagi (1937) on the relationship of sex hormones to the growth of various organ tissues of the rabbit indicated that male hormones stimulated spleen and hemopoietic tissues, but inhibited dental pulp and testicular tissue, while female hormones stimulated ovarian and testicular tissue but inhibited dental pulp, spleen, and blood marrow.

In another early study (Haam and Cappel, 1940) on fibroblasts in tissue culture, estrogen affected some growth stimulation with some increase of the mitotic index. At the same time, progesterone greatly reduced the mitotic index and the growth rate, while testosterone showed greatest depression of growth with relatively little decrease in the mitotic index.

Estradiol is the principal member of the estrogens. A concentration of 1:100,000 to 1:1,000,000 produced many mitotic disturbances with numerous metaphase arrests in developing amphibian embryos (Bodenstein, 1954). It is believed to have a pronounced effect on the nucleic acids of cells and the cells may be partially or totally protected by adding RNA to the medium.

Estradiol has been shown to be a mitotic poison to all elements of bone marrow culture. Given in minute doses, it has a leukopoietic effect in animals; only if given in large doses does it have a leukopenic effect (Schlag and Boll, 1951). It may be that the leukopoietic effect is not due to the direct action of the estradiol.

von Möllendorff (1942) has also shown it to act as a mitotic poison on fibroblasts in tissue culture. However, the dosage of estradiol required for its action as a mitotic poison is approximately 30,000 times

that required for its estrogenic effect, the latter being 0.033 gamma per 20 gram mouse (Lettré, 1943a). Disodium estradiol phosphate is somewhat more potent and inhibits growth at a dosage of 60 gamma per cc. This has been verified by Knake (1951), who found a dose of 75 to 100 gamma per cc. to be an effective mitotic poison in vitro.

Estradiol has a limited hemolytic capacity on human type O red blood corpuscles (Tateno and Kilbourne, 1954). No effect was shown by the benzoate form against Ehrlich ascites tumor (Sugiura, 1953).

Estrone appears to be the least effective of the naturally occurring estrogens as a mitotic poison (von Möllendorff, 1942a). It showed only a mild effect against fibroblasts (Lettré, 1943a) and was incapable of producing bridging effects in mitotic cells of Walker rat carcinosarcoma (Schmahl, 1954). Nor could it offer a protective action to tuberculin hypersensitive cells against the purified protein derivatives of tuberculin (Leahy and Morgan, 1952).

Estriol induced a slight inhibition of the mitotic rate at concentrations of 1:50,000 on fibroblasts and also some anaphase and telophase disturbances (von Möllendorff, 1942b).

Equilin and Equilenin are relatively inactive as mitotic poisons. However, their amino compounds show some activity. 7,17-diamino-3-hydroxy-estratrien-1,3,5 produced vacuolization of fibroblasts at a concentration of 3 gamma per cc., and the 7,17-diamino-3-monomethyl-ether-estratrien-1,3,5 acts as a mitotic poison on chick heart fibroblasts at concentrations of 1:1,000,000 (Lettré, 1943b).

Progesterone reduces the mitotic index and has been shown to interfere with chromosome distribution and chromosome division (von Möllen-

dorff, 1942). This effect is produced over a wide range of concentration to a rather moderate degree. Progesterone and all its derivatives were ineffective against growth of Ehrlich ascites carcinoma (Sugiura, 1953).

The androgens have been shown to possess only mild antimitotic activity and rather high concentrations are needed. Mitosis may proceed in spite of some metaphase disturbances.

Methyltestosterone was the most active of this group, while testosterone, the next most active member, was only about one-fourth as active. The antimitotic activity of the male hormones appears to be unrelated to their hormonal potency but is, rather, a side effect (von Möllendorff, 1942).

The relative antimitotic activity of the various androgen compounds present in concentrations of 1:50,000 as indicated by metaphase disturbances was in the following order: methyltestosterone 82%; testosterone 21%; transdihydroandrosterone 20%; 3-trans-17-trans-androstendiol 15.4%; androstendion 13.3%; methyl-dihydrotestosterone 0%; transdihydrotestosterone 0%; transandrosterone 0%; 3-trans-17-trans-androstanediol 0%; androstanedione 0%.

Stilbylamine derivatives are active as antimitotic agents (Lettré and Delitzsch, 1944). 4'methoxystilbylamine is an active mitotic poison for chick heart fibroblasts at a concentration of 5 micrograms per cc. of media. The methoxy group is present in the B-phenyl residue. The stilbylamine group (α,β-diphenylethylamine) is essential if the compound is to act as a mitotic poison. The 3'-4'-dioxyethylene (Lettré and Delitzsch, 1952) is four times as antimitotic as the dioxymethylene derivatives. The antimitotic activity of homologous 4'-alkoxy-stilbylamines varies with the

length of the hydrocarbon chain attached at the oxygen position (Lettre et al, 1952).

When R = CH_3 activity is shown at 4 gamma/cc.

When R = C_2H_5 activity is shown at 0.4-0.5 gamma/cc.

When R = C_3H_7 activity is shown at 0.8 gamma/cc.

When R = C_4H_9 activity is shown at 1.8 gamma/cc.

Also in the C_2H_5derivative, the levoform is 100 times as active as the dextroform.

Bivalent phenols as 4-4'dioxy-α,β-diethylstilben or diethylstilbestrol in concentrations of 0.5 microgram per cc. of medium is an effective antimitotic agent on sea urchin eggs, Paracentrotus lividus (Druckrey, Danneberg, and Schmahl, 1952). This is over 30 times as effective as colchicine. Used in vitro, this compound exhibits a marked specificity as a chemotherapeutic agent against prostatic carcinoma (Druckrey and Rasbe, 1952). Whether it acts via the anterior pituitary or directly on prostatic cells or both is not definitely known.

Diethylstilbestrol (Lettré, 1943a) is also widely employed in the treatment of cancers of the breast. This compound acts as a mitotic poison at concentrations of 100 micrograms per cc. against chick fibroblast, while 150 micrograms per cc. inhibits growth entirely. Ten to 25 micrograms per cc. showed no action.

In regards to cytological effects produced by stilbestrol, it was noted by Thomann (1942) that a concentration of 1:60,000 produced a 500% rise in mitotic figures within five hours. Mitotic mechanism disturbances were initiated in early metaphase with the production of eccentrically and random positioned chromosomes, and individually displaced chromosomes,

as well as clumped chromosome masses. In early telophase, daughter chromosome groups appeared to lie abnormally close together. This continued into late telophase, with abnormal cleavage. Diethylstilbestrol also induces in vitro hemolysis (Tateno and Kilbourne, 1954) of human O red blood corpuscles. This compound also proved very active as a fungistatic agent (Bocobo, Curtis, and Harrell, 1953).

Stilbestrol showed marked effect on development of amphibian embryos (Bodenstein, 1954). There were numerous mitotic disturbances and many metaphase arrests. The effect was at a maximum in the organ showing the greatest proliferation. It is thought that hormones, unlike the mustards, have a marked effect on ribonucleic acid of cells and that cells may be partially or totally protected by adding ribosenucleic acid to the medium about the cells.

When 20 mg. per rat of Na salt of stilbestrol-diphosphate is injected for 7 days into animals innoculated with Walker carcinoma, it produces a chromosome bridging effect in the anaphase and telophase, but it does not influence the tumor growth (Schmahl, 1954). Diethylstilbestrol diproprionate, however, proved ineffective when injected subcutaneously in mice carrying Ehrlich ascites carcinoma (Sugiura, 1953).

Adrenocortical Steroids

11-Dehydrocorticosterone, or Compound A. Heilman (1945a) found that when added to cultures of lymphocytes, Compound A showed little migratory inhibitive effect, even at concentrations of 1:20,000, but when combined with Compound E, it produced marked degeneration of the cells. The migration rate of macrophages cultured from rabbit spleen was stimu-

lated 18 to 31% on the addition of 5.0 to 60.0 gamma per cc. of Compound A (Heilman, 1945b). At the same time there was a moderate inhibition of growth of fibroblasts in one-third of the cultures grown in a medium containing 5.0 gamma per cc. of Compound A. It was also demonstrated by Feldman (1950) to have a moderately rapid injurious effect on all leucocytic types of cells grown in tissue culture.

Administration of 5.0 mg. per day for 7 days in 2- to 3-month old guinea pigs increased the lymphocyte count in blood and it stimulated granulocyte formation in bone marrow (Yoffey, Ancill, Hold, Owen-Smith, and Herdan, 1954).

11-Dehydro-17-hydroxycorticosterone, or Compound E, or Cortisone. A concentration of 1.25 to 50.0 gamma per cc. of Compound E produced significant inhibitions of lymphocytic migration, but this inhibition was not affected by high or low concentration. It also produced considerable degeneration of small lymphocytes (Heilman, 1945a). Stimulation of migration was observed in large wandering cells in cultures of rabbit spleen where 2.5 to 60.0 gamma per cc. induced a 10 to 41% increase in the migratory rate. However, 2.5 gamma per cc. produced a moderate inhibition of fibroblast growth in one-third of the cultures (Heilman, 1945b). It showed no injurious effect on leucocytes (Feldman, 1950). Five mg. per day for 7 days in 2- to 3-month old guinea pigs stimulated red blood cell formation in bone marrow (Yoffey et al, 1954).

Holden, Segal, and Ryby (1951) found that cortisone acetate inhibited fibroblast growth and leucocyte migration when used at a concentration of 4×10^{-6}. Cortisone inhibits the synthesis of chondroitin sulfate by embryonic and wound tissue and suppresses sulfate fixation but

not migration (Layton, 1951). Probably this is in part its action in inhibiting healing of wounds. It has been shown that daily injection of 0.0005 mg. of cortisone acetate inhibits healing by mitotic arrest but does not inhibit migratory activity of cells in traumatized area of Triturus viridescens (Manner, 1955).

The toxicity of various cortisone preparations on embryonic chick tissues varies with the acid radicle attached (Ruskin, Pomerat, and Ruskin, 1951). The range between the least inhibitory dose to total inhibition was 2.35 to 150.0 gamma per cc. for cortisone acetate, 875.0 to 7,000.0 gamma per cc. for cortisone tricarballylate, and 3,750.0 to 15,000.0 per cc. for cortisone sulfate. It exhibited no inhibition of migration of cellular elements of human buffy coat or of migration of chick embryo fibroblasts (Baldridge, Kligman, Lipnik, and Phillsbury, 1951), and it was identical with Compound F in its ability to inhibit the cytotoxic action of purified protein derivative of tuberculin on macrophages of splenic tissue of tubercular guinea pigs (Leahy and Morgan, 1952).

17-Hydroxycorticosterone, or Compound F. When Compound F was administered at a concentration of 100 gamma per cc., it inhibited the cytotoxic action of purified protein derivative of tuberculin on macrophages of splenic tissue of tubercular guinea pigs. Twenty-four hours of pretreatment with Compound F was needed before the addition of PPD (Leahy and Morgan, 1952).

Subcutaneous injection of 37.5 mg. per Kg. per day of the acetate form into tumor-bearing mice showed marked destruction of Ehrlich ascites tumor (Sugiura, 1953).

Desoxycorticosterone (DOC), or Compound Q. Cornman (1951) found that DOC produces damage to fibroblasts. A concentration of 0.02 mg. of DOC per cc. produces visible changes within 16 hours, 0.03 mg. per cc. produces visible changes within 6 to 7 hours. 0.1 mg. per ml. did not produce permanent damage to endothelial cells in 25 hours and recovery followed. Fibroblasts did not recover, but lost their smooth contours and became granular and narrowed to one-half to one-third their width. If cortisone was added in a concentration of 0.05 to 0.15 mg. per ml. to DOC solution, it acted as a synergist and accentuated the effect.

Concentrations of 1:20,000 to 1:500,000 of DOC increases the number of chromosome lags and displacements in cultures of rabbit fibroblasts (von Möllendorff, 1942) without mitotic inhibition. Concentrations of 1:10,000 to 1:20,000 inhibit mitosis. While the rate is inhibited, the mitotic disturbances are not dependent upon concentration. DOC showed no effect on lymphocytes in the intact animal (Feldman, 1950).

Like diethylstilbestrol, DOC acetate induces in vitro hemolysis of human O red blood cells (Tateno and Kilbourne, 1954). However, it showed no effect when 500.0 mg. were injected into animals with Ehrlich ascites carcinoma (Sugiura, 1953).

DOC glycoside showed no protection against the cytotoxic action of purified protein derivative of tuberculin on macrophages of splenic tissues of tuberculous guinea pigs (Leahy and Morgan, 1952).

Selection of Solvent for Steroids

Since steroidal compounds are rather difficult to dissolve in an aqueous medium, solvents of various types may be employed and numerous

references to these may be found in the literature.

It is to be cautioned that in experiments in which steroid effects on mitosis are being studied, a solvent should be selected which will have a minimum effect on cell division, so that the possibility of a false reading will be reduced to a minimum. The degree of this activity varies with the type of solvent.

It has been shown by Rosenfeld and Weinberg (1932) that a 0.3 to 0.4 molal solution of ether or a 0.01 to 0.02 molal solution of chloroform will produce nuclear changes in one to three minutes. Ether has also been demonstrated (Rosenfeld, 1931) to produce a reversible liquification of cytoplasm during mitosis. There is a reversal of mitosis from anaphase and metaphase with a possible production of a tetraploid nucleus. Binucleated cells were also produced. Ether has been shown to cause metaphase prolongation even when a spindle is present (Kemp and Jinel, 1930), and also faulty separation of chromosomes (Haecker, 1900). Ethylene glycol also produces faulty separation (Östergren, 1944a).

Ethyl alcohol (Ladell, 1938) in low concentrations may actually stimulate the growth of explants. A concentration of 2% may produce initial inhibition of growth followed by stimulation.

Benzene, which is toxic to bone marrow in the intact animal, showed no effect on bone marrow cultures (Harrison and Randall, 1947). Its toxic effect in intact animals is reported as being due to its oxidation to a dihydric phenol, which is the toxic agent.

Acetone has been frequently employed, but it also shows some degree of injurious and inhibitory effect on cultures, and this becomes rapidly accentuated if the concentration of acetone rises above 2.0%

Ruskin, Pomerat, and Ruskin, 1951; von Möllendorff, 1942).

Perhaps the least toxic vehicle for these purposes and which is an excellent solvent for steroids and at the same time readily miscible in aqueous solution is propylene glycol (Pomerat, 1953). It can comprise as much as 15% of the culture media before it reaches total inhibitory concentrations.

CHAPTER III

METHODS OF PROCEDURE

In studying the action of a chemical compound such as a drug or hormone on the growth of a tissue, it has been customary to use an intact animal, especially in the final stages of investigation. However, tissue culture offers certain advantages in that the individual intact cell may be studied and the mechanism of action of any specific compound on the life processes of the cell may be determined, at least to a degree, by observations made on the cell following exposure to such compounds. Another advantage of employing tissue culture methods is that results may be obtained within a relatively short period of 24 to 48 hours. Tissue culture is rapidly becoming an important tool in numerous fields of biological investigation, in bioassay and chemotherapy, and in various fundamental studies. This method lends itself readily to studies on cellular growth and mitosis and it has been employed frequently in determining the mechanism of action of various compounds on the stimulation or inhibition of cell division. The information derived has found further application in our understanding of such processes as wound healing and inhibition and arrest of neoplasms.

Preparation of Cultures

Standard tissue culture procedures were employed in the course of

these investigations. Hanging drop cultures were prepared, using explants of chick embryo hearts of 8 to 10 days' incubation. The culture media employed were fluid in nature and were prepared by grinding up the embryos and mixing one part by volume of the brei obtained to two parts by volume of Tyrode's solution.

The steroidal compounds were dissolved in propylene glycol so that 1 cc. of the original stock solution would contain 10 mg. of the compound. While some compounds dissolved quite readily, others were stored in an incubator overnight to facilitate their solution. Further dilutions of the stock solution were prepared with propylene glycol in the following way: 0.1 cc. of propylene glycol was placed in each of a series of sterile and chemically clean screw top vials, using a 1.0 cc. tuberculin syringe. The vials were numbered so that identification was possible at a later date. By use of a 0.25 cc. tuberculin syringe with a one inch 21 gauge needle, the stock solution was thoroughly mixed by drawing it into the syringe and forcing it out again 20 times. This was done to distribute the concentration of the dissolved steroid as uniformly as possible throughout the solution. It was then transferred to vial No. 1. Using a fresh 0.25 cc. syringe, 0.1 cc. was drawn up and transferred to vial No. 2, containing 0.9 cc. propylene glycol. This was mixed in the same fashion as before and, with a clean syringe, further dilutions were made until concentrations of the steroid in the solution contained in the various vials equalled:

Stock solution #1 - 10.0 mg. per cc. propylene glycol

Stock solution #2 - 1.0 mg. per cc. propylene glycol

Stock solution #3 - 0.1 mg. per cc. propylene glycol

Stock solution #4 - 0.010 mg. per cc. propylene glycol

Stock solution #5 - 0.001 mg. per cc. propylene glycol

A series of sterile Kahn serological test tubes were prepared to receive the culture media containing the various dilutions of the steroid. Using graduated hematological pipettes, dilutions were made so that each concentration of the steroid solution was diluted 100 times with embryo extract. These dilutions were placed in the marked Kahn tubes and thoroughly mixed by pumping up and down with a syringe as before.

The following culture media variants were prepared:

No. 1 - plain embryo extract

No. 2 - embryo extract with 1% propylene glycol

No. 3 - embryo extract with 1% stock solution #1

No. 4 - embryo extract with 1% stock solution #2

No. 5 - embryo extract with 1% stock solution #3

No. 6 - embryo extract with 1% stock solution #4

No. 7 - embryo extract with 1% stock solution #5

The weight of the steroid in medias No. 3 to 7 was 0.001; 0.0001; 0.000001; 0.0000001; and 0.00000001 mg. per ml. respectively.

Some comparative studies were made using the well-known antimitotic compound colchicine. Dilutions were made to 1:100,000,000. This colchicine was obtained from General Biological Supply House, Chicago, as Colchicine Alkaloid U.S.P.

Two types of propylene glycol were employed. A less purified type was employed in the earlier screening studies. In the later and more detailed cytological studies a chemically pure brand was used.

Two types of studies were made. In the first series the relative

potency of the compound was determined. Ten cultures were prepared of each dilution media and incubated for 48 hours and fixed. Cultures in both series were always prepared in the afternoon hours from 2 to 6 p.m. and fixation on termination of the growth period was also carried out during this same time period.

Methods of Fixation and Staining

Fixation and staining of the cultures was done by several techniques:

(1) Fixation in Bouin's solution followed by Iron Hematoxylin stain,

(2) Bouin's and hematoxylin-eosin method,

(3) Aceto-orcein method, and

(4) Special methods for cytological studies

  (a) Geither Technique

  (b) Kedrovsky Technique.

Measurements

The explants were made as uniformly in size as possible, and circumferential tracings were prepared of the original explant. Tracings were also made of the outgrowth at the termination of each experiment by using micro-projection, and such magnification that 1 mm. on the culture would be equal to 1 inch on the tracing.

Measurements of the tracings of the explant and the outgrowth were made with a planimeter that recorded areas in square inches. By substituting the word "millimeter" for "inches", the actual area of the culture in square millimeters was automatically obtained.

Comparative ratios were calculated for each of the series and the growth index obtained by comparing the ratios of the experimental groups with that of the control.

This technique of measurement, first employed by Ebeling (1921), is frequently used. To obtain the comparative growth ratio (R) for a series, the total outgrowth area (G) was divided by the total explant area (E) so that

$$\frac{\text{Area of Total outgrowth}}{\text{Area of Explant}} = \text{Comparative growth ratio}$$

or

$$\frac{G}{E} = R$$

R obtained for the control series was designated as Rc. That of propylene glycol control was Rp. Those of the experimental series were designated as R1 for the group containing a steroid concentration of 10^{-4}, R2 for the group containing a steroid concentration of 10^{-5}, R3 for the group containing a steroid concentration of 10^{-6}, R4 for the group containing a concentration of 10^{-7}, and so on.

The relative growth index for each group was obtained by dividing the value obtained for each of the ratios by the ratio of the control. Thus

$$\frac{Rc}{Rc} = Ic = 1.00,$$

The growth index for the control group is always equal to unity.

$\frac{R1}{Rc} = I_1$, the growth index for the steroid concentration of 10^{-4}

$\frac{R2}{Rc} = I_2$, the growth index for the steroid concentration of 10^{-5}

and so on.

The various values for I were then graphed.

In the second series of studies involving cytological studies, cultures were prepared in the usual manner with the exception that only undiluted embryo extract was employed in the media during the first 24 hours. After permitting the cultures to grow for this period, a good zone of proliferation containing many dividing cells was obtained. The cultures were then unsealed and the old medium was rinsed off with Tyrode's solution and replaced with fresh media containing dilutions of the steroidal compounds in fresh embryo extract, such as were employed in the first series. Three to five cultures were prepared with each dilution. These cultures were then resealed and incubated for another 24 hours.

After mounting the cultures as permanent preparations, cell counts were made to establish a ratio between

(1) resting cells and mitotic cells

(2) various phases of mitosis

(3) normal mitoses

(4) abnormal mitoses.

From these data the mitotic index was calculated by dividing the values of the mitotic counts into the total cell count, and it was possible to determine the frequency of the incidence of

(a) normal mitoses

(b) abnormal mitoses

(c) total mitoses.

The following compounds were studied in Series I:

(a) using the standard grade of propylene glycol as solvent:

1. Equilenin
2. 17-Dihydroequilenin-17B
3. 3-Methylequilenin
4. 16-Keto-equilenin
5. Estradiol
6. Estradiol-3,16a
7. Estradiol-3,16B
8. Epi-estradiol (B-estradiol)
9. 3-Methyl-16-keto-estradiol
10. 16-Keto-estradiol
11. Estrone
12. Estrone-16
13. 3-Methylestrone-16
14. 3-Methylestrone-17
15. 16-Keto-estrone
16. Estriol
17. Estriol glucoronide-Na
18. Diethylstilbestrol

(b) using chemically pure propylene glycol as solvent:

1. Colchicine
2. Diethylstilbestrol
3. 16-Keto-estradiol
4. 3-Methylestradiol-3,16a
5. 3-Esthylestradiol-3,16B

6. 3-Methyl-16-keto-estradiol
7. 3-Ethyl-16-keto-estradiol
8. 16-Keto-estrone
9. 3-Ethylestrone-16
10. 3-Methylestrone-17
11. 3-Ethylestrone-17
12. 3-Methyl-16-keto-estrone
13. 3-Ethyl-16-keto-estrone

In Series II differential mitotic counts were made of cells treated for 24 hours with the following compounds:

1. Colchicine
2. Diethylstilbestrol
3. 16-Keto-estradiol
4. 3-Methylestradiol-3,16a
5. 3-Methyl-16-keto-estradiol
6. 3-Ethyl-16-keto-estradiol
7. 16-Keto-estrone
8. 3-Methyl-16-keto-estrone

Control studies were also made on the propylene glycol solvents employed, both old unrefined and new chemically pure.

A great variety of methods have been employed to ascertain the influence of various substances on the growth and mitotic activity of cells in tissue culture. Some of these are crude, others well refined.

One method frequently employed (Haam and Cappel, 1940) is to prepare scaled tracings of the explant and zone of outgrowth on uniformly thick paper by means of a camera ludica. These tracings are then cut out

and weighed. Another method is to obtain the average radial outgrowth (Heilman, 1945) by averaging the distance to which proliferation occurred around the periphery.

Growth rate may be determined in mass cultures of cells suspended in fluid medium (Earle, Bryant, and Schilling, 1954) by obtaining the wet weight of cells cultured for various periods of time in various modified media. Another method consisted of counting 8 fields of cells under high dry magnification of the outgrowth of cells and enumerating the total number of cells and all mitotic and pyknotic figures (Plummer, Wright, Antikajian, and Weintraub, 1952). Quantitative determination of the desoxyribose nucleic acids (DNA) in mass cultures is probably the most accurate method by which growth increase may be determined by chemical means (Block and Godman, 1955), as interphase cells contain a constant amount of DNA.

For actual study of mitotic processes and the time involved, the best method is the use of cinematography with time-lapse motion pictures. By this method (von Möllendorff, 1942) it is possible to obtain the mitotic index as well as the time required for the various phases of mitosis.

CHAPTER IV

OBSERVATIONS

In Tables 1 to 38 may be seen the statistical analysis of the data on the growth of cultures as obtained by measurement with a planimeter. The first column of figures represents the area of the original explanted pieces of tissue. The next column represents the area of the actual growth. The third column represents the ratio (R) of the area of growth (G) to the area of the original explant (E) and is obtained by the division of the first value by the second, so that

$$R = \frac{G}{E}$$

The last column represents the growth index (I) and is obtained by taking the values of the growth ratios (R) obtained for the various experimental dilutions and making a comparison with the value obtained for the control. Thus, by dividing the value of the experimental ratio (Rx) by that of the control (Rc), we obtain the growth index (I); i.e.,

$$\frac{Rx}{Rc} = I.$$

By this method of calculation, the value of the growth index (I) for the controls will always be equal to unity, while the values of the various experimental groups will be greater or less than unity, depending on whether stimulation or inhibition of growth occurred. The information

below the statistical data is an approximation of the sizes of the dosage of the compound needed to produce

(a) detectable inhibition

(b) 50% inhibition

(c) total inhibition.

The values are stated as micrograms per milliliter of medium and they were obtained by the application of slide rule calculations to the various values of I. Thus, in Table 1, the least inhibitory dosage was more than 1:10,000,000, or 0.1 microgram per ml. of medium. Fifty percent inhibition was produced by a concentration between 1:1,000,000 and 1:10,000,000; with the aid of a sliding scale this value is approximately 0.7 microgram per ml. of medium. Finally, the dosage required to produce total inhibition was 1:100,000, or 10.0 micrograms per ml. of medium.

Statistical analysis revealed that the cultures treated with the various equilenin and estradiol compounds (Tables 1 to 10) showed inhibition at the higher concentrations and frequently some stimulation at the lower concentrations.

The estrone compounds (Tables 11 to 15) displayed more persistent inhibitory action even at the greater dilutions. The greatest inhibitory action was observed in cultures treated with 3-methylestrone-17, where concentrations of 0.01 micrograms per ml. of medium produced more than 50% inhibition.

This compared well with the action of diethylstilbestrol (Table 18,) where 50% inhibition was produced by a dose of 0.1 microgram per ml. of medium.

The estriol compounds (Tables 16 and 17) showed no inhibitory

action, but rather were stimulatory.

The Effect of Propylene Glycol on Proliferation of Cells

The persistent total inhibition of growth in the first group of experiments, in which a standard grade of propylene glycol was employed, contrasted with the absence of this action in the later experiments where a chemically pure brand was used, led to comparative studies on the action of these two grades of solvents alone. The data of these experiments may be found in Tables 19 to 25.

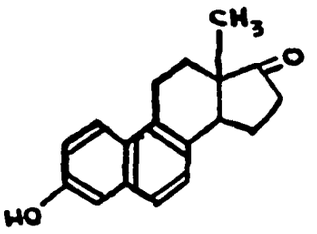
In the first experiments, represented by Tables 1 to 22, the steroidal compounds were dissolved in standard grade propylene glycol. It may be noted that almost invariably no growth occurred where the concentration of the steroid was 1:10,000 and frequently none occurred at 1:100,000.

In contrast to this, the experiments represented by Tables 23 and 24 and Tables 26 to 38, all show some growth even at the highest concentration of 1:10,000. The propylene glycol employed in this later group was chemically pure. The persistent inhibition observed in the first group is indicative of synergistic action affected by impurities in the propylene glycol of standard grade.

Control experiments employing propylene glycol alone showed considerable inhibition in the higher concentrations. This effect also appeared to increase with the age of the propylene glycol. The first control experiment showed a reduction of about 26.0% in cultures containing 1.0% propylene glycol, while similar control experiments run at later

(text continued on page 64)

EQUILENIN



THE STRUCTURAL FORMULA OF EQUILENIN

TABLE 1

THE ACTION OF EQUILENIN ON THE OUTGROWTH OF EMBRYONIC CHICK HEART FIBROBLASTS IN TISSUE CULTURE

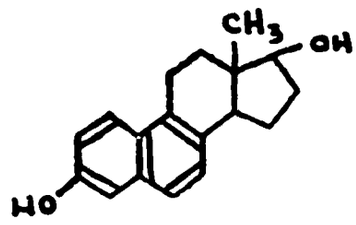
	Explant Area (E)	Growth Area (G)	Growth Ratio (R) $R = \frac{G}{E}$	Growth Index (I) $I = \frac{Rx}{Rc}$
Control	20.50	33.78	1.65	1.00
Propylene Glycol	11.57	10.06	0.87	0.53
10^{-4}		0.00		
10^{-5}		0.00		
10^{-6}	7.54	2.22	0.29	0.17
10^{-7}	9.24	25.28	2.76	1.67
10^{-8}	8.51	11.68	1.37	0.83

Least inhibitory dose 0.1 microgram per ml.

Half-lethal dose 0.7 microgram per ml.

Lethal dose 10.0 micrograms per ml.

17-DIHYDROEQUILENIN-17B



THE STRUCTURAL FORMULA OF
17-DIHYDROEQUILENIN-17B

TABLE 2

THE ACTION OF 17-DIHYDROEQUILENIN-17B ON THE OUTGROWTH OF EMBRYONIC CHICK HEART FIBROBLASTS IN TISSUE CULTURE

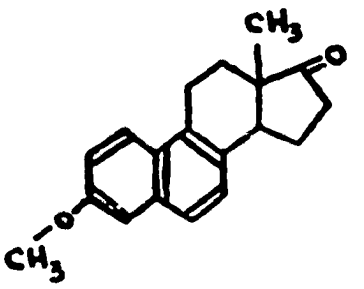
	Explant Area (E)	Growth Area (G)	Growth Ratio (R) $R = \frac{G}{E}$	Growth Index (I) $I = \frac{Rx}{Rc}$
Control	28.23	79.66	2.82	1.00
Propylene Glycol	7.67	13.58	1.77	0.63
10^{-4}		0.00		
10^{-5}	7.31	8.27	1.13	0.40
10^{-6}	11.28	59.02	5.23	1.85
10^{-7}	12.42	42.68	3.44	1.22

Least inhibitory dose < 1.0 microgram per ml.

Half-lethal dose 7.0 micrograms per ml.

Lethal dose 100.0 micrograms per ml.

3-METHYLEQUILENIN



THE STRUCTURAL FORMULA OF 3-METHYLEQUILENIN

TABLE 3

THE ACTION OF 3-METHYLEQUILENIN ON THE OUTGROWTH OF EMBRYONIC CHICK HEART FIBROBLASTS IN TISSUE CULTURE

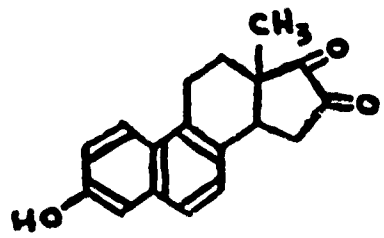
	Explant Area (E)	Growth Area (G)	Growth Ratio (R) $R = \frac{G}{E}$	Growth Index (I) $I = \frac{Rx}{Rc}$
Control	20.50	33.78	1.65	1.00
Propylene Glycol	11.57	10.06	0.87	0.53
10^{-4}		0.00		
10^{-5}		0.00		
10^{-6}	7.51	2.56	0.34	0.21
10^{-7}	6.18	8.31	1.34	0.81
10^{-8}	6.30	10.58	1.68	1.20

Least inhibitory dose 0.1 microgram per ml.

Half-lethal dose 0.5 microgram per ml.

Lethal dose 10.0 micrograms per ml.

16-KETO-EQUILENIN



THE STRUCTURAL FORMULA OF 16-KETO-EQUILENIN

TABLE 4

THE ACTION OF 16-KETO-EQUILENIN ON THE OUTGROWTH OF EMBRYONIC CHICK HEART FIBROBLASTS IN TISSUE CULTURE

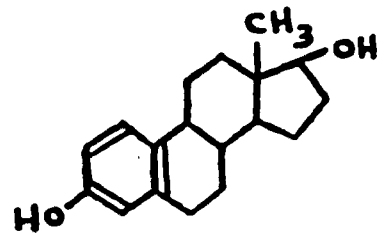
	Explant Area (E)	Growth Area (G)	Growth Ratio (R) $R = \frac{G}{E}$	Growth Index (I) $I = \frac{Rx}{Rc}$
Control	6.60	18.20	2.76	1.00
Propylene Glycol	8.32	19.05	2.29	0.83
10^{-4}		0.00		
10^{-5}	7.58	14.23	1.88	0.68
10^{-6}	7.33	22.10	3.01	1.09
10^{-7}	7.39	29.66	4.01	1.77

Least inhibitory dose 1.0 microgram per ml.

Half-lethal dose 25.0 micrograms per ml.

Lethal dose 100.0 micrograms per ml.

ESTRADIOL



THE STRUCTURAL FORMULA OF ESTRADIOL

TABLE 5

THE ACTION OF ESTRADIOL ON THE OUTGROWTH OF EMBRYONIC CHICK HEART FIBROBLASTS IN TISSUE CULTURE

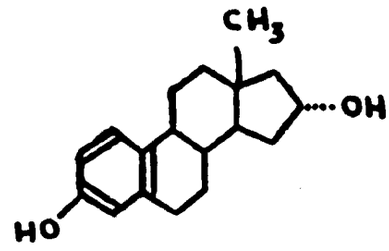
	Explant Area (E)	Growth Area (G)	Growth Ratio (R) $R = \frac{G}{E}$	Growth Index (I) $I = \frac{Rx}{Rc}$
Control	14.40	44.90	3.12	1.00
Propylene Glycol	8.32	18.13	2.18	0.70
10^{-4}		0.00		
10^{-5}	11.21	5.99	0.53	0.17
10^{-6}	13.15	42.86	3.26	1.04
10^{-7}	7.60	19.85	2.61	0.83

Least inhibitory dose < 1.0 microgram per ml.

Half-lethal dose 6.0 micrograms per ml.

Lethal dose 100.0 micrograms per ml.

ESTRADIOL-3,16a



THE STRUCTURAL FORMULA OF ESTRADIOL-3,16a

TABLE 6

THE ACTION OF ESTRADIOL-3,16a ON THE OUTGROWTH OF EMBRYONIC CHICK HEART FIBROBLASTS IN TISSUE CULTURE

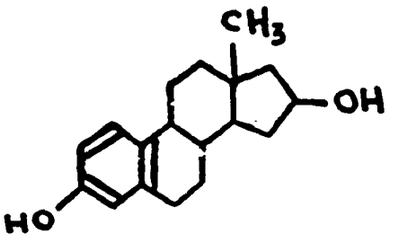
	Explant Area (E)	Growth Area (G)	Growth Ratio (R) $R = \frac{G}{E}$	Growth Index (I) $I = \frac{Rx}{Rc}$
Control	12.47	24.26	1.95	1.00
Propylene Glycol	8.02	11.06	1.38	0.71
10^{-3}		0.00		
$5x10^{-3}$	7.06	2.79	0.39	0.20
$25x10^{-3}$	6.56	1.52	0.23	0.11
$125x10^{-3}$	10.34	19.35	1.87	0.95
$625x10^{-3}$	6.85	25.56	3.73	1.91
$3,125x10^{-3}$	8.44	13.84	1.64	0.84
$15,625x10^{-3}$	7.70	9.66	1.25	0.64

Least inhibitory dose 8.0 micrograms per ml.

Half-lethal dose 78.0 micrograms per ml.

Lethal dose 1000.0 micrograms per ml.

ESTRADIOL-3,16B



THE STRUCTURAL FORMULA OF ESTRADIOL-3,16B

TABLE 7

THE ACTION OF ESTRADIOL-3,16B ON THE OUTGROWTH OF EMBRYONIC CHICK HEART FIBROBLASTS IN TISSUE CULTURE

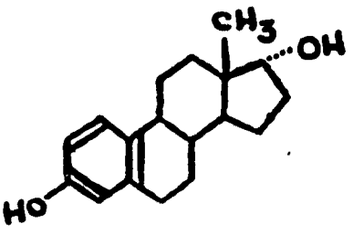
	Explant Area (E)	Growth Area (G)	Growth Ratio (R) $R = \frac{G}{E}$	Growth Index (I) $I = \frac{Rx}{Rc}$
Control	11.72	41.82	3.50	1.00
Propylene Glycol	8.26	11.97	1.45	0.41
10^{-4}		0.00		
10^{-5}	16.25	4.33	0.26	0.07
10^{-6}	16.72	87.00	5.20	1.40
10^{-7}	12.90	29.44	2.30	0.65

Least inhibitory dose 0.1 microgram per ml.

Half-lethal dose 6.0 - 7.0 micrograms per ml.

Lethal dose 10.0 micrograms per ml.

EPI-ESTRADIOL (B-ESTRADIOL)



THE STRUCTURAL FORMULA OF
EPI-ESTRADIOL (B-ESTRADIOL)

TABLE 8

THE ACTION OF EPI-ESTRADIOL (B-ESTRADIOL) ON THE OUTGROWTH OF EMBRYONIC CHICK HEART FIBROBLASTS IN TISSUE CULTURE

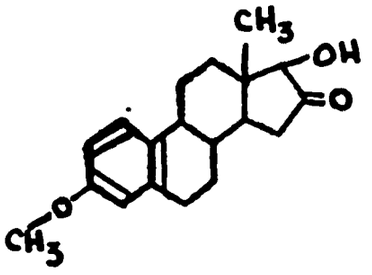
	Explant Area (E)	Growth Area (G)	Growth Ratio (R) $R = \frac{G}{E}$	Growth Index (I) $I = \frac{Rx}{Rc}$
Control	13.60	10.60	0.75	1.00
Propylene Glycol	9.03	4.96	0.55	0.74
10^{-4}		0.00		
10^{-5}	9.70	5.60	0.58	0.77
10^{-6}	32.90	17.70	0.54	0.72
10^{-7}	23.50	18.20	0.77	1.03

Least inhibitory dose 1.0 microgram per ml.

Half-lethal dose 35.0 micrograms per ml.

Lethal dose 100.0 micrograms per ml.

3-METHYL-16-KETO-ESTRADIOL



THE STRUCTURAL FORMULA OF
3-METHYL-16-KETO-ESTRADIOL

TABLE 9

THE ACTION OF 3-METHYL-16-KETO-ESTRADIOL ON THE OUTGROWTH OF EMBRYONIC CHICK HEART FIBROBLASTS IN TISSUE CULTURE

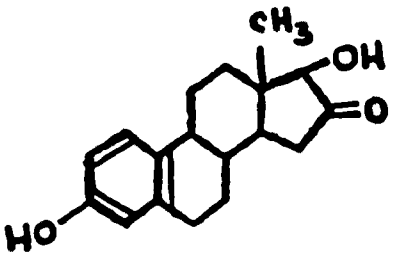
	Explant Area (E)	Growth Area (G)	Growth Ratio (R) $\frac{G}{E}$	Growth Index (I) $\frac{Rx}{Rc}$
Control	28.23	79.66	2.82	1.00
Propylene Glycol	7.67	13.40	1.77	0.63
10^{-4}		0.00		
10^{-5}	7.34	8.71	1.19	0.42
10^{-6}	9.32	38.00	4.08	1.44
10^{-7}	8.38	44.98	5.37	1.90

Least inhibitory dose $<$ 1.0 microgram per ml.

Half-lethal dose 9.0 micrograms per ml.

Lethal dose 100.0 micrograms per ml.

16-KETO-ESTRADIOL



THE STRUCTURAL FORMULA OF 16-KETO-ESTRADIOL

TABLE 10

THE ACTION OF 16-KETO-ESTRADIOL ON THE OUTGROWTH OF EMBRYONIC CHICK HEART FIBROBLASTS IN TISSUE CULTURE

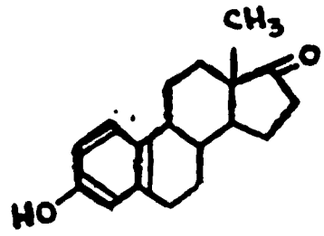
	Explant Area (E)	Growth Area (G)	Growth Ratio (R) $R = \frac{G}{E}$	Growth Index (I) $I = \frac{Rx}{Rc}$
Control	6.60	18.20	2.76	1.00
Propylene Glycol	8.32	19.05	2.29	0.83
10^{-4}		0.00		
10^{-5}	5.56	0.56	0.10	0.037
10^{-6}	7.71	2.25	0.29	0.106
10^{-7}	6.21	20.36	3.28	1.19

Least inhibitory dose $<$ 0.1 microgram per ml.

Half-lethal dose $>$ 1.0 microgram per ml.

Lethal dose 100.0 micrograms per ml.

ESTRONE



THE STRUCTURAL FORMULA OF ESTRONE

TABLE 11

THE ACTION OF ESTRONE ON THE OUTGROWTH OF EMBRYONIC CHICK HEART FIBROBLASTS IN TISSUE CULTURE

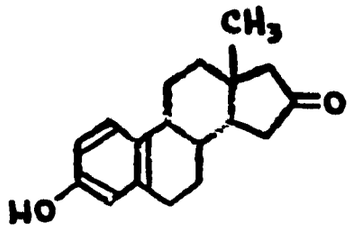
	Explant Area (E)	Growth Area (G)	Growth Ratio (R) $R = \frac{G}{E}$	Growth Index (I) $I = \frac{Rx}{Rc}$
Control	10.92	28.51	2.61	1.00
Propylene Glycol	8.94	13.52	1.59	0.60
10^{-4}		0.00		
10^{-5}	10.22	28.35	2.02	0.77
10^{-6}	9.90	26.30	2.65	1.02
10^{-7}	8.00	17.62	2.20	0.84

Least inhibitory dose $<$ 1.0 microgram per ml.

Half-lethal dose 12.0 micrograms per ml.

Lethal dose 100.0 micrograms per ml.

ESTRONE-16



THE STRUCTURAL FORMULA OF ESTRONE-16

TABLE 12

THE ACTION OF ESTRONE-16 ON THE OUTGROWTH OF EMBRYONIC CHICK HEART FIBROBLASTS IN TISSUE CULTURE

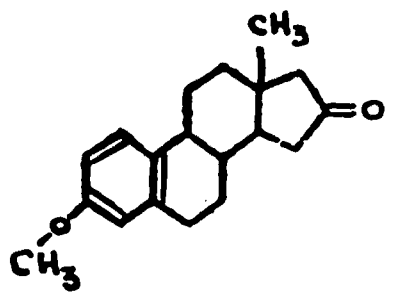
	Explant Area (E)	Growth Area (G)	Growth Ratio (R) $R = \frac{G}{E}$	Growth Index (I) $I = \frac{Rx}{Rc}$
Control	36.32	161.28	4.44	1.00
Propylene Glycol	8.45	25.85	3.06	0.69
10^{-4}		0.00		
10^{-5}	6.21	1.63	0.26	0.06
10^{-6}	11.74	36.10	3.08	0.69
10^{-7}	6.36	17.62	2.79	0.63

Least inhibitory dose > 1.0 microgram per ml.

Half-lethal dose 30.0 micrograms per ml.

Lethal dose 100.0 micrograms per ml.

3-METHYLESTRONE-16



THE STRUCTURAL FORMULA OF 3-METHYLESTRONE-16

TABLE 13

THE ACTION OF 3-METHYLESTRONE-16 ON THE OUTGROWTH OF EMBRYONIC CHICK HEART FIBROBLASTS IN TISSUE CULTURE

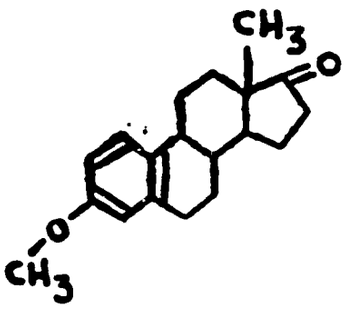
	Explant Area (E)	Growth Area (G)	Growth Ratio (R) $R = \frac{G}{E}$	Growth Index (I) $I = \frac{Rx}{Rc}$
Control	36.32	161.28	4.44	1.00
Propylene Glycol	8.45	25.85	3.06	0.69
10^{-4}		0.00		
10^{-5}	8.24	36.30	4.40	0.99
10^{-6}	8.30	34.98	4.21	0.95
10^{-7}	7.58	27.46	3.67	0.82

Least inhibitory dose 10.0 micrograms per ml.

Half-lethal dose 50.0 micrograms per ml.

Lethal dose 100 .0 micrograms per ml.

3-METHYLESTRONE-17



THE STRUCTURAL FORMULA OF 3-METHYLESTRONE-17

TABLE 14

THE ACTION OF 3-METHYLESTRONE-17 ON THE OUTGROWTH OF EMBRYONIC CHICK HEART FIBROBLASTS IN TISSUE CULTURE

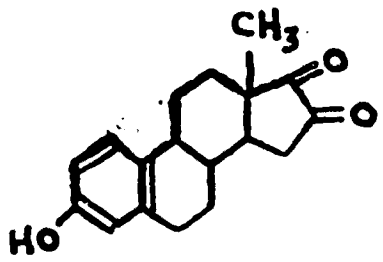
	Explant Area (E)	Growth Area (G)	Growth Ratio (R) $R = \frac{G}{E}$	Growth Index (I) $I = \frac{Rx}{Rc}$
Control	20.50	33.78	1.65	1.00
Propylene Glycol	11.57	10.06	0.87	0.53
10^{-4}		0.00		
10^{-5}		0.00		
10^{-6}	4.62	0.35	0.08	0.05
10^{-7}	7.58	2.32	0.31	0.19
10^{-8}	10.68	6.06	0.57	0.34

Least inhibitory dose >0.1 microgram per ml.

Half-lethal dose 0.1 microgram per ml.

Lethal dose 10.0 micrograms per ml.

16-KETO-ESTRONE



THE STRUCTURAL FORMULA OF 16-KETO-ESTRONE

TABLE 15

THE ACTION OF 16-KETO-ESTRONE ON THE OUTGROWTH OF EMBRYONIC CHICK HEART FIBROBLASTS IN TISSUE CULTURE

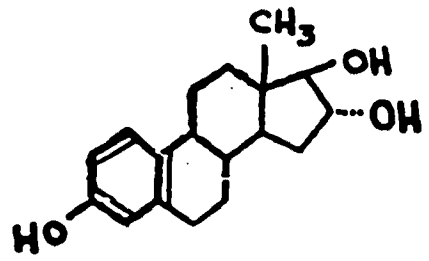
	Explant Area (E)	Growth Area (G)	Growth Ratio (R) $R = \frac{G}{E}$	Growth Index (I) $I = \frac{Rx}{Rc}$
Control	26.01	107.83	4.14	1.00
Propylene Glycol	8.21	27.83	3.39	0.82
10^{-4}		0.00		
10^{-5}		0.00		
10^{-6}	7.84	11.17	1.43	0.34
10^{-7}	9.82	32.29	3.29	0.88

Least inhibitory dose >0.1 microgram per ml.

Half-lethal dose 0.7 microgram per ml.

Lethal dose 10.0 micrograms per ml.

ESTRIOL



THE STRUCTURAL FORMULA OF ESTRIOL

TABLE 16

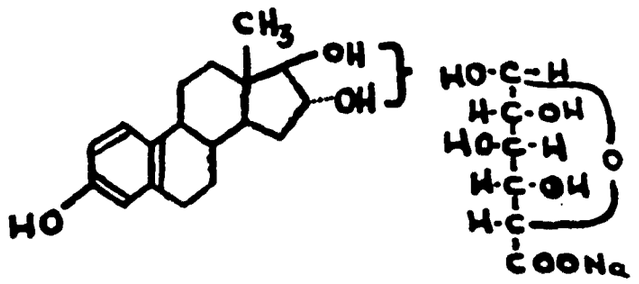
THE ACTION OF ESTRIOL ON THE OUTGROWTH OF EMBRYONIC CHICK HEART FIBROBLASTS IN TISSUE CULTURE

	Explant Area (E)	Growth Area (G)	Growth Ratio (R) $R = \frac{G}{E}$	Growth Index (I) $I = \frac{Rx}{Rc}$
Control	14.02	14.59	1.04	1.00
Propylene Glycol	13.76	10.13	0.73	0.70
1/10,000	10.65	17.90	1.68	1.60
1/50,000	11.56	54.34	4.70	4.50
1/250,000	15.21	40.44	2.66	2.56
1/1,250,000	11.16	19.09	1.71	1.65
1/6,250,000	15.52	24.22	1.57	1.51

Least inhibitory dose < 100.0 micrograms per ml.

Lethal dose < 100.0 micrograms per ml.

ESTRIOL GLUCURONIDE-Na



THE STRUCTURAL FORMULA OF ESTRIOL GLUCURONIDE-Na

TABLE 17

THE ACTION OF ESTRIOL GLUCURONIDE-Na ON THE OUTGROWTH OF EMBRYONIC CHICK HEART FIBROBLASTS IN TISSUE CULTURE

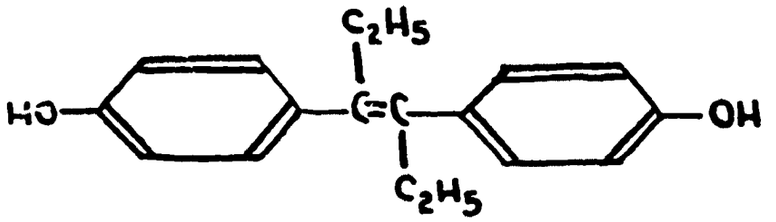
	Explant Area (E)	Growth Area (G)	Growth Ratio (R) $R = \frac{G}{E}$	Growth Index (I) $I = \frac{Rx}{Rc}$
Control	9.46	32.54	3.40	1.00
Propylene Glycol	8.19	21.30	2.60	0.78
10^{-4}		0.00		
10^{-5}	9.45	33.35	3.50	1.03
10^{-6}	13.42	79.18	5.20	1.50
10^{-7}	11.74	77.50	6.50	1.91

Least inhibitory dose 8.0 micrograms per ml.

Half-lethal dose 52.0 micrograms per ml.

Lethal dose 100.0 micrograms per ml.

DIETHYLSTILBESTROL



THE STRUCTURAL FORMULA OF DIETHYLSTILBESTROL

TABLE 18

THE ACTION OF DIETHYLSTILBESTROL ON THE OUTGROWTH OF EMBRYONIC CHICK HEART FIBROBLASTS IN TISSUE CULTURE

	Explant Area (E)	Growth Area (G)	Growth Ratio (R) $R = \frac{G}{E}$	Growth Index (I) $I = \frac{Rx}{Rc}$
Control	6.50	18.20	2.76	1.00
Propylene Glycol	8.32	19.05	2.29	0.83
10^{-4}		0.00		
10^{-5}		0.00		
10^{-6}	10.91	6.76	0.62	0.22
10^{-7}	10.77	15.89	1.45	0.52

Least inhibitory dose > 0.1 microgram per ml.

Half-lethal dose 0.1 microgram per ml.

Lethal dose 10.0 micrograms per ml.

PROPYLENE GLYCOL

$CH_3-CHOH-CH_2OH$

THE STRUCTURAL FORMULA OF PROPYLENE GLYCOL

TABLE 19

THE ACTION OF STANDARD PROPYLENE GLYCOL ON THE OUTGROWTH OF EMBRYONIC CHICK HEART FIBROBLASTS IN TISSUE CULTURE

	Explant Area (E)	Growth Area (G)	Growth Ratio (R) $R = \frac{G}{E}$	Growth Index (I) $I = \frac{Rx}{Rc}$
Control	15.31	23.21	1.51	1.00
10^{-1}		0.00		
10^{-2}	9.87	11.09	1.12	0.74
10^{-3}	10.23	11.15	1.09	0.72
10^{-4}	8.25	11.93	1.44	0.95
10^{-5}	7.23	11.58	1.60	1.06

TABLE 20

THE ACTION OF STANDARD PROPYLENE GLYCOL ON THE OUTGROWTH OF EMBRYONIC CHICK HEART FIBROBLASTS IN TISSUE CULTURE

	Explant Area (E)	Growth Area (G)	Growth Ratio (R) $R = \frac{G}{E}$	Growth Index (I) $I = \frac{Rx}{Rc}$
Control	26.15	86.88	3.32	1.00
10^{-1}		0.00		
10-2	11.51	1.72	0.15	0.05
10^{-3}	10.83	19.47	1.79	0.54
10^{-4}	9.40	25.97	2.76	0.83

PROPYLENE GLYCOL

$CH_3\text{-}CHOH\text{-}CH_2OH$

THE STRUCTURAL FORMULA OF PROPYLENE GLYCOL

TABLE 21

THE ACTION OF STANDARD PROPYLENE GLYCOL ON THE OUTGROWTH OF EMBRYONIC CHICK HEART FIBROBLASTS IN TISSUE CULTURE

	Explant Area (E)	Growth Area (G)	Growth Ratio (R) $R = \frac{G}{E}$	Growth Index (I) $I = \frac{Rx}{Rc}$
Control	7.79	39.27	5.04	1.00
10^{-2}		0.00		
10^{-3}	4.16	16.10	3.86	0.76
10^{-4}	4.89	20.37	4.16	0.82
10^{-5}	4.40	28.00	6.36	1.26

TABLE 22

THE ACTION OF STANDARD PROPYLENE GLYCOL ON THE OUTGROWTH OF EMBRYONIC CHICK HEART FIBROBLASTS IN TISSUE CULTURE

	Explant Area (E)	Growth Area (G)	Growth Ratio (R) $R = \frac{G}{E}$	Growth Index (I) $I = \frac{Rx}{Rc}$
Control	7.79	39.27	5.04	1.00
10^{-2}		0.00		
10^{-3}	4.92	12.04	2.44	0.48
10^{-4}	7.74	45.44	5.09	1.01
10^{-5}	4.96	37.86	7.63	1.51

PROPYLENE GLYCOL

$CH_3-CHOH-CH_2OH$

THE STRUCTURAL FORMULA OF PROPYLENE GLYCOL

TABLE 23

THE ACTION OF CHEMICALLY PURE PROPYLENE GLYCOL ON THE OUTGROWTH OF EMBRYONIC CHICK HEART FIBROBLASTS IN TISSUE CULTURE

	Explant Area (E)	Growth Area (G)	Growth Ratio (R) $R = \frac{G}{E}$	Growth Index (I) $I = \frac{Rx}{Rc}$
Control	7.33	29.02	3.96	1.00
1.0%	4.96	36.07	7.25	1.96
0.2%	7.73	47.32	6.12	1.54
0.04%	7.47	38.25	5.10	1.29
0.008%	8.19	65.89	8.04	2.03

TABLE 24

THE ACTION OF CHEMICALLY PURE PROPYLENE GLYCOL ON THE OUTGROWTH OF EMBRYONIC CHICK HEART FIBROBLASTS IN TISSUE CULTURE

	Explant Area (E)	Growth Area (G)	Growth Ratio (R) $R = \frac{G}{E}$	Growth Index (I) $I = \frac{Rx}{Rc}$
Control	7.33	29.02	3.96	1.00
1.0%	7.40	19.26	2.60	0.65
0.2%	6.89	61.25	8.77	2.21
0.04%	6.77	20.31	3.00	0.75
0.008%	8.64	51.35	5.94	1.50

dates showed an augmentation of this effect. This apparent toxic effect diminished on further dilution and in higher dilutions actually produced a stimulatory effect to a fair degree.

In Table 25 is the data showing the difference in the action on growth of 1.0% propylene glycol. The standard grade of propylene glycol produced an average growth index of 0.60. This indicates a 40.0% inhibition of growth below normal. The chemically pure propylene glycol acted as a growth stimulant in all but two cases. The average growth index was 1.29, which indicates a 29.0% stimulation above normal.

The second series of experiments, where chemically pure propylene glycol was employed, showed a stimulatory effect in all but two instances. The average inhibition of 1.0% propylene glycol of standard grade in the first series of experiments was approximately 40.0%, while the chemically pure propylene glycol present in the same concentration acted as a stimulatory factor and increased the outgrowth approximately 29.0%. It is felt that the synergistic activity of the standard grade of propylene glycol is due to some contaminant, probably a break-down product. It is also interesting to note that the chemically pure propylene glycol appears to act as a stabilizer, as the percentage of abnormal mitotic figures in cultures in media containing 1.0% propylene glycol is markedly less than in those grown in embryo extract alone.

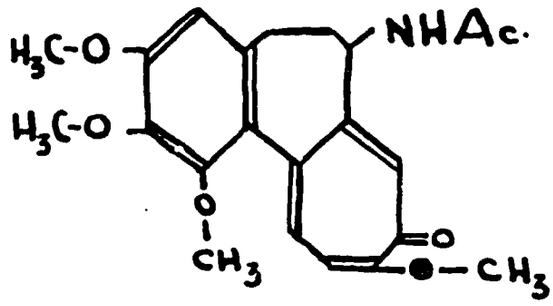
In the later series (Tables 26 to 38, in which the chemically pure propylene glycol was employed as solvent) the action of the estradiol compounds was greater than that of the estrones. A number approximated the degree of inhibitory action of diethylstilbestrol. Several exceeded the inhibitory action of colchicine. (text continued on page 79)

TABLE 25

COMPARISON OF GROWTH INDICES OF EXPERIMENTAL CONTROL CULTURES GROWN WITH 1% SOLUTION OF PROPYLENE GLYCOL IN THE MEDIA

Standard Grade Propylene Glycol		Chemically Pure Propylene Glycol
a. 0.53	j. 0.83	a. 1.83
b. 0.82	k. 0.70	b. 1.53
c. 0.69	l. 0.76	c. 0.66
d. 0.78	m. 0.74	d. 1.27
e. 0.70	n. 0.63	e. 1.21
f. 0.83	l. 0.74	f. 1.20
g. 0.67	p. 0.05	g. 1.96
h. 0.71	q. 0.00	h. 0.65
i. 0.63	r. 0.00	
Total	10.81	10.31
Average	0.60	1.29

COLCHICINE



THE STRUCTURAL FORMULA OF COLCHICINE

TABLE 26

THE ACTION OF COLCHICINE ON THE OUTGROWTH OF EMBRYONIC CHICK HEART FIBROBLASTS IN TISSUE CULTURE

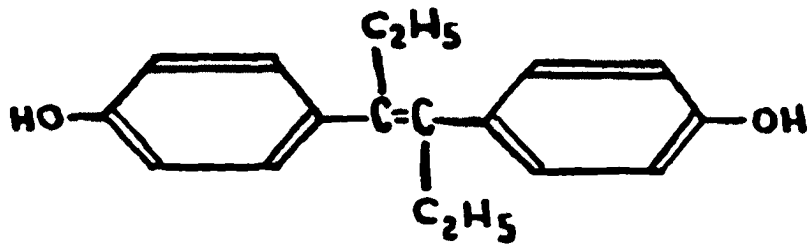
	Explant Area (E)	Growth Area (G)	Growth Ratio (R) $R = \frac{G}{E}$	Growth Index (I) $I = \frac{Rx}{Rc}$
Control	16.87	51.53	3.05	1.00
Propylene Glycol	16.40	91.57	5.58	1.83
10^{-4}	7.39	2.49	0.33	0.11
10^{-5}	10.85	18.91	1.74	0.57
10^{-6}	18.95	45.56	2.41	0.79
10^{-7}	6.24	12.49	2.00	0.65
10-8	15.01	35.03	2.33	0.76

Least inhibitory dose < 0.001 micrograms per ml.

Half-lethal dose 20.0 micrograms per ml.

Lethal dose 100.0 micrograms per ml.

DIETHYLSTILBESTROL



THE STRUCTURAL FORMULA OF DIETHYLSTILBESTROL

TABLE 27

THE ACTION OF DIETHYLSTILBESTROL ON THE OUTGROWTH OF EMBRYONIC CHICK HEART FIBROBLASTS IN TISSUE CULTURE

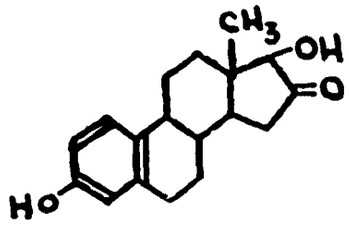
	Explant Area (E)	Growth Area (G)	Growth Ratio (R) $R = \frac{G}{E}$	Growth Index (I) $I = \frac{Rx}{Rc}$
Control	7.45	23.79	3.19	1.00
Propylene Glycol	4.85	23.83	4.91	1.53
10^{-4}	3.57	2.74	0.77	0.24
10^{-5}	5.55	4.12	0.74	0.23
10^{-6}	3.81	3.78	0.99	0.31
10^{-7}	3.32	2.95	0.86	0.27
10^{-8}	0.93	1.20	1.29	0.40

Least inhibitory dose > 0.01 microgram per ml.

Half-lethal dose > 0.01 microgram per ml.

Lethal dose 100.0 micrograms per ml.

16-KETO-ESTRADIOL



THE STRUCTURAL FORMULA OF 16-KETO-ESTRADIOL

TABLE 28

THE ACTION OF 16-KETO ESTRADIOL ON THE OUTGROWTH OF EMBRYONIC CHICK HEART FIBROBLASTS IN TISSUE CULTURE

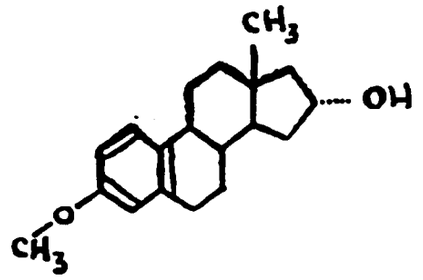
	Explant Area (E)	Growth Area (G)	Growth Ratio (R) $R = \frac{G}{E}$	Growth Index (I) $I = \frac{Rx}{Rc}$
Control	4.88	23.18	4.75	1.00
Propylene Glycol	4.56	24.98	5.48	1.15
10^{-4}	4.15	6.98	1.90	0.40
10^{-5}	2.26	5.09	2.25	0.47
10^{-6}	5.56	24.78	4.45	0.94
10^{-7}	5.88	32.25	5.52	1.16

Least inhibitory dose < 0.1 microgram per ml.

Half-lethal dose 7.0 microgram per ml.

Lethal dose <100.0 micrograms per ml.

3-METHYLESTRADIOL-3,16a



THE STRUCTURAL FORMULA OF
3-METHYLESTRADIOL-3,16a

TABLE 29

THE ACTION OF 3-METHYLESTRADIOL-3,16α ON THE OUTGROWTH OF EMBRYONIC CHICK HEART FIBROBLASTS IN TISSUE CULTURE

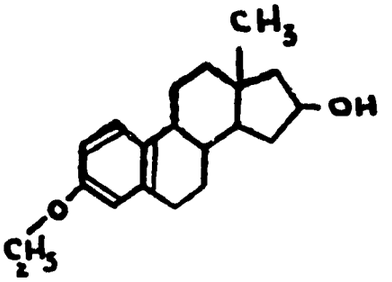
	Explant Area (E)	Growth Area (G)	Growth Ratio (R) $R = \frac{G}{E}$	Growth Index (I) $I = \frac{Rx}{Rc}$
Control	5.57	23.19	4.16	1.00
Propylene Glycol	7.23	19.85	2.75	0.66
10^{-4}	5.22	3.37	0.64	0.15
10^{-5}	5.89	27.06	4.61	1.10
10^{-6}	7.04	36.38	5.16	1.24
10^{-7}	5.83	18.14	3.11	0.75

Least inhibitory dose < 9.0 micrograms per ml.

Half-lethal dose 70.0 micrograms per ml.

Lethal dose < 100.0 micrograms per ml.

3-ETHYLESTRADIOL-3,16B



THE STRUCTURAL FORMULA OF
3-ETHYLESTRADIOL-3,16B

TABLE 30

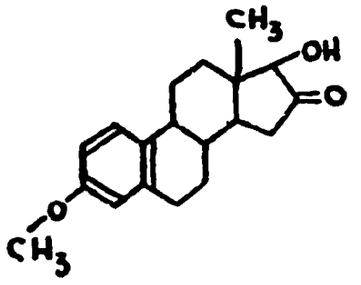
THE ACTION OF 3-ETHYLESTRADIOL-3,16B ON THE OUTGROWTH OF EMBRYONIC CHICK HEART FIBROBLASTS IN TISSUE CULTURE

	Explant Area (E)	Growth Area (G)	Growth Ratio (R) $R = \frac{G}{E}$	Growth Index (I) $I = \frac{Rx}{Rc}$
Control	4.32	22.60	5.23	1.00
Propylene Glycol	4.62	29.01	6.29	1.20
10^{-4}	5.37	14.02	2.63	0.50
10^{-5}	6.77	41.23	6.09	1.16
10^{-6}	5.65	19.45	3.49	0.66
10^{-7}	4.34	18.09	4.17	0.79
10^{-8}	2.80	7.50	2.69	0.51

Least inhibitory dose $<$ 30.0 micrograms per ml.

Half-lethal dose 100.0 micrograms per ml.

3-METHYL-16-KETO-ESTRADIOL



THE STRUCTURAL FORMULA OF
3-METHYL-16-KETO-ESTRADIOL

TABLE 31

THE ACTION OF 3-METHYL-16-KETO-ESTRADIOL ON THE OUTGROWTH OF EMBRYONIC CHICK HEART FIBROBLASTS IN TISSUE CULTURE

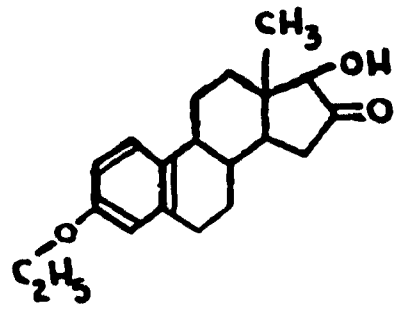
	Explant Area (E)	Growth Area (G)	Growth Ratio (R) $R = \frac{G}{E}$	Growth Index (I) $I = \frac{Rx}{Rc}$
Control	4.88	23.14	4.75	1.00
Propylene Glycol	4.56	24.98	5.48	1.15
10^{-4}	3.49	1.44	0.41	0.09
10^{-5}	3.14	3.27	1.04	0.22
10^{-6}	2.56	8.73	3.43	0.72
10^{-7}	3.84	12.46	3.24	0.68

Least inhibitory dose $>$ 0.1 microgram per ml.

Half-lethal dose 4.0 micrograms per ml.

Lethal dose $<$ 100.0 micrograms per ml.

3-ETHYL-16-KETO-ESTRADIOL



THE STRUCTURAL FORMULA OF
3-ETHYL-16-KETO-ESTRADIOL

TABLE 32

THE ACTION OF 3-ETHYL-16-KETO-ESTRADIOL ON THE OUTGROWTH OF EMBRYONIC CHICK HEART FIBROBLASTS IN TISSUE CULTURE

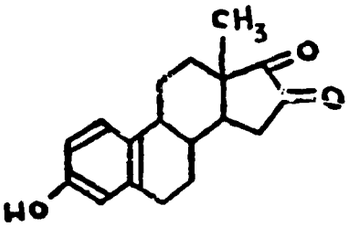
	Explant Area (E)	Growth Area (G)	Growth Ratio (R) $R = \frac{G}{E}$	Growth Index (I) $I = \frac{Rx}{Rc}$
Control	5.43	31.64	5.83	1.00
Propylene Glycol	5.03	37.24	7.41	1.27
10^{-4}	5.07	5.98	1.18	0.21
10^{-5}	7.37	9.61	1.30	0.24
10^{-6}	5.30	10.30	1.94	0.33
10^{-7}	4.48	15.62	3.49	0.59
10^{-8}	1.06	3.20	3.01	0.51

Least inhibitory dose $>$ 0.01 microgram per ml.

Half-lethal dose 0.4 microgram per ml.

Lethal dose $<$ 100.0 micrograms per ml.

16-KETO-ESTRONE



THE STRUCTURAL FORMULA OF 16-KETO-ESTRONE

TABLE 33

THE ACTION OF 16-KETO-ESTRONE ON THE OUTGROWTH OF EMBRYONIC CHICK HEART FIBROBLASTS IN TISSUE CULTURE

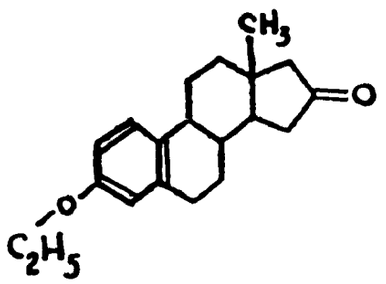
	Explant Area (E)	Growth Area (G)	Growth Ratio (R) $R = \frac{G}{E}$	Growth Index (I) $I = \frac{Rx}{Rc}$
Control	4.88	23.18	4.75	1.00
Propylene Glycol	4.56	24.98	5.48	1.15
10^{-4}	5.97	6.43	1.08	0.23
10^{-5}	8.37	24.50	2.95	0.62
10^{-6}	9.23	38.52	4.17	0.88
10^{-7}	7.30	22.76	3.12	0.66

Least inhibitory dose > 0.1 microgram per ml.

Half-lethal dose 20.0 micrograms per ml.

Lethal dose < 100.0 micrograms per ml.

3-ETHYLESTRONE-16



THE STRUCTURAL FORMULA OF 3-ETHYLESTRONE-16

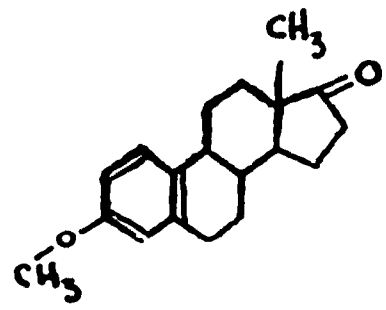
TABLE 34

THE ACTION OF 3-ETHYLESTRONE-16 ON THE OUTGROWTH OF EMBRYONIC CHICK HEART FIBROBLASTS IN TISSUE CULTURE

	Explant Area (E)	Growth Area (G)	Growth Ratio (R) $R = \frac{G}{E}$	Growth Index (I) $I = \frac{Rx}{Rc}$
Control	5.43	31.64	5.83	1.00
Propylene Glycol	5.03	37.24	7.41	1.27
10^{-4}	7.03	43.67	6.21	1.06
10^{-5}	5.82	45.78	7.86	1.34
10^{-6}	7.89	53.85	6.82	1.16
10^{-7}	5.18	45.38	8.76	1.50
10^{-8}	5.44	36.44	6.70	1.14

Lethal Dose $<$100.0 micrograms per ml.

3-METHYLESTRONE-17



THE STRUCTURAL FORMULA OF 3-METHYLESTRONE-17

TABLE 35

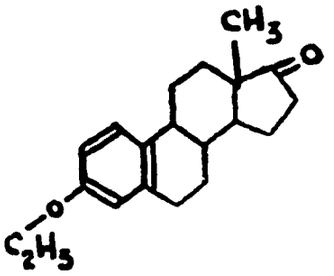
THE ACTION OF 3-METHYLESTRONE-17 ON THE OUTGROWTH OF EMBRYONIC CHICK HEART FIBROBLASTS IN TISSUE CULTURE

	Explant Area (E)	Growth Area (G)	Growth Ratio (R) $R = \frac{G}{E}$	Growth Index (I) $I = \frac{Rx}{Rc}$
Control	4.32	22.60	5.23	1.00
Propylene Glycol	4.62	29.01	6.29	1.20
10^{-4}	8.62	36.83	4.50	0.86
10^{-5}	4.96	25.99	5.24	1.00
10^{-6}	7.32	43.86	5.99	1.14
10^{-7}	6.72	25.33	3.77	0.72
10^{-8}	5.24	17.85	3.41	0.65

Least inhibitory dose 10.0 micrograms per ml.

Half-lethal dose 100.0 micrograms per ml.

3-ETHYLESTRONE-17



THE STRUCTURAL FORMULA OF 3-ETHYLESTRONE-17

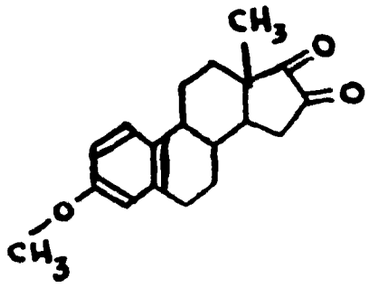
TABLE 36

THE ACTION OF 3-ETHYLESTRONE-17 ON THE OUTGROWTH OF EMBRYONIC CHICK HEART FIBROBLASTS IN TISSUE CULTURE

	Explant Area (E)	Growth Area (G)	Growth Ratio (R) $R = \frac{G}{E}$	Growth Index (I) $I = \frac{Rx}{Rc}$
Control	9.57	42.37	4.37	1.00
Propylene Glycol	5.63	29.40	5.22	1.21
10^{-4}	9.84	34.92	3.55	0.81
10^{-5}	6.78	26.82	3.95	0.90
10^{-6}	10.02	46.76	4.66	1.06
10^{-7}	6.87	27.30	3.97	0.90

Least inhibitory dose $<$1.0 microgram per ml.

3-METHYL-16-KETO-ESTRONE



THE STRUCTURAL FORMULA OF
3-METHYL-16-KETO-ESTRONE

TABLE 37

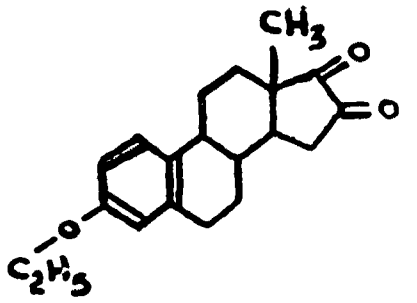
THE ACTION OF 3-METHYL-16-KETO-ESTRONE ON THE OUTGROWTH OF EMBRYONIC CHICK HEART FIBROBLASTS IN TISSUE CULTURE

	Explant Area (E)	Growth Area (G)	Growth Ratio (R) $R = \frac{G}{E}$	Growth Index (I) $I = \frac{Rx}{Rc}$
Control	5.57	23.19	4.16	1.00
Propylene Glycol	7.23	19.85	2.75	0.66
10^{-4}	5.77	13.98	2.44	0.58
10^{-5}	4.71	17.68	3.75	0.90
10^{-6}	contaminated			
10^{-7}	contaminated			

Least inhibitory dose < 6.0 micrograms per ml.

Half-lethal dose 200.0 micrograms per ml.

3-ETHYL-16-KETO-ESTRONE



THE STRUCTURAL FORMULA OF
3-ETHYL-16-KETO-ESTRONE

TABLE 38

THE ACTION OF 3-ETHYL-16-KETO-ESTRONE ON THE OUTGROWTH OF EMBRYONIC CHICK HEART FIBROBLASTS IN TISSUE CULTURE

	Explant Area (E)	Growth Area (G)	Growth Ratic (R) $R = \frac{G}{E}$	Growth Index (I) $I = \frac{Rx}{Rc}$
Control	9.67	42.37	4.37	1.00
Propylene Glycol	5.83	29.40	5.22	1.21
10^{-4}	11.88	28.44	2.39	0.54
10^{-5}	6.83	23.24	3.40	0.76
10^{-6}	9.62	55.08	5.73	1.31
10^{-7}	7.51	53.89	7.17	1.64
10^{-8}	4.66	34.23	7.34	1.65

Least inhibitory dose 6.0 micrograms per ml.

Half-lethal dose < 100.0 micrograms per ml.

Lethal dose < 100.0 micrograms per ml.

Observations on Aberrant Mitotic Figures

A wide variety of distortions of mitotic figures and their degree of frequency was observed in the various series of cultures. The control cultures also contained some aberrations; however, the frequency of these was greatly elevated in the experimental series.

Among the phenomena observed are such things as fragmentation of chromatin material (Photographs 23 and 25, Plate IV), stubbing effect on chromosomes (Photograph 30, Plate V, Photograph 39, Plate VII), agglutination or sticking together of chromatin and chromosomes (Photograph 12, Plate II, Photographs 16 and 17, Plate III), lagging of chromosomes (Photograph 19, Plate III), disruption of chromosomes (Photograph 33, Plate VI), various degrees of pyknosis (Photographs 40, 41, 42, 43, Plate VII, Photographs 48 and 49, Plate VII), and failures in the reconstruction of the nucleus (Photograph 47, Plate VIII).

In many of the nuclei fragments of chromosomes had retracted to form small dense hyperchromic spherules (Photograph 13, Plate II). This effect was more pronounced where chromosomes had become interrupted during the course of their formation so that the nuclear area was filled with numerous more or less uniformly-sized particles. This phenomena was quite frequent in metaphase, but the particles formed were few in number and not uniform in size and shape (Photographs 23, 24, 25, Plate IV). In some cells it appeared as though one or more of the chromosomes became heavily beaded (Photograph 38, Plate VII), which may have been an indication of the state just prior to fragmentation or an early stage of shrinkage of the chromosome.

In a number of instances the point of fragmentation of the chro-

mosome appeared to be the kinetochore (Photograph 33, Plate VI), and one arm of the chromosome remained attached to the spindle apparatus and was drawn away, while the other arm remained behind.

Another effect observed was that of the formation of short stubby chromosomes induced by a shrinking of the filamentous chromosomes (Photograph 30, Plate V). This was most frequently observed in metaphase and frequently the entire metaphase plate could be seen to be composed of a group of uniformly sized short peglike bodies. This stubbing was also very apparent in some instances where such chromosomes were left behind by the lagging effect following spindle dissociation. The shrinking and contraction of the chromosome leading to the formation of a rather pyknotic structure may have been initiated by a syneretic process brought on by disturbances of water balance of these cellular organelles as a result of the action of the steroids.

Stickiness or agglutination of chromosomes is frequently seen when chromosomes fail to separate following metaphase cleavage. While some of the chromosomes may separate normally, one or more may fail to do so and may remain in the interzonal region between the two poleward moving groups of chromosomes in anaphase, or they may be drawn to one or the other of the daughter groups after these have reached their destination. In some instances the surface character of the chromosome appears to be ragged and not as sharply delineated as in normal chromosomes, perhaps due to their more adhesive nature. This stickiness may give rise to chromosome bridges between the two daughter groups, and even deletion of one or more chromosomes from the reconstituted cell, thus producing a condition of aneuploidy.

In some metaphase plates, confluence of the peripheral ends of chromosome arms may produce a stellate pattern (Photograph 28, Plate V), generally termed "star metaphases", and also doughnut-shaped ringlike patterns. Confluence of chromosomes in anaphase gives rise to figures with marked dimentional reduction.

Pyknosis is another characteristic of severely damaged cells and was encountered frequently and in varying degrees of intensity (Photographs 20, 21, 22, Plate IV). It is a result of the inability of the chromatin material as well as the chromosomes to become properly organized so that they may enter the next phase of development. There may be a confluence of chromosomes and chromatin material due to the stickiness of their surface. This then produces a vacuolated-like body with a dense reticulum of chromatin bands by which further confluence and agglutination decreases in volume and becomes a small hyperchromatic knotlike body. Pyknotic nuclei were observed to follow a slightly different sequence of events (Photographs 20, 21, 22, Plate IV). After the chromosomes had become organized to form a metaphase plate, there was a disruption of the spindle mechanism and a failure of response by the chromosomes. The chromosomes appeared to become crowded together into a smaller space and then, due to their sticky surfaces, they began to adhere to one another and gradually flowed together to form a small densely stainable body. Pyknosis may also be initiated in later stages, such as anaphase (Photographs 45 and 46, Plate VIII), telophase, and in the reconstruction phase, but the frequency is very small.

In some cultures, where there was a great deal of cellular damage, the cell rounded up and the chromatin became dispersed as a group of

several irregular-sized bodies that stain quite densely with nuclear stains. This situation came about by the clumping together of several groups of chromosomes which became pyknotic after further coalescence.

Chromosome lags appear when the spindle fiber becomes disorganized, stretched, or is unable to contract at the same rate as the remaining fibers (Photograph 27, Plate V, Photograph 37, Plate VI). As a result, the chromosome to which the spindle fiber is attached lags behind the others on its entry of metaphase or in anaphase.

Such lags may result in an unequal number of chromosomes in daughter nuclei and chromosome deletions may occur. Deletions may also result in instances where chromosomes lag behind due to a break in the attached spindle fiber or the dissociation of the chromosome from the fiber at the kinetochore.

Another abnormality that was noted fairly frequently was a slight lateral tilting of one of the groups of daughter chromosome at an angle away from the common axis (Photograph 44, Plate VIII). Apart from this tilt effect, no other defect was detected. The actual tilt is a result of resistance being encountered by the chromosome in one sector of the cleaved plate during anaphase. This is caused by a weakening of spindle fibers controlling that specific sector so that one side of the migrating plate of chromosomes lags behind.

Multipolar cells are also encountered (Photograph 34, Plate VI). These may form a greater number of asters than just the two which are found in the normal mitotic cells. As many as six asters have been counted in one cell. This effect is produced by the division of the centrosome into more than two parts with the subsequent organization of

additional spindles. The number of chromosomes which become associated with the several spindles may vary, some having only as few as one or two. If cell division should successfully proceed to a conclusion, it would of course result in a decrease in the number of chromosomes in all the daughter cells.

Difficulties in reconstruction of the new cells have also been noted. In a number of instances reconstitution of the nuclei occurred without cleavage of the cytoplasm, so that binucleated cells developed. In other cases, cleavage of cytoplasm occurred, but the nuclei of these cells were pyknotic (Photograph 48, Plate VIII). At least one cell was encountered where one nucleus was reconstituted in a normal fashion while its sister nucleus became pyknotic (Photograph 47, Plate VIII).

The frequency of aberrant mitotic cells was much higher in cultures exposed to higher concentrations of the steroidal compounds, and here pyknotic cells were generally more numerous. In the more dilute concentrations the principal abnormality was disjunction and lagging of chromosomes. The most sensitive phase in the mitotic cycle was the metaphase stage. While a very small number of the aberrant states were encountered in the other phases of mitosis, the number, however, was negligible in comparison to that found in metaphase.

Observations of Steroidal Action on Mitosis

The second series of studies were made employing a number of this group of compounds. Cell counts and mitoses were made of all cultures in this series. The various mitotic phases of cells in division were counted, as well as the number of aberrant mitoses. The statistical data of these

studies are presented in their analyzed form in Tables 39 to 46.

The upper half of the table lists the following statistical data and analysis:

Column 1 - The total number of resting and mitotic cells counted in order to yield the mitotic cell count listed in columns 2, 3, and 4.

Column 2 - The number of normal mitoses encountered.

Column 3 - The number of abnormal mitoses encountered.

Column 4 - The total number of mitoses encountered.

Columns 5, 6, and 7 show the mitotic indices obtained by dividing the total cell count of column 1 by the cell counts of normal, abnormal, and sum total mitoses as found in columns 2, 3, and 4. This procedure then produces

Column 5 - The average number of cells counted that contained 1 normal mitosis.

Column 6 - The average number of cells counted that contained 1 abnormal mitosis.

Column 7 - The average number of cells counted that contained 1 mitosis, whether normal or abnormal.

In the lower half of each table is listed the actual distribution of mitoses in the various phases including the abnormal metaphases.

The upper figure of each horizontal division represents the actual number of mitotic cells in the various phases and the lower figure indicates its value on a percentage basis given to the nearest first decimal place.

(text continued on page 109)

TABLE 39

ACTION OF COLCHICINE ON THE MITOTIC INDEX AND THE MITOTIC DISTRIBUTION IN CHICK EMBRYO FIBROBLASTS

A. THE MITOTIC INDEX

	Total Cell Count (1)	Mitotic Cell Count			Mitotic Index		
		Normal (2)	Abnormal (3)	Total (4)	Normal (5)	Abnormal (6)	Total (7)
Control	55,448	405	27	432	136.9	2053.8	128.0
Propylene Glycol	40,762	265	16	281	304.2	2547.8	145.0
10^{-4}		all abnormal mitoses					
10^{-5}	19,411	156	76	232	124.4	255.5	83.6
10^{-6}	45,380	292	85	377	155.4	533.9	120.0
10^{-7}	60,009	389	39	428	140.2	1538.6	140.0
10^{-8}	17,961	92	14	106	195.1	1282.9	169.4

By dividing the total cell count in column 1 by the mitotic cell counts in columns 2, 3, and 4, we obtain the mitotic indices as given in columns 5, 6, and 7.

B. THE MITOTIC DISTRIBUTION

	Prophase	Metaphase		Anaphase	Telophase
		Normal	Abnormal		
Control	92 21.3%	134 31.1%	27 6.2%	7 16.6%	107 24.7%
Propylene Glycol	46 16.3%	134 48.2%	16 5.1%	51 18.1%	36 12.3%
10^{-4}			100.0%		
10^{-5}	23 9.9%	79 34.0%	76 32.7%	36 15.5%	18 7.9%
10^{-6}	60 15.9%	113 30.4%	85 22.2%	79 20.9%	40 10.6%
10^{-7}	65 15.3%	283 42.4%	39 9.1%	106 24.9%	35 8.3%
10^{-8}	21 19.8%	39 36.8%	14 13.2%	24 22.6%	8 7.8%

The upper figure in each space represents the number of mitoses counted; the lower figure gives their percentage value.

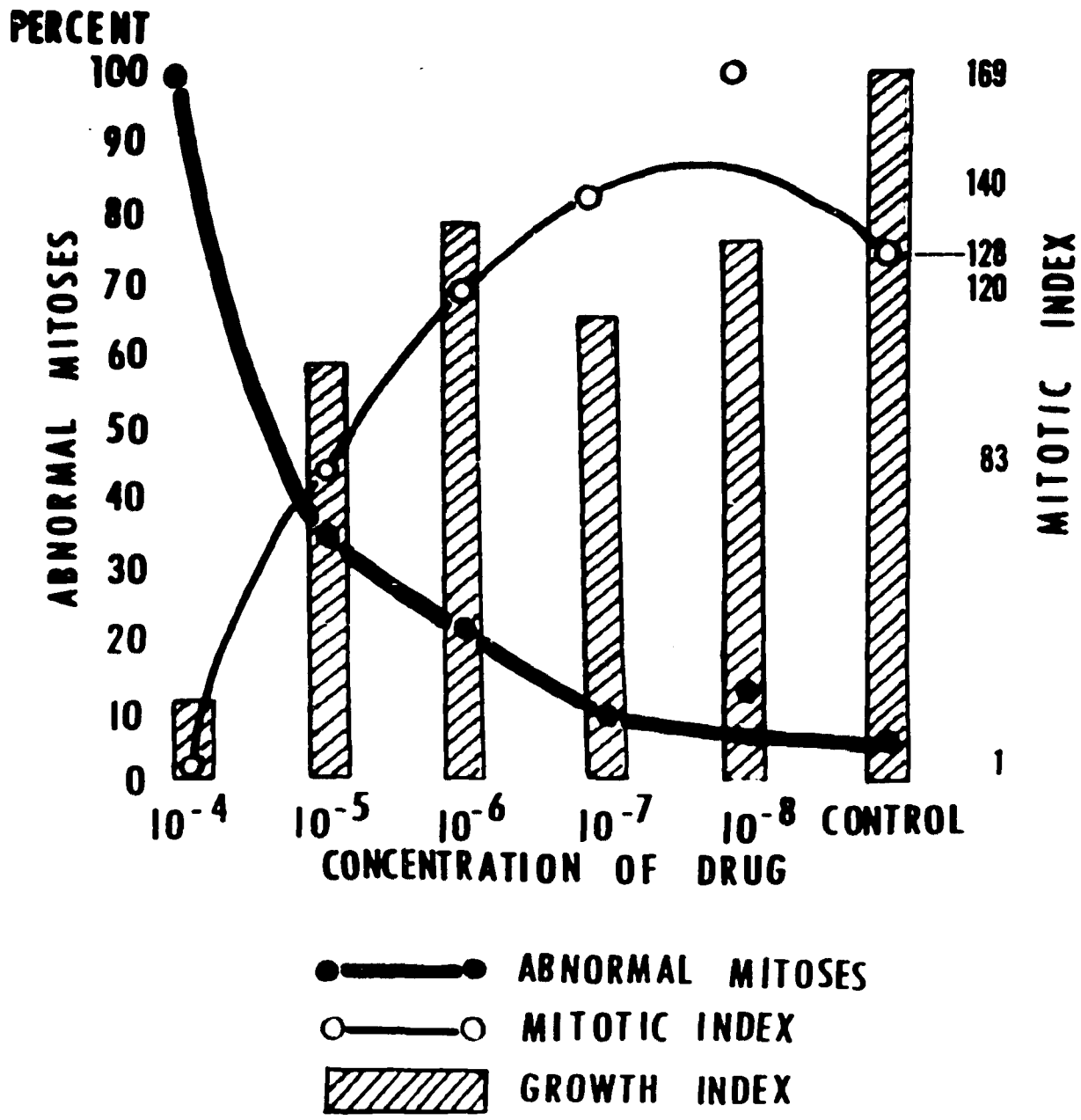


FIGURE 1 THE ACTION OF COLCHICINE ON GROWTH AND MITOSIS OF CHICK EMBRYO FIBROBLASTS

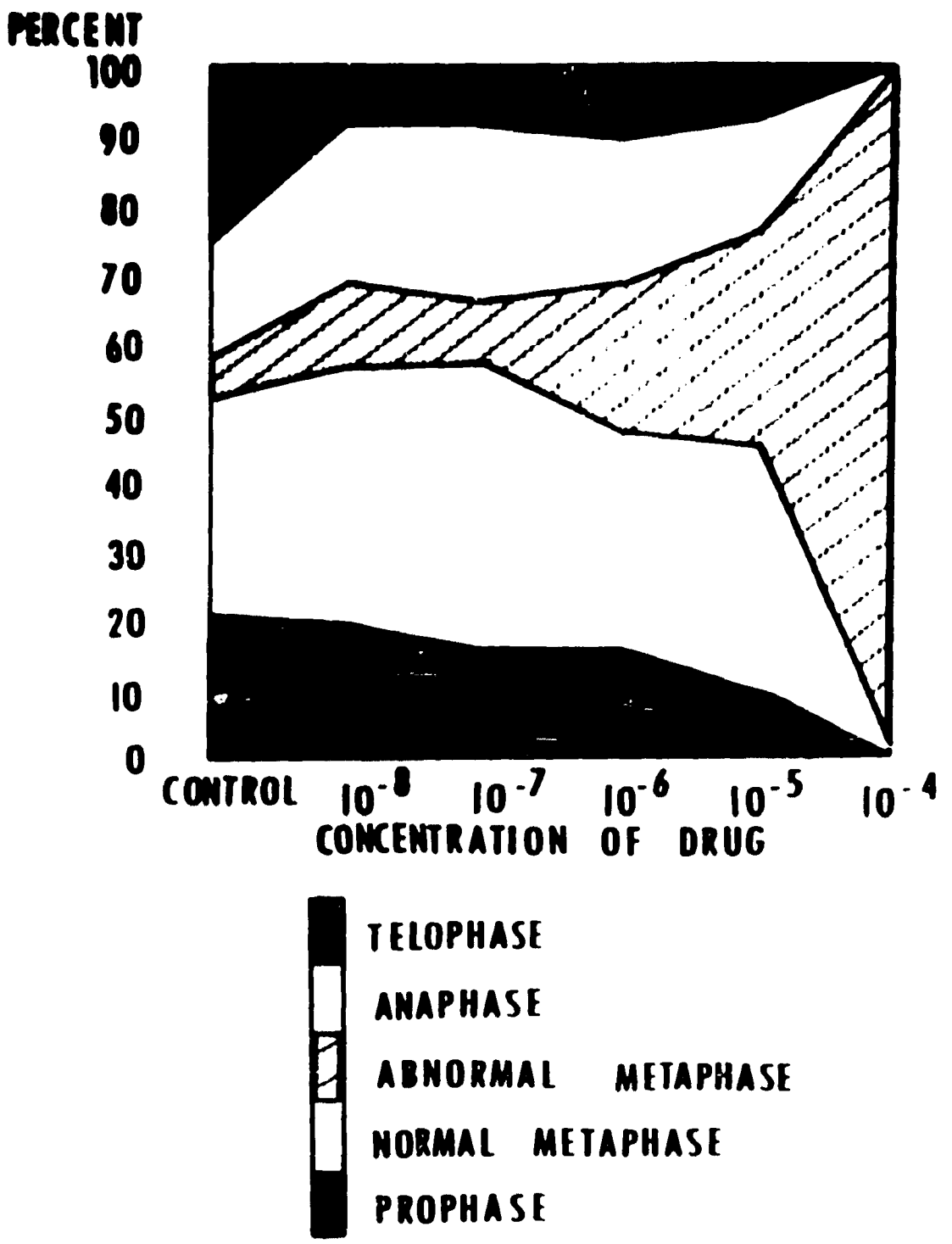


FIGURE 2 THE ACTION OF COLCHICINE ON THE MITOTIC DISTRIBUTION OF CHICK EMBRYO FIBROBLASTS

TABLE 40

ACTION OF DIETHYLSTILBESTROL ON THE MITOTIC INDEX AND THE MITOTIC DISTRIBUTION IN CHICK EMBRYO FIBROBLASTS

A. THE MITOTIC INDEX

	Total Cell Count (1)	Mitotic Cell Count			Mitotic Index		
		Normal (2)	Abnormal (3)	Total (4)	Normal (5)	Abnormal (6)	Total (7)
Control	24,812	97	3	100	265.9	8270.7	248.12
10^{-4}	10,905	10	301	311	1090.5	36.23	35.07
10^{-5}	14,415	13	401	419	800.7	35.95	34.4
10^{-6}	11,121	28	752	780	397.1	14.72	14.13
10^{-7}	18,862	171	89	260	110.3	212.9	72.5
10^{-8}	21,063	114	20	134	183.9	1053.1	157.1

By dividing the total cell count in column 1 by the mitotic cell counts in columns 2, 3, and 4, we obtain the mitotic indices as given in columns 5, 6, and 7.

B. THE MITOTIC DISTRIBUTION

	Prophase	Metaphase		Anaphase	Telophase
		Normal	Abnormal		
Control	15 15%	48 48%	3 3%	5 5%	29 29%
10^{-4}	2 0.6%	5 1.6%	301 96.8%	0 0%	3 1.0%
10^{-5}	7 1.7%	7 1.7%	401 95.8%	4 0.8%	0 0%
10^{-6}	23 2.9%	3 0.4%	752 96.2%	2 0.5%	0 0%
10^{-7}	70 26.9%	70 26.9%	89 34.3%	20 7.7%	11 4.2%
10^{-8}	24 17.6%	54 40.5%	20 14.7%	27 20.4%	9 6.8%

The upper figure in each space represents the number of mitoses counted; the lower figure gives their percentage value.

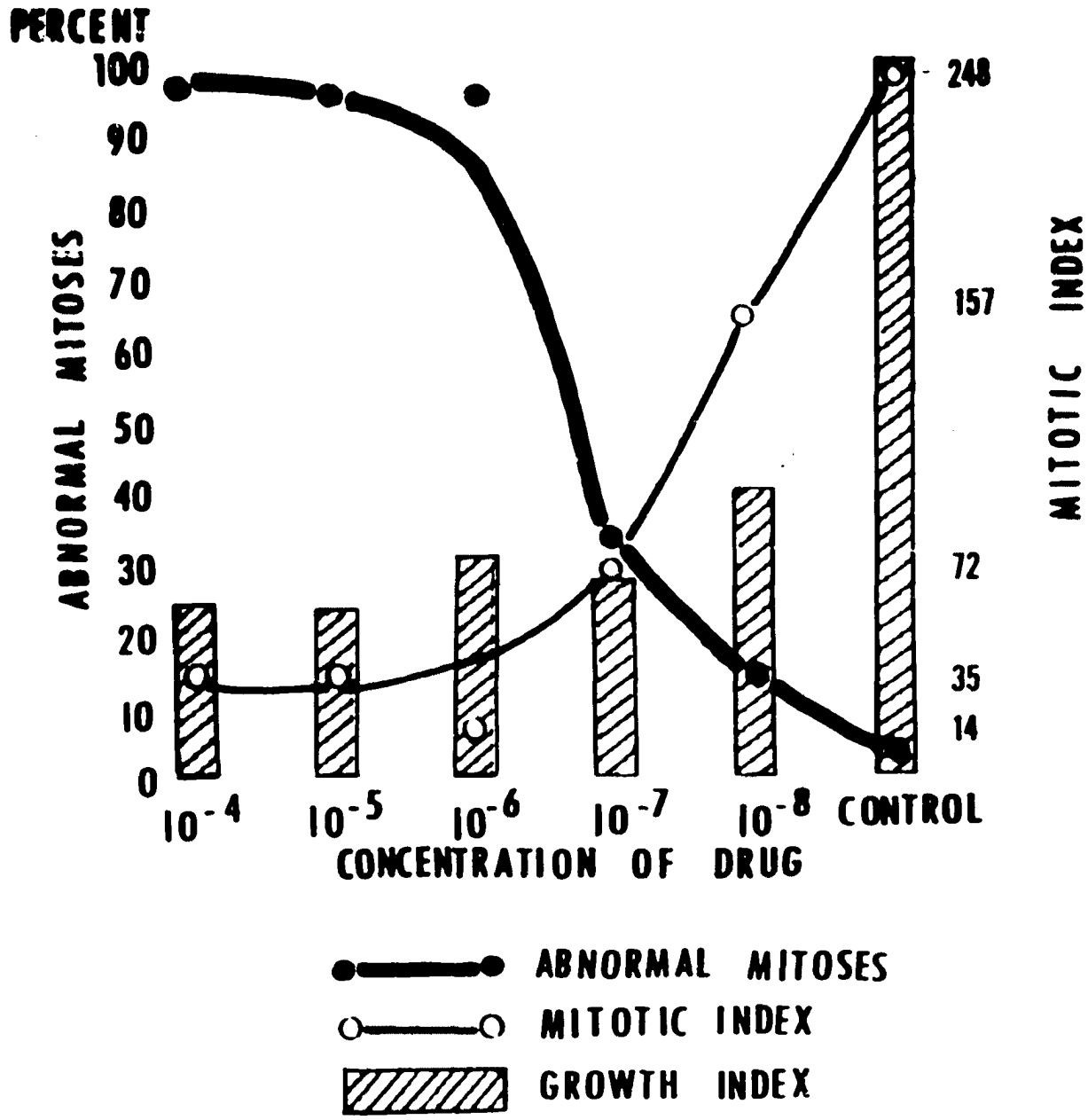


FIGURE 3 THE ACTION OF DIETHYLSTILBESTROL ON GROWTH AND MITOSIS OF CHICK EMBRYO FIBROBLASTS

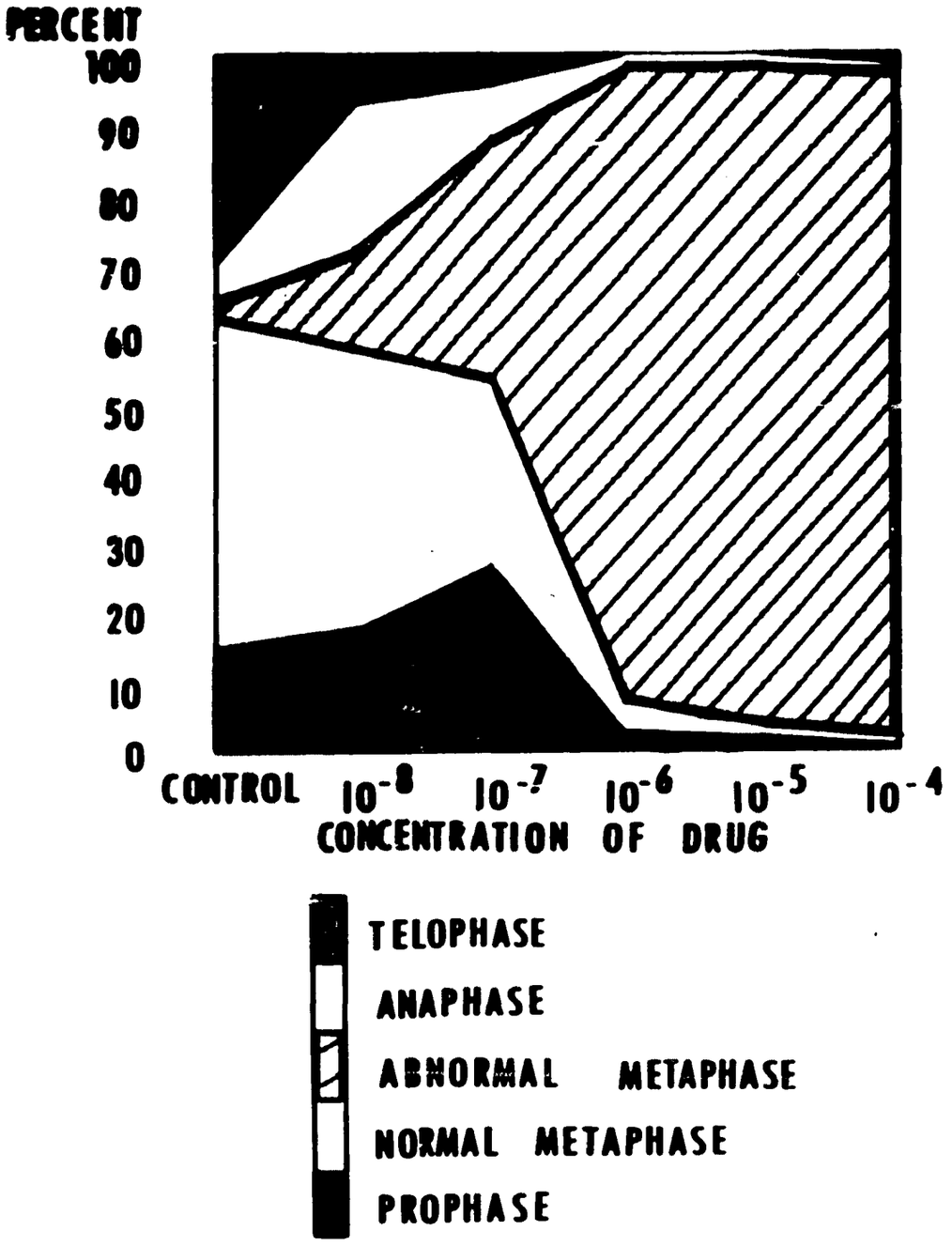


FIGURE 4 THE ACTION OF DIETHYLSTILBESTROL ON THE MITOTIC DISTRIBUTION OF CHICK EMBRYO FIBROBLASTS

TABLE 41

ACTION OF 16-KETO-ESTRADIOL ON THE MITOTIC INDEX AND THE MITOTIC DISTRIBUTION IN CHICK EMBRYO FIBROBLASTS

A. THE MITOTIC INDEX

	Total Cell Count (1)	Mitotic Cell Count			Mitotic Index		
		Normal (2)	Abnormal (3)	Total (4)	Normal (5)	Abnormal (6)	Total (7)
Control	27,249	60	9	69	454.1	3027.6	394.9
Propylene Glycol	55,190	74	8	82	748.5	6898.7	673.0
10^{-4}	31,536	33	334	367	955.9	94.4	85.7
10^{-5}	42,981	21	124	145	2046.7	346.6	296.4
10^{-6}	20,584	18	94	112	1143.5	218.5	183.8
10^{-7}	36,115	47	99	146	768.3	364.8	247.4

By dividing the total cell count in column 1 by the mitotic cell counts in columns 2, 3, and 4, we obtain the mitotic indices as given in columns 5, 6, and 7.

B. THE MITOTIC DISTRIBUTION

	Prophase	Metaphase		Anaphase	Telophase
		Normal	Abnormal		
Control	3 4.3%	22 32.0%	9 12.9%	11 15.9%	24 34.9%
Propylene Glycol	8 9.7%	25 30.6%	8 9.7%	17 20.9%	24 29.1%
10^{-4}	1 0.3%	2 0.6%	334 91.0%	3 0.8%	27 7.3%
10^{-5}	0 0.0%	4 2.7%	124 85.6%	7 4.8%	10 6.9%
10^{-6}	2 1.8%	5 4.5%	94 83.9%	5 4.4%	6 5.4%
10^{-7}	2 1.4%	14 10.0%	99 67.4%	8 5.4%	23 15.7%

The upper figure in each space represents the number of mitoses counted; the lower figure gives their percentage value.

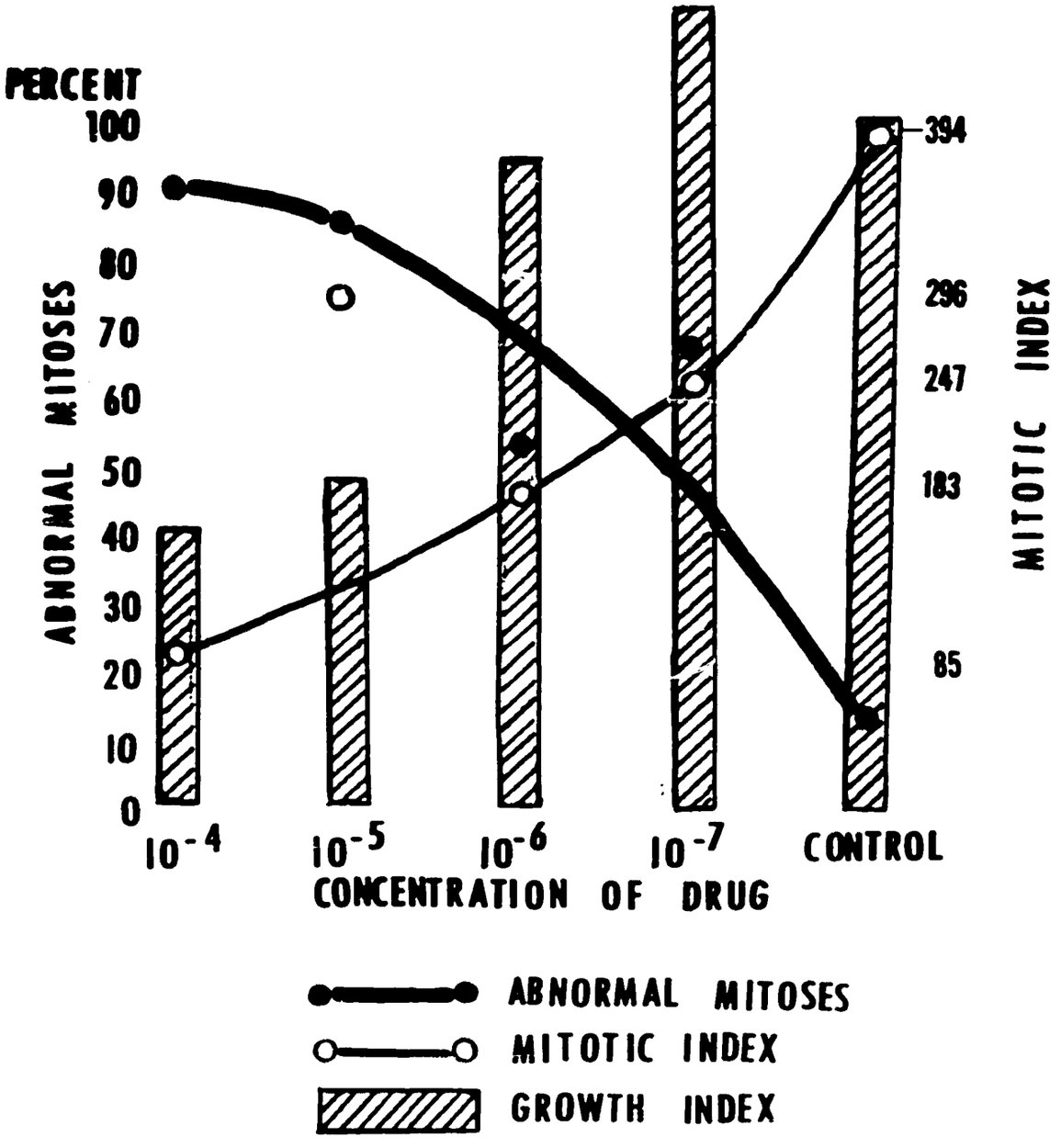


FIGURE 5 THE ACTION OF 16-KETO-ESTRADIOL ON GROWTH AND MITOSIS OF CHICK EMBRYO FIBROBLASTS

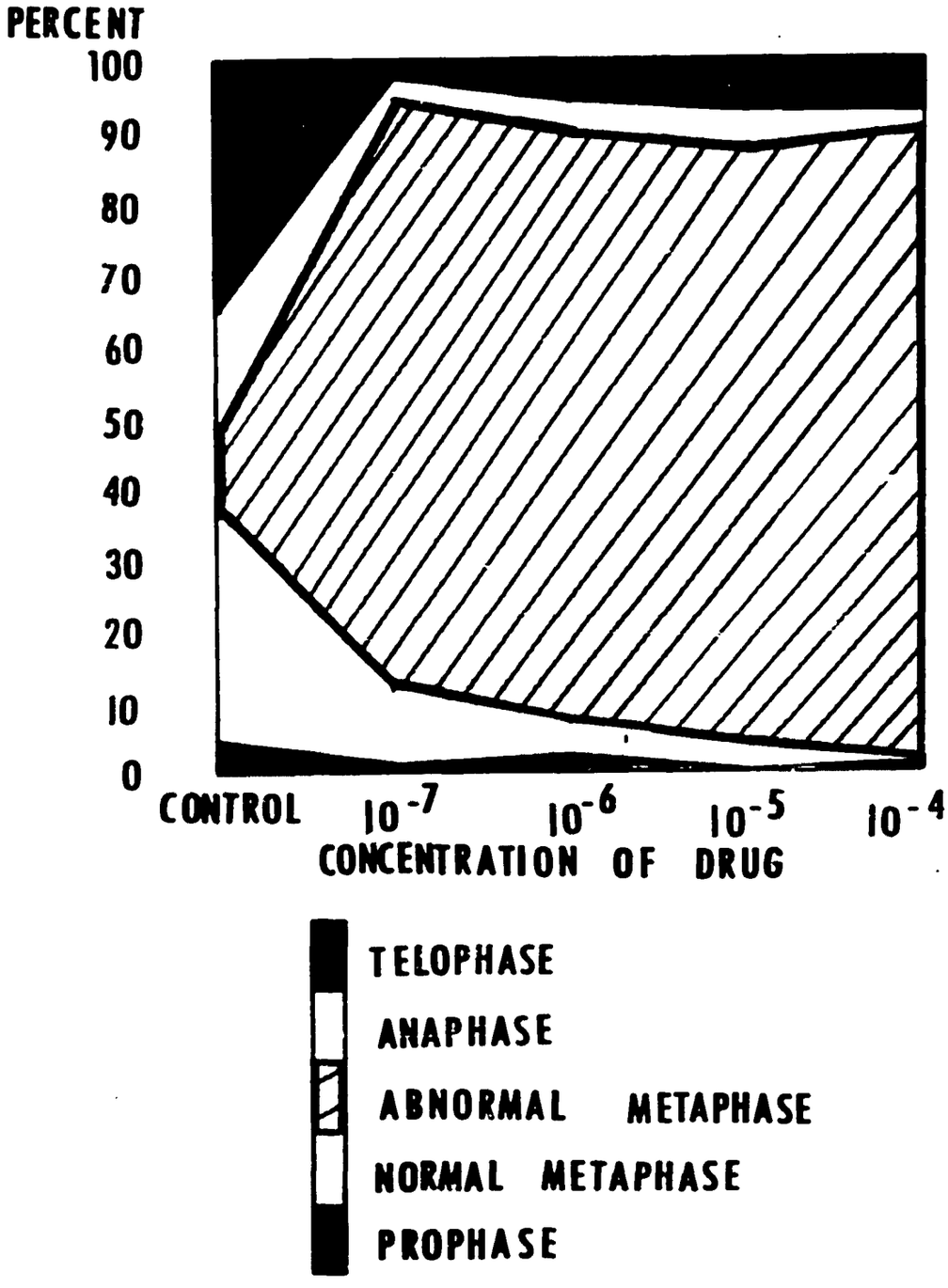


FIGURE 6 THE ACTION OF 16-KETO- ESTRADIOL ON THE MITOTIC DISTRIBUTION OF CHICK EMBRYO FIBROBLASTS

TABLE 42

ACTION OF 3-METHYLESTRADIOL-3,16a ON THE MITOTIC INDEX AND THE MITOTIC DISTRIBUTION IN CHICK EMBRYO FIBROBLASTS

A. THE MITOTIC INDEX

	Total Cell Count (1)	Mitotic Cell Count			Mitotic Index		
		Normal (2)	Abnormal (3)	Total (4)	Normal (5)	Abnormal (6)	Total (7)
Control	51,875	162	14	176	320.2	3705.3	300.1
Propylene Glycol	47,382	122	15	137	388.4	3159.8	345.9
10^{-4}	39,952	382	208	690	104.5	129.7	57.9
10^{-5}	47,840	641	441	1082	74.6	108.4	43.3
10^{-6}	27,685	123	21	144	225.0	1318.3	192.2
10^{-7}	22,549	101	27	128	223.2	464.7	176.1

By dividing the total cell count in column 1 by the mitotic cell counts in columns 2, 3, and 4, we obtain the mitotic indices as given in columns 5, 6, and 7.

B. THE MITOTIC DISTRIBUTION

	Prophase	Metaphase		Anaphase	Telophase
		Normal	Abnormal		
Control	16 9.9%	74 42.1%	14 7.9%	44 25.0%	28 15.1%
Propylene Glycol	20 14.6%	53 39.0%	15 10.9%	27 19.6%	22 16.2%
10^{-4}	28 4.0%	283 40.7%	308 44.9%	35 5.1%	36 5.2%
10^{-5}	30 2.8%	496 45.8%	441 40.8%	79 7.3%	36 3.3%
10^{-6}	20 13.9%	44 30.5%	21 14.6%	28 19.5%	31 21.5%
10^{-7}	27 21.1%	33 25.5%	27 21.1%	22 16.9%	19 15.4%

The upper figure in each space represents the number of mitoses counted; the lower figure gives their percentage value.

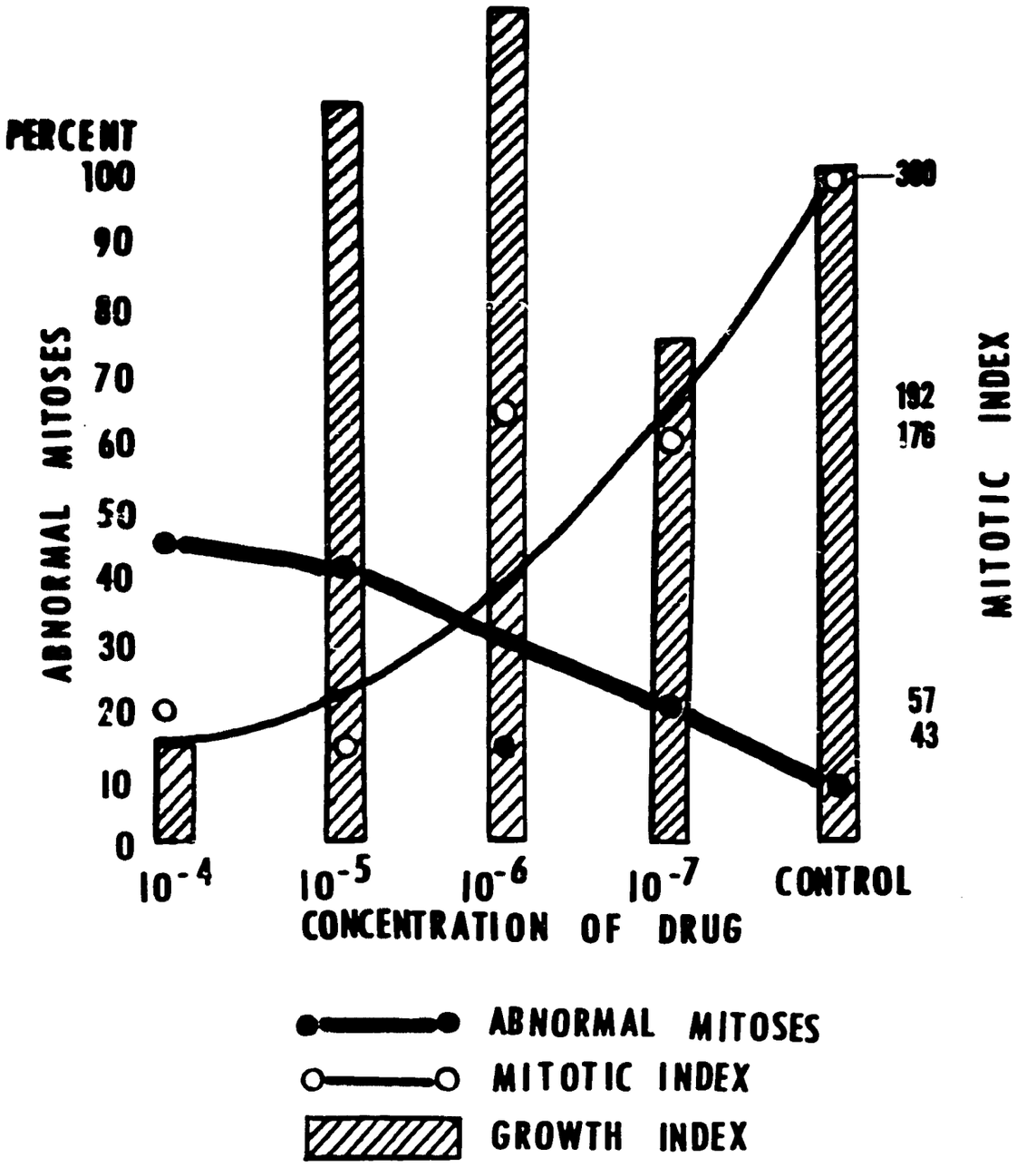


FIGURE 7 THE ACTION OF 3-METHYLESTRADIOL - 3,16α ON GROWTH AND MITOSIS OF CHICK EMBRYO FIBROBLASTS

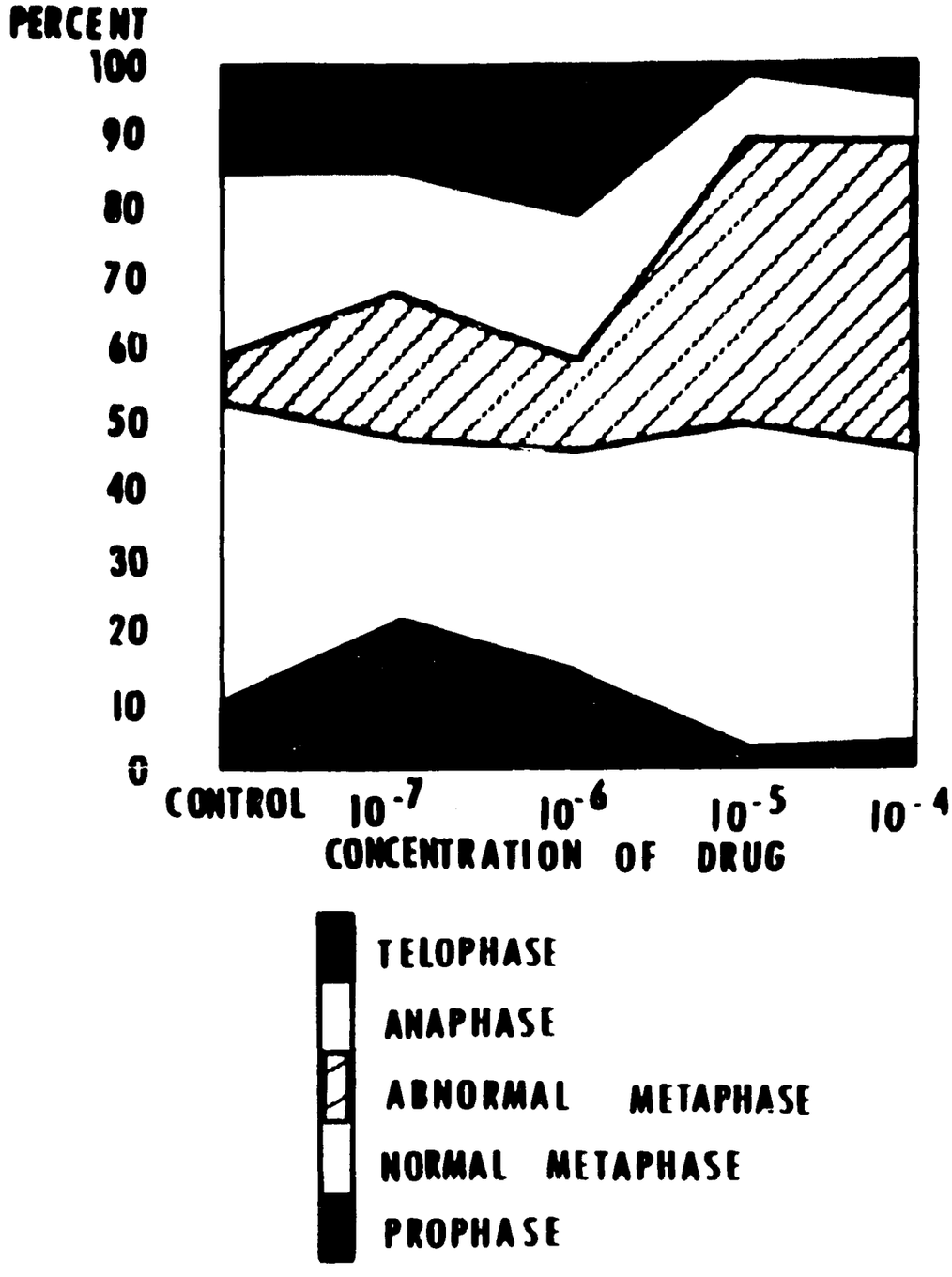


FIGURE 8 THE ACTION OF 3-METHYLESTRADIOL - 3,16α ON THE MITOTIC DISTRIBUTION OF CHICK EMBRYO FIBROBLASTS

TABLE 43

ACTION OF 3-METHYL-16-KETO-ESTRADIOL ON THE MITOTIC INDEX AND THE MITOTIC DISTRIBUTION IN CHICK EMBRYO FIBROBLASTS

A. THE MITOTIC INDEX

	Total Cell Count (1)	Mitotic Cell Count			Mitotic Index		
		Normal (2)	Abnormal (3)	Total (4)	Normal (5)	Abnormal (6)	Total (7)
Control	27,249	60	9	69	454.1	3027.6	394.9
Propylene Glycol	55,190	74	8	82	748.5	6893.7	673.0
10^{-4}	18,746	5	468	473	3669.2	40.0	39.2
10^{-5}	39,270	41	386	427	957.7	101.7	91.2
10^{-6}	32,667	26	265	291	1256.4	123.2	112.2
10^{-7}	25,491	50	179	229	509.8	142.4	111.3

By dividing the total cell count in column 1 by the mitotic cell counts in columns 2, 3, and 4, we obtain the mitotic indices as given in columns 5, 6, and 7.

B. THE MITOTIC DISTRIBUTION

	Prophase	Metaphase		Anaphase	Telophase
		Normal	Abnormal		
Control	3 4.3%	22 32.0%	9 12.9%	11 15.9%	24 34.9%
Propylene Glycol	8 9.7%	25 30.6%	8 9.7%	17 20.9%	24 29.1%
10^{-4}	3 0.6%	0 0.0%	473 99.0%	1 0.2%	1 0.2%
10^{-5}	4 1.0%	7 1.5%	386 90.5%	9 2.1%	21 4.9%
10^{-6}	0 0.0%	3 1.1%	265 91.0%	10 3.4%	13 4.5%
10^{-7}	2 1.9%	31 14.2%	178 77.7%	9 3.6%	6 2.4%

The upper figure in each space represents the number of mitoses counted; the lower figure gives their percentage value.

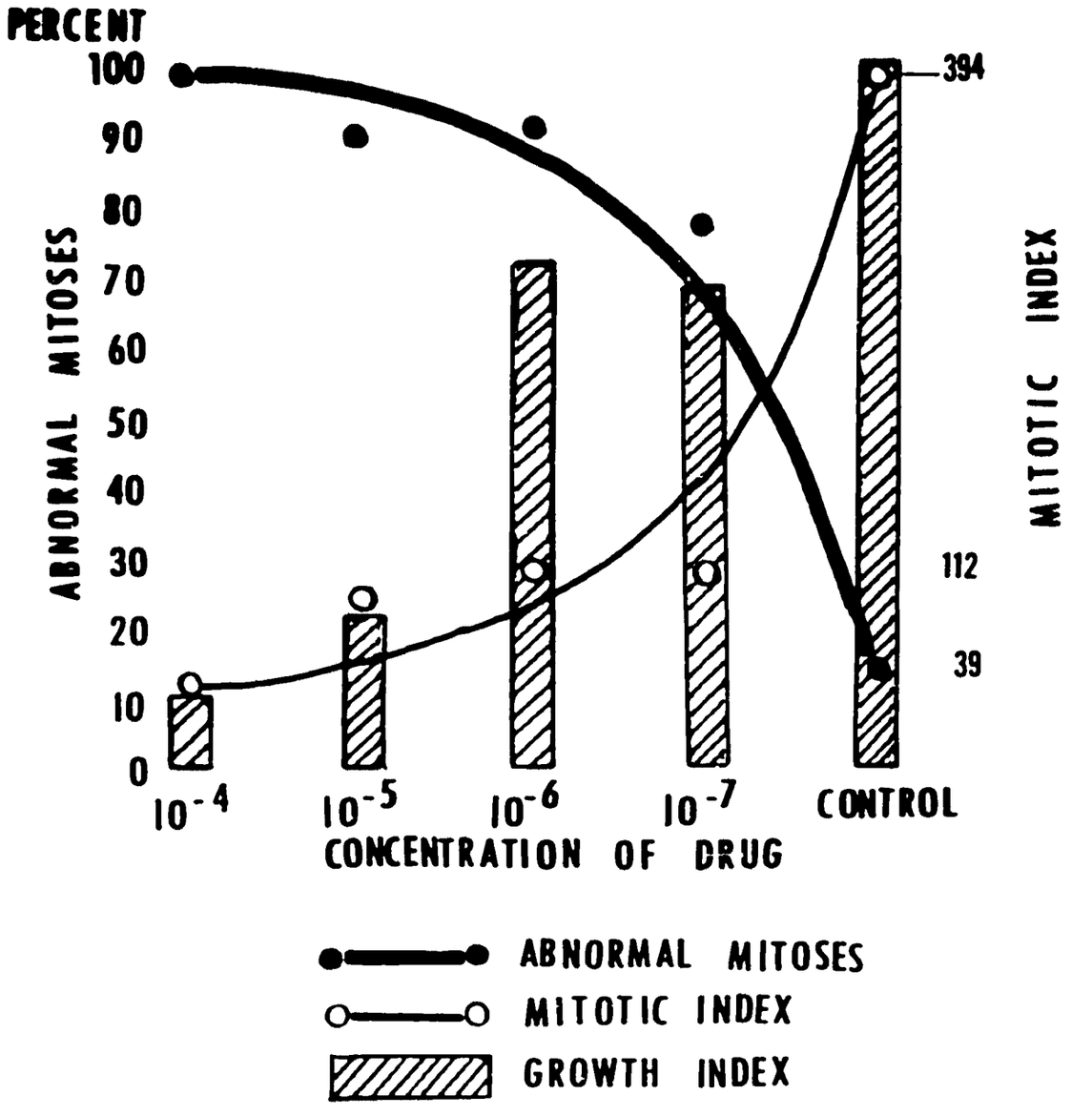


FIGURE 9 THE ACTION OF 3-METHYL-16-KETO-ESTRADIOL ON GROWTH AND MITOSIS OF CHICK EMBRYO FIBROBLASTS

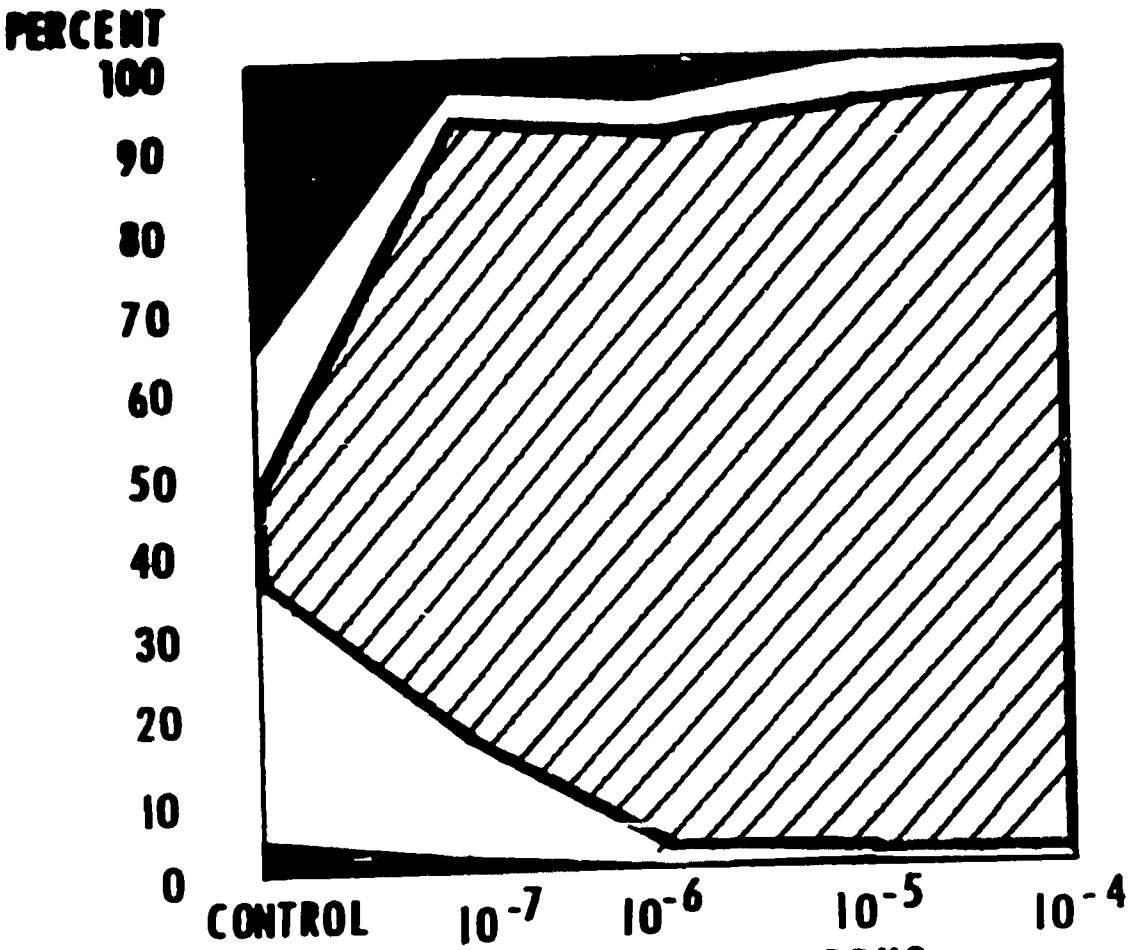


FIGURE 10 THE ACTION OF 3-METHYL-16-KETO-ESTRADIOL ON THE MITOTIC DISTRIBUTION OF CHICK EMBRYO FIBROBLASTS

TABLE 44

ACTION OF 3-ETHYL-16-KETO-ESTRADIOL ON THE MITOTIC INDEX AND THE MITOTIC DISTRIBUTION IN CHICK EMBRYO FIBROBLASTS

A. THE MITOTIC INDEX

	Total Cell Count (1)	Mitotic Cell Count			Mitotic Index		
		Normal (2)	Abnormal (3)	Total (4)	Normal (5)	Abnormal (6)	Total (7)
Control	28,076	190	22	212	147.7	1276.2	132.4
Propylene Glycol	45,505	223	30	253	204.0	1516.8	179.8
10^{-4}	4,454	8	155	163	556.8	28.7	27.3
10^{-5}	10,032	36	702	738	278.7	14. 3	13.6
10^{-6}	12,126	1	144	145	12126.0	84.2	83.7
10^{-7}	15,612	63	74	137	247.8	210.9	113.9
10^{-8}	23,849	114	30	144	209.2	794.9	165.6

By dividing the total cell count in column 1 by the mitotic cell counts in columns 2, 3, and 4, we obtain the mitotic indices as given in columns 5, 6, and 7.

B. THE MITOTIC DISTRIBUTION

	Prophase	Metaphase		Anaphase	Telophase
		Normal	Abnormal		
Control	31 14.6%	66 31.1%	22 10.4%	43 20.3%	50 23.6%
Propylene Glycol	30 11.8%	93 36.8%	30 11.8%	46 18.2%	54 21.4%
10^{-4}	1 0.6%	4 2.4%	155 95.1%	2 1.3%	1 0.6%
10^{-5}	5 0.7%	8 1.1%	702 95.1%	10 1.4%	13 1.7%
10^{-6}	0 0.0%	1 0.7%	144 99.3%	0 0.0%	0 0.0%
10^{-7}	10 7.3%	28 20.4%	74 54.0%	16 11.5%	9 6.6%
10^{-8}	15 10.5%	54 36.0%	30 20.8%	25 17.3%	24 16.6%

The upper figure in each space represents the number of mitoses counted; the lower figure gives their percentage value.

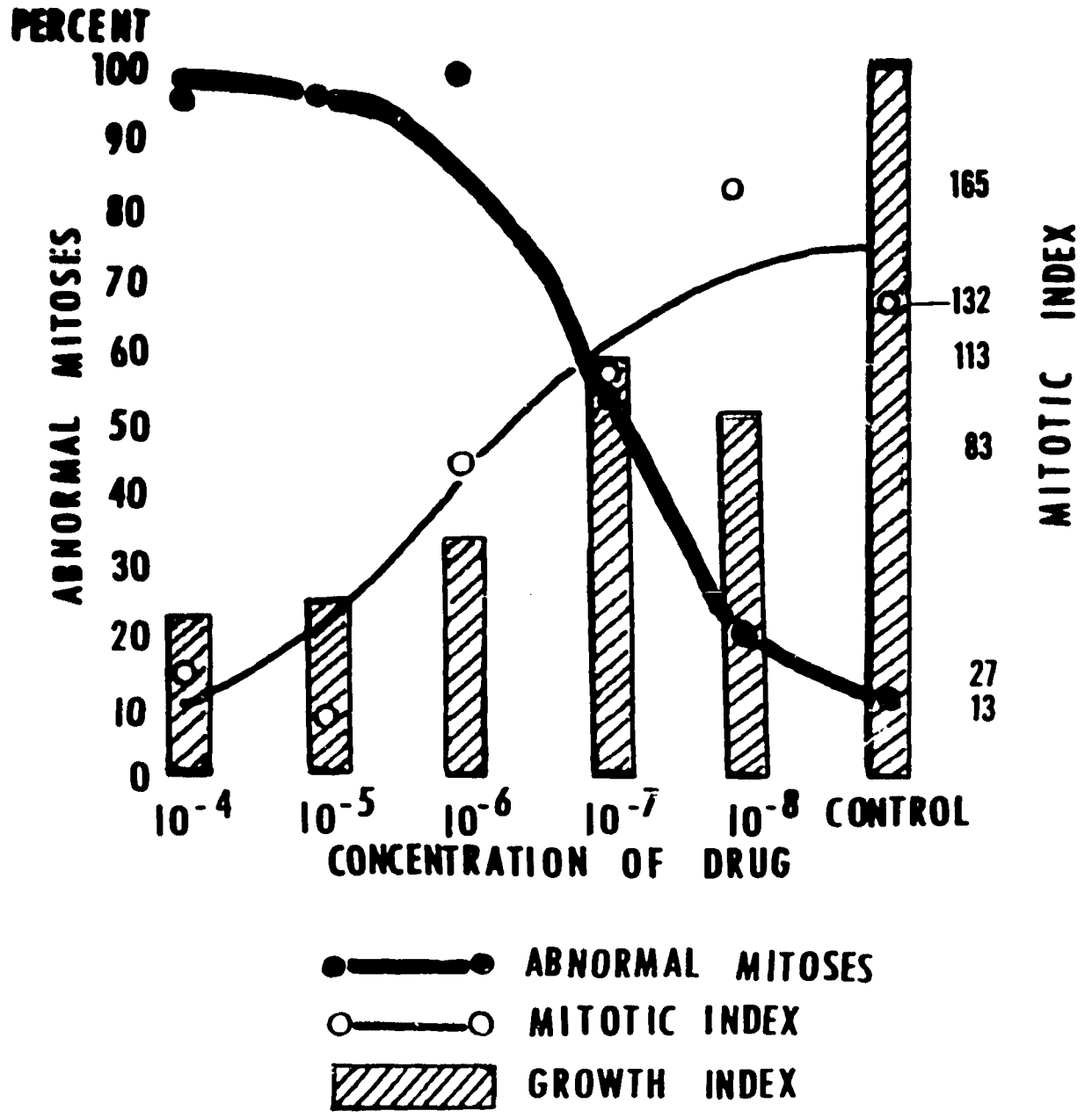


FIGURE 11 THE ACTION OF 3-ETHYL-16-KETO-ESTRADIOL ON GROWTH AND MITOSIS OF CHICK EMBRYO FIBROBLASTS

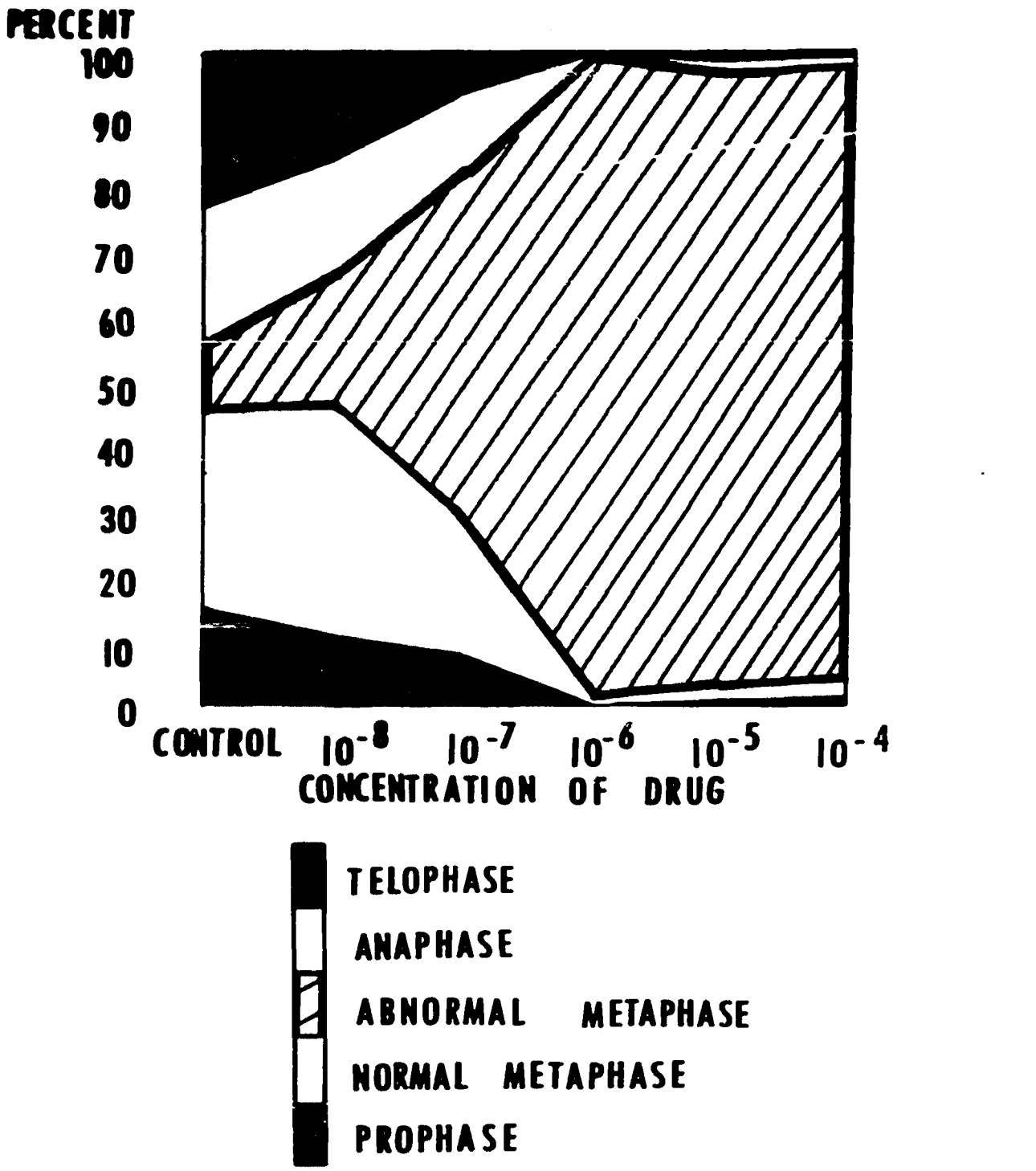


FIGURE 12 THE ACTION OF 3-ETHYL-16-KETO-ESTRADIOL ON THE MITOTIC DISTRIBUTION OF CHICK EMBRYO FIBROBLASTS

TABLE 45

ACTION OF 16-KETO-ESTRONE ON THE MITOTIC INDEX AND THE MITOTIC DISTRIBUTION IN CHICK EMBRYO FIBROBLASTS

A. THE MITOTIC INDEX

	Total Cell Count (1)	Mitotic Cell Count			Mitotic Index		
		Normal (2)	Abnormal (3)	Total (4)	Normal (5)	Abnormal (6)	Total (7)
Control	27,249	60	9	69	454.1	3027.6	394.9
Propylene Glycol	55,190	74	8	82	748.5	6898.7	673.0
10^{-4}	47,779	35	193	228	1365.1	247.5	209.5
10^{-5}	11,448	10	101	111	1144.8	113.3	103.1
10^{-6}	13,841	15	39	54	922.7	354.9	256.3
10^{-7}	39,620	63	130	193	628.8	304.8	205.3

By dividing the total cell count in column 1 by the mitotic cell counts in columns 2, 3, and 4, we obtain the mitotic indices as given in columns 5, 6, and 7.

B. THE MITOTIC DISTRIBUTION

	Prophase	Metaphase		Anaphase	Telophase
		Normal	Abnormal		
Control	3 4.3%	22 32.0%	9 12.9%	11 15.9%	24 34.9%
Propylene Glycol	8 9.7%	25 30.6%	8 9.7%	17 20.9%	24 29.1%
10^{-4}	2 0.9%	4 1.8%	193 84.6%	13 5.7%	16 7.0%
10^{-5}	2 1.8%	1 0.9%	101 90.8%	0 0.0%	7 6.3%
10^{-6}	1 1.8%	7 13.0%	39 72.2%	0 0.0%	7 12.8%
10^{-7}	2 1.3%	14 7.3%	130 67.3%	19 9.8%	28 14.5%

The upper figure in each space represents the number of mitoses counted; the lower figure gives their percentage value.

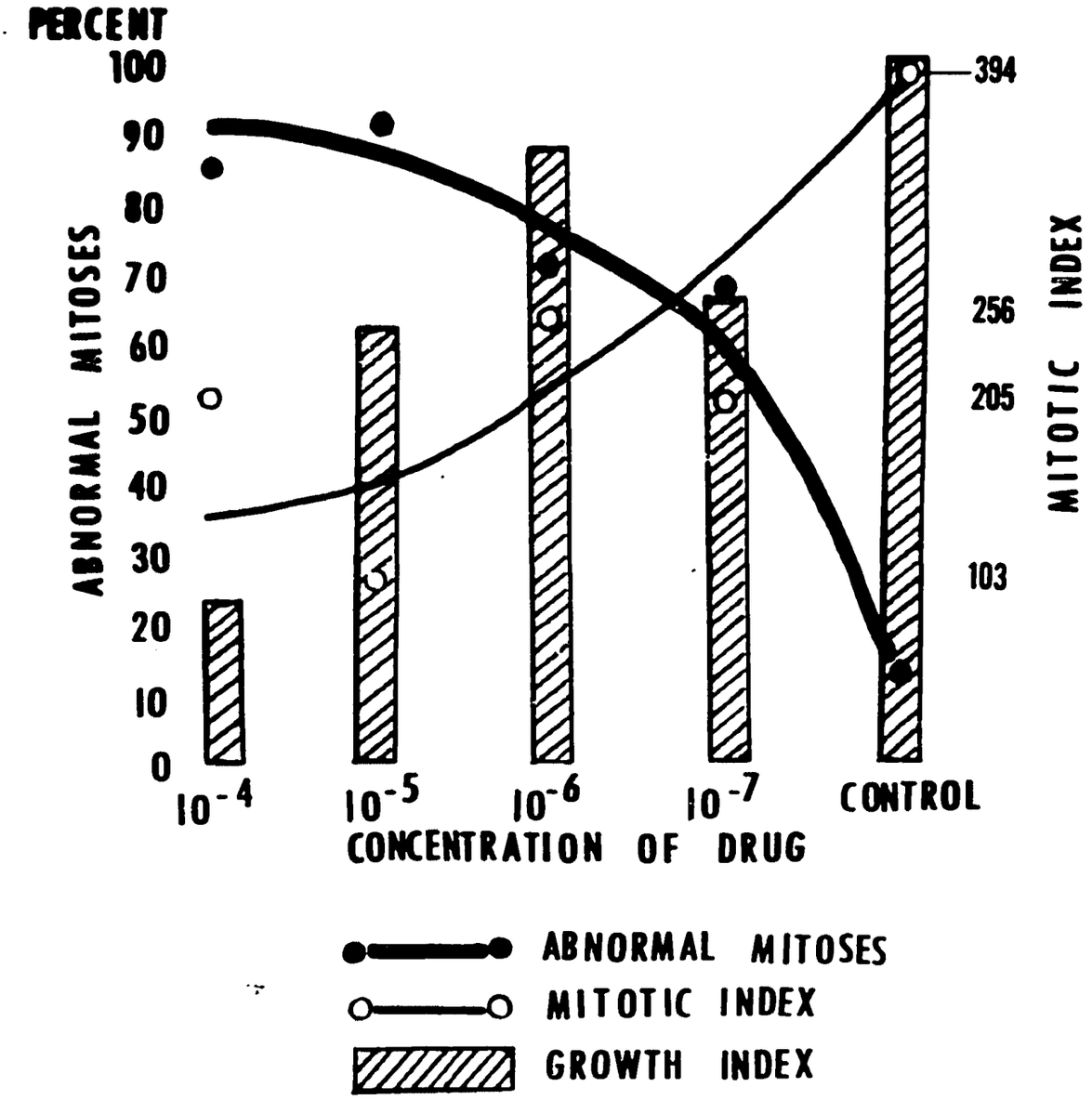


FIGURE 13 THE ACTION OF 16-KETO-ESTRONE ON GROWTH AND MITOSIS OF CHICK EMBRYO FIBROBLASTS

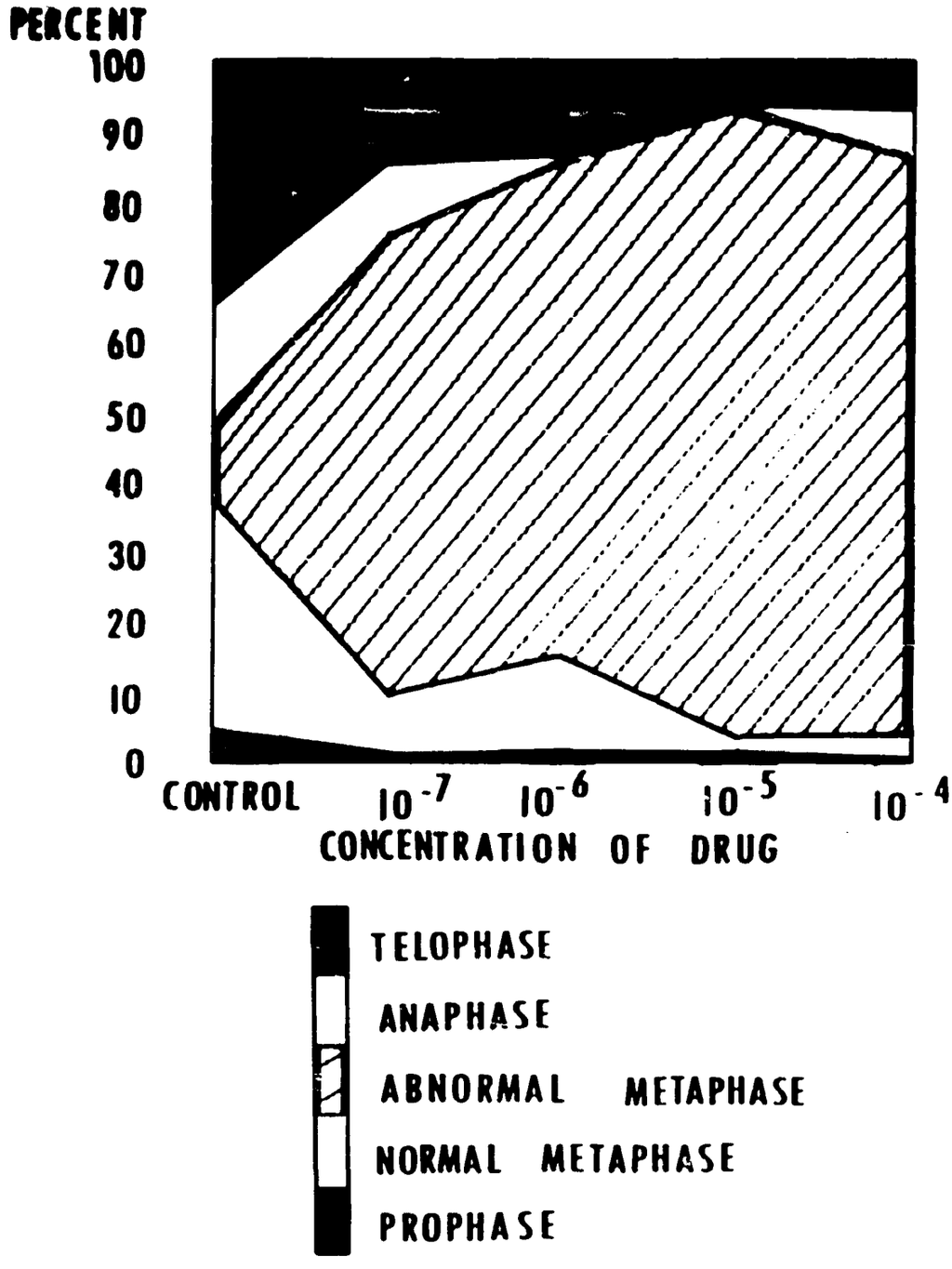


FIGURE 14 THE ACTION OF 16-KETO-ESTRONE ON THE MITOTIC DISTRIBUTION OF CHICK EMBRYO FIBROBLASTS

TABLE 46

ACTION OF 3-METHYL-16-KETO-ESTRONE ON THE MITOTIC INDEX AND THE MITOTIC DISTRIBUTION IN CHICK EMBRYO FIBROBLASTS

A. THE MITOTIC INDEX

	Total Cell Count (1)	Mitotic Cell Count			Mitotic Index		
		Normal (2)	Abnormal (3)	Total (4)	Normal (5)	Abnormal (6)	Total (7)
Control	51,875	162	14	176	320.2	3705.8	300.1
Propylene Glycol	47,392	122	15	137	388.4	3158.8	345.9
10^{-4}	57,606	207	51	258	278.3	1129.5	223.2
10^{-5}	30,475	92	11	103	331.2	2770.4	295.8
10^{-6}	54,483	254	26	280	214.5	2095.5	194.6
10^{-7}	55,910	243	22	265	230.0	2541.4	210.9

By dividing the total cell count in column 1 by the mitotic cell counts in columns 2, 3, and 4, we obtain the mitotic indices as given in columns 5, 6, and 7.

B. THE MITOTIC DISTRIBUTION

	Prophase	Metaphase		Anaphase	Telophase
		Normal	Abnormal		
Control	16 9.9%	74 42.1%	14 7.9%	44 25.0%	28 15.1%
Propylene Glycol	20 14.6%	53 39.0%	15 10.9%	27 19.6%	22 16.2%
10^{-4}	31 12.0%	77 29.9%	51 19.7%	41 15.9%	58 22.5%
10^{-5}	14 13.7%	46 44.6%	11 10.7%	18 17.3%	14 13.7%
10^{-6}	35 12.5%	104 37.1%	26 9.3%	49 17.5%	66 23.6%
10^{-7}	23 8.7%	107 40.4%	22 8.2%	48 17.1%	65 25.6%

The upper figure in each space represents the number of mitoses counted; the lower figure gives their percentage value.

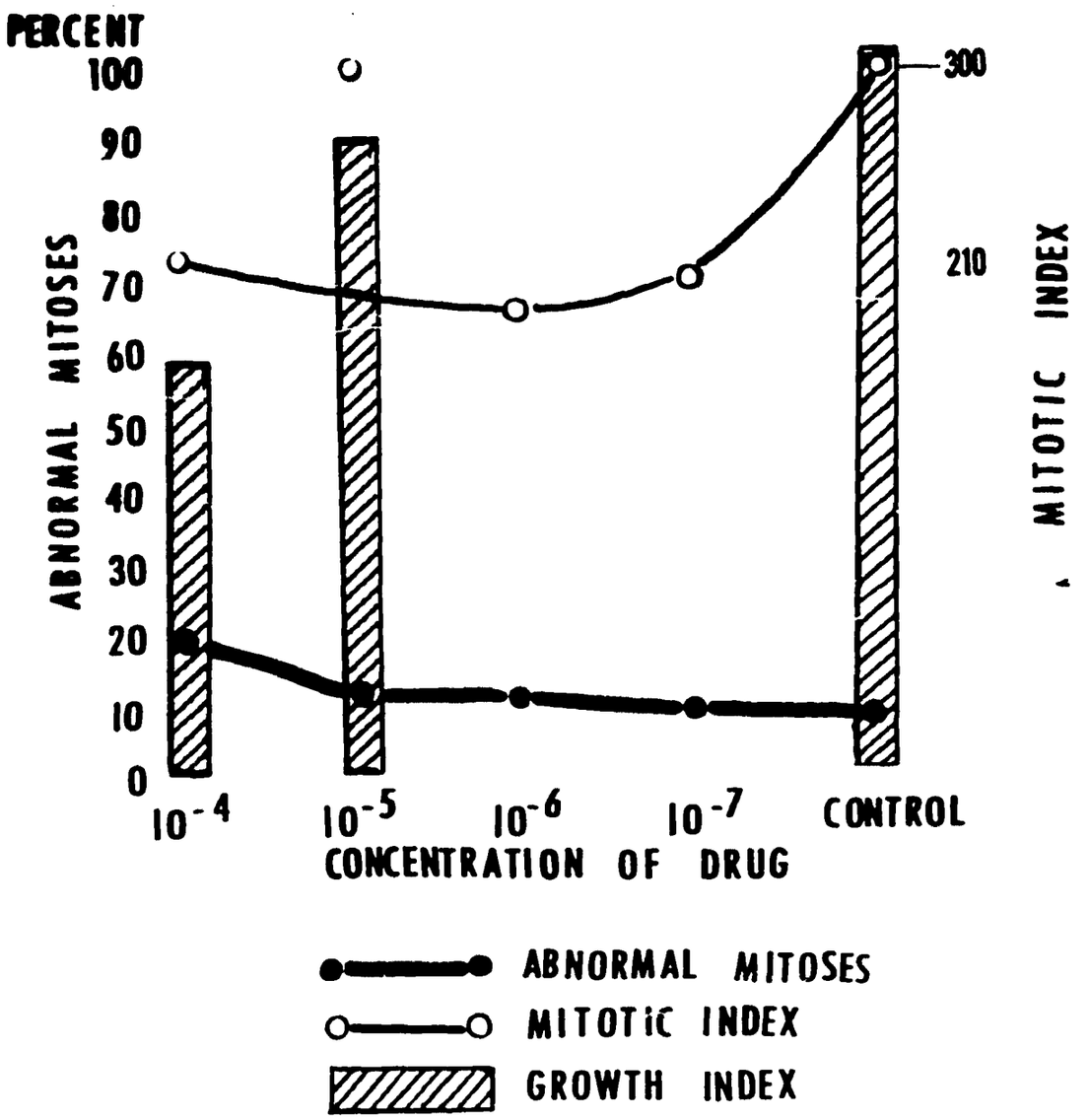


FIGURE 15 THE ACTION OF 3-METHYL-16-KETO-ESTRONE ON GROWTH AND MITOSIS OF CHICK EMBRYO FIBROBLASTS

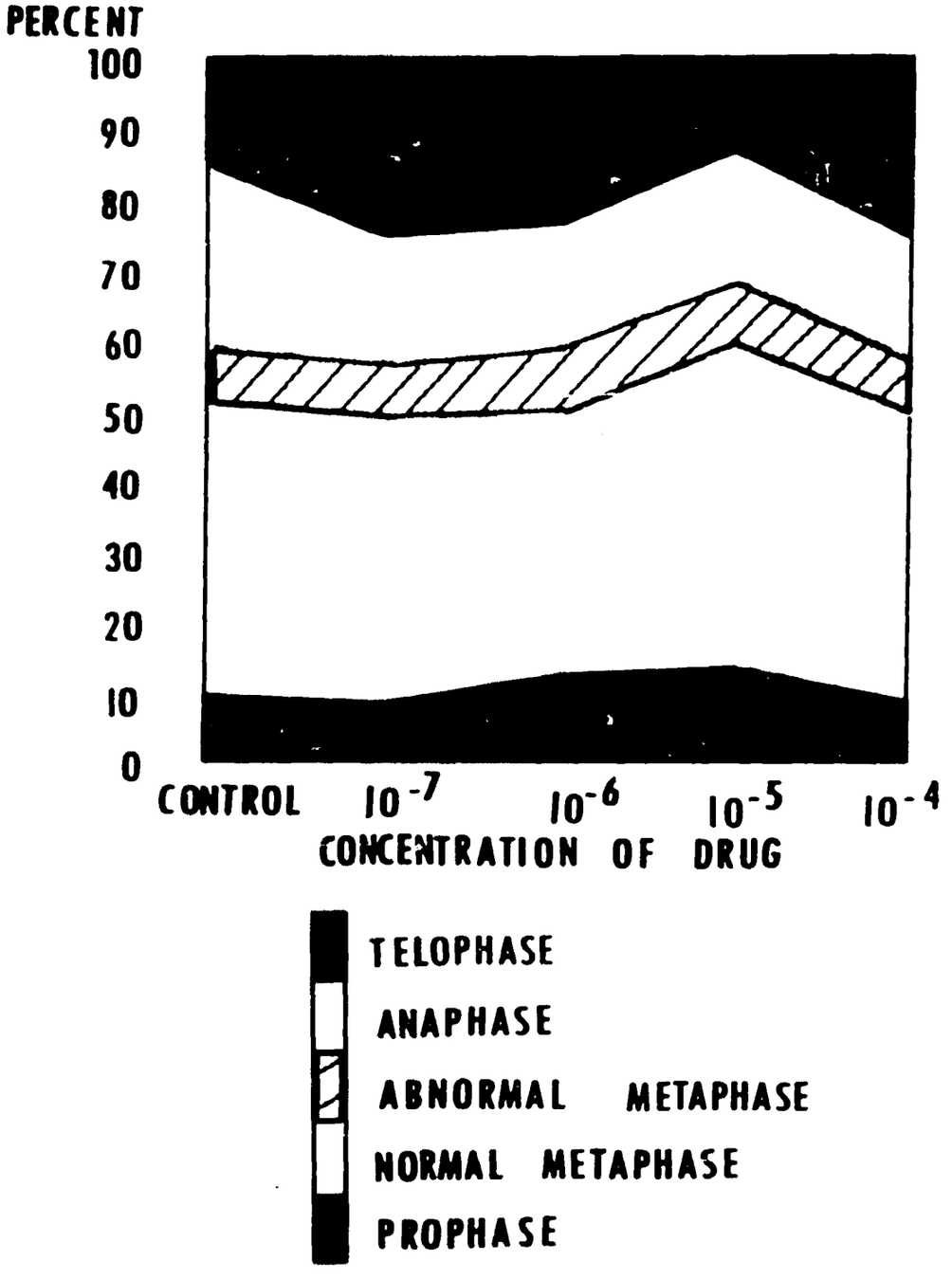


FIGURE 16 THE ACTION OF 3-METHYL-16-KETO-ESTRONE ON THE MITOTIC DISTRIBUTION OF CHICK EMBRYO FIBROBLASTS

It will be noted that these mitotic indices reveal the following information:

(a) The incidence of the number of normal mitoses is low in the cultures treated with a high concentration of the steroidal compounds, while the inverse is true in the incidence of abnormal mitoses.

(b) The incidence of mitoses of both normal and abnormal type is more frequent in the counts of cells treated with higher concentrations of the compounds.

(c) The difference in the mitotic indices between that of the control group and that of the group containing 1:10,000 of the steroid in the experimental group in the majority of cases is proportional to approximately the antimitotic action of that steroid.

The differences observed in the values of the mitotic indices of cultures as we go from one concentration of the experimental compound to the next are much more marked in those compounds which show greater antimitotic action, and much less in compounds that are less active.

Comparison of the relative potency of the various compounds as to their ability to inhibit growth, produce abnormal mitosis, and alter the mitotic index may be seen on the graphs in Figures 1 to 16.

The Metaphase/Prophase Ratio

The following Table 47 shows the relation of the metaphase/prophase ratios of mitotic cells treated with the various concentrations of the compounds studied.

It may be noted that there is a tendency for a marked decline in the values of the M/P ratios as we pass from cells exposed to higher

TABLE 47

THE METAPHASE/PROPHASE RATIO

	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	Control
Colchicine	*	6.6	3.3	4.9	2.6	1.7
Diethylstilbestrol	153.0	58.3	32.6	2.3	3.05	3.4
3-Ethyl-16-keto-estradiol	159.0	142.0	*	10.2	2.7	2.8
16-Keto-estradiol	336.0	*	49.5	56.5		10.3
3-Methyl-16-keto-estradiol	157.7	99.2	*	104.5		10.3
16-Keto-estrone	88.5	51.0	46.0	72.0		10.3
3-Methylestradiol-3,16a	21.1	31.2	3.2	2.2		5.5
3-Methyl-16-keto-estrone	4.2	4.08	3.64	5.65		5.5

*These values could not be obtained as no prophase divisions were encountered.

concentrations of steroids to those exposed to lower concentrations. This is particularly true in case of the more active antimitotic compounds, such as 3-ethyl-16-keto-estradiol, 16-keto-estradiol, and diethylstilbestrol.

The gradient of this decline approaches the horizontal in the less active steroids, such as 3-methyl-16-keto-estrone. The values of the ratios were also much higher in the more active steroids. This is indicative of suppression of the metaphase stage of division by producing an extension of the time required for the completion of this phase, or even causing the arrest of mitoses in this stage of division.

The absolute lack of any prophase stages in four isolated groups of cultures is also an indication of some suppression of the actual in-

itiation of prophase, so that some poisoning effect may already be present in the interphase or resting cells.

CHAPTER V

DISCUSSION

In the first series of studies (see Tables 1 to 18), the steroids were dissolved in propylene glycol of standard grade. Almost invariably no growth of cells was obtained at concentrations of 1:10,000 and frequently none occurred at 1:100,000. Furthermore, the controls which were done with propylene glycol alone invariably demonstrated some inhibition. It is believed that some toxic contaminent was present in the raw propylene glycol which produced this action and that this was synergistic in its ability to augment greatly the inhibitory action of the steroids at even very low concentrations. That such action is possible has been shown by Lettré (1954). The synergistic component is believed to be accumulative in its formation, since the last two control experiments employing propylene glycol alone showed greater toxicity than those done one and two years before (see Tables 19 to 22).

In contrast to this, when chemically pure propylene glycol was obtained, no toxic effect was noted (see Tables 23 and 24); in fact, the controls containing 1.0% propylene glycol which were run with the various steroids indicated a stimulatory effect of approximately 10% to 15%. Apparently the toxic factor was not present in the chemically pure solvent. The stimulatory action may have resulted from the utilization of the

glycol by the cells as a source of nutriment. According to Ladell (1938) ethyl alcohol is capable of acting as a growth stimulant at low concentrations, and it may be that chemically pure propylene glycol may produce a similar effect.

The synergistic effect on growth inhibition and on antimitotic action which was so evident in the experiment employing estrone-17 dissolved in the standard grade of propylene glycol was entirely lacking when the chemically pure propylene glycol was employed with this same steroid.

The Metaphase/Prophase Ratio

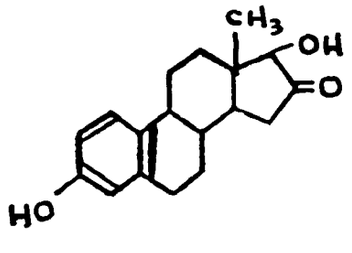
Analysis of the data indicates that a significant increase in the value of the metaphase/prophase ratio (M/P) occurs with an increase in the concentration of the steroids as well as their relative antimitotic capacity. The average M/P for control cultures was 4.7, with a range that varies from 1.7 to 10.3. Reference to the finding of Fardon and Prince (1952) reveals that in computing the M/P of normal epithelial cells from the crypts of Lieberkuhn, they arrived at an average of 3.5 with a range that varied from 0.1 to 8.0.

The sudden response of the mitotic tissue culture cell to the action of the various steroids employed in these studies is reflected in the sharp rise of the M/P ratio. This increase is indicative of c-mitosis in which there is spindle inhibition which first prolongs the metaphase stage in the lower concentrations and then produces metaphase arrest in the higher concentrations. This ratio was increased 56.8 times with 3-ethyl-16-keto-estradiol at 1:10,000 and persisted at almost this level at concentrations of 1:1,000,000.

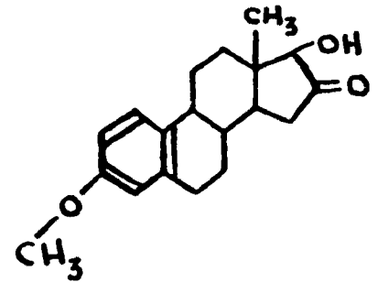
In the least active steroid employed, 3-methyl-16-keto-estrone, the M/P ratio actually dropped to a value slightly below that of the control; this is perhaps indicative of a slight prolongation of prophase, but no very significant metaphase disturbance.

The Relationship of Dosage to Antimitotic Action

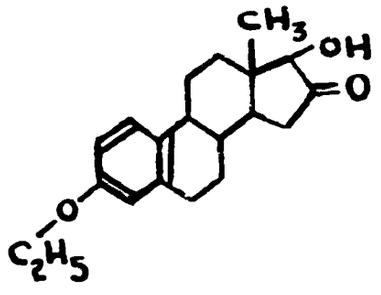
While all compounds employed in these studies produced an increase in the percentage of abnormal mitoses that paralleled their concentration in the media, there were certain steroids that were less dependent upon their concentrations in their mitotic poisoning action. This is particularly evident in a number of the steroids with the 16-keto configuration, namely, 16-keto-estradiol, 3-ethyl-16-keto-estradiol, 3-methyl-16-keto-estradiol, and 16-keto-estrone. All four of these steroids produced



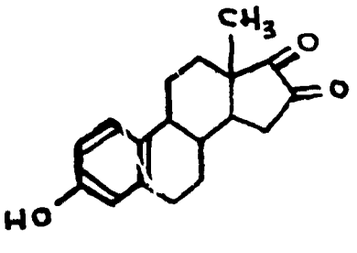
16-keto-estradiol



3-methyl-16-keto-estradiol



3-ethyl-16-keto-estradiol



16-keto-estrone

mitotic arrests and disturbances in over 50% of the cells in mitosis at a concentration of 1:10,000,000. This stands in agreement with the observations by von Möllendorff (1942) in which he showed that in their

antimitotic action, estrogenic steroids are less dependent upon their concentration than are those of the androgenic series.

The dosage of the various epimers of estradiol required for antimitotic action compares well with those given by Bodenstein (1954), who reported that a concentration of 1:100,000 to 1:1,000,000 produced numerous metaphase arrests and other types of disturbances.

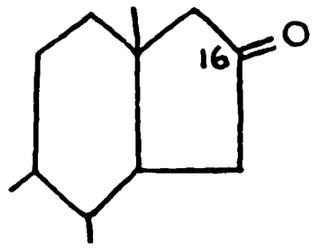
The variations encountered in the degree of antimitotic activity at the higher concentrations of some of the more active compounds may have been produced by factors other than merely experimental deviations. The ability of the cells to absorb the steroid molecules and the ability of the cells to metabolize or detoxify them may require some consideration. However, the capacity of the steroid and its metabolites to interfere with internal cellular processes, particularly that of cell division, is the factor that will receive principal consideration here.

The slopes of the curves which indicate the decline of antimitotic action with decrease in concentration also reflect the degree of refractility that the tissues exhibited to the various compounds. 3-Methyl-estradiol-3,16a and 3-methyl-16-keto-estrone in particular displayed refractility by the tissue.

The Relationship of Structural Configuration to Antimitotic Action

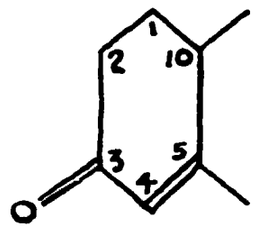
Unsaturated Bonds

Structural configuration and the presence of unsaturated bonds are related to the ability of a steroid to induce antimitotic activity. The presence of a ketone at C_{16} position (C_{16}=O) was a common character-



Ketone group at carbon 16 position

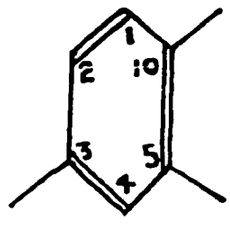
istic of five estrogen members, four of which displayed marked antimitotic activity. von Möllendorff (1942) showed that a ketone at C_3 position (C_3=O), together with a double bond between position C_4 and C_5 (C_4=C_5)



Ketone group at carbon 3 position

were common characteristics in androgens, such as methyltestosterone and testosterone, which produced a high percentage of mitotic disturbances in tissue culture.

All of the estrogens studied for their action on mitosis have unsaturated bonds in the first benzene ring between positions C_1 and C_2, C_3 and C_4, C_5 and C_{10}.



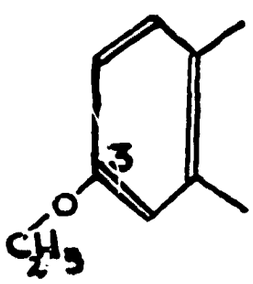
Unsaturated bonds in the first benzene ring

The greater effective action demonstrated by the unsaturated 16-keto steroids named is also in agreement with the findings of von Möllendorff (1941) that steroidal compounds, in which carbon atoms are saturated by hydrogenation, are ineffective as mitotic poisons. This perhaps accounts for the activity and usefulness of diethylstilbestrol as an antimitotic agent.

Carcinogenic agents have also beenknown to give rise to mitotic disturbances, and it was postulated by von Möllendorff (1941) that while cells may successfully detoxify some of the naturally occurring steroidal compounds by the process of hydrogenation, they are perhaps either incapable of dealing with carcinogens, or the enzymes that normally perform this function are absent or inhibited in cells that show a predisposition to the development of a neoplastic state.

Side Chains

The presence of side chains may also be a factor in the antimitotic capacity of certain steroidal compounds. Ether side chains were present on C_3 position in four of the compounds employed. Of these, two headed the list of compounds studied in their ability to act as antimi-



Ethyl ether side chain at carbon 3 position

totic agents and growth inhibitors. 3-Ethyl-16-keto-estradiol proved the most active, and this was immediately followed by 3-methyl-16-keto-

estradiol. The longer side chain of the ethyl ether may have been a factor in the display of greater antimitotic action of that steroid, while the methyl ether form, shortened by one methylene group, $-CH_2-$, was measurably less effective in its action. Seifriz (1951) has presented data which indicate that the toxicity of the alcohol series to cytoplasm increasss with the length of the molecular chain. This was further verified by Lettré et al (1952).

The estradiol configuration with an alcohol radical at position C_{17} in the majority of instances was more active than the estrone config-



Alcohol group at carbon 17 position

uration with a ketone at this position. Similar findings are reported by von Möllendorff (1942). In 3-methyl-estradiol-3,16a, however, the inhibitory action was markedly reduced and it is believed that the steroisomerism as evidenced by the alcohol at position C_{17} with the α form may have rendered this molecule less potent in its action. Since the α configuration normally is frequently encountered in excreted forms, it may be that the cells do not absorb it in sufficient amounts to be affected, or it is at least partially inert.

While no rigid rule can be established at this time as to the exact relationship that exists between the structural configuration of a steroid and its effectiveness as an antimitotic agent, there is consider-

able evidence that such a relationship does exist, and that by modification of its structure by the addition of side chains or varying its degree of saturation its potency may be altered.

CHAPTER VI

CONCLUSIONS

1. The several steroidal compounds employed in these studies displayed a capacity to interfere with the mitotic process in varying degrees.

2. This action was more evident in the estradiol epimers than in those of the estrone series. Some were considerably more potent than colchicine.

3. The action was initiated by the inhibition of the spindle mechanism and the production of c-mitoses, prolongation of metaphase, and metaphasic arrests.

4. The structural configuration of the steroids has a bearing on the potency of their antimitotic action. Their potency is enhanced by the presence of unsaturated bonds between the carbon atoms of the first benzene ring, the presence of a ketone at C_{16} and an alcohol group at C_{17}, the presence of ether side chains at position C_3, and the length of such side chains.

5. The action of a steroid with relatively mild inhibitory capacity may be enhanced by the synergistic action of another compound showing some inhibitory characteristics.

6. A number of the steroids, particularly those of the 16-keto

series, displayed an extension of a high degree of antimitotic action which was not entirely dependent on their concentration.

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EXPLANATION OF PLATES

All photographs on the following plates are of fibroblasts grown in tissue cultures employing eight- to ten-day old chick embryo heart tissue as explants. The photographic assembly consisted of a DeJur enlarger-Richoflex camera combination in conjunction with a Bausch and Lomb microscope fitted with 10x, 43x, and 93x oil immersion objectives and a 10x hyperplane occular lens. Köhler illumination was employed, using a Bausch and Lomb ribbon filament lamp fitted with a Corning polished glass filter #4015 (colorimeter yellow green). Exposures were made on Eastman Kodak Verichrome film #120 and processed in Eastman Microdol developer. Enlargements were prepared on #3 Kodabromide white glossy paper. An optical micrometer scale was employed to determine the final magnification on the enlarged print.

PLATE I

Photographs 1 and 2. Normal nuclei of cells in interphase. Nuclear membrane (NM) is clearly visible. Each nucleus contains two nucleoli. Aceto-orcein stain. Magnification x1000.

Photograph 3. Normal interphase nucleus with a single nucleolus. Aceto-orcein stain. Magnification x1000.

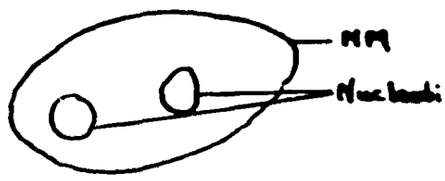
Photograph 4. Normal cell in interphase. Note two vacuoles (V) in cytoplasm. Aceto-orcein stain. Magnification x500.

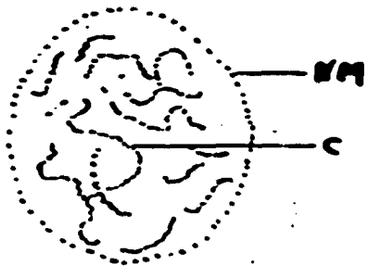
Photograph 5. Normal cell in early prophase. Chromosomes (C) are forming as long filaments and are distributed at random. The nuclear membrane (NM) is in the process of disappearing. Aceto-orcein stain. Magnification x2500.

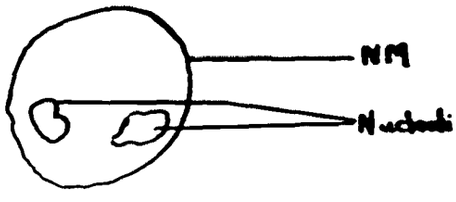
Photograph 6. Normal metaphase with chromosomes (C) arranged in equatorial plate. Aceto-orcein stain. Magnification x2500.

Photograph 7. Normal metaphase with the equatorial plate (EP) viewed from the side. Aceto-orcein stain. Magnification x2500.

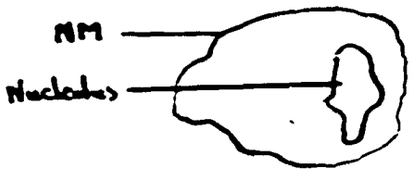
Photograph 8. Normal anaphase showing the daughter chromosomes (DC) being drawn toward the poles of the cell. Aceto-orcein stain. Magnification x2500.


NM
Nucleoli


NM
C


NM
Nucleoli


C


NM
Nucleolus


EP


V
V


DC
DC

PLATE I

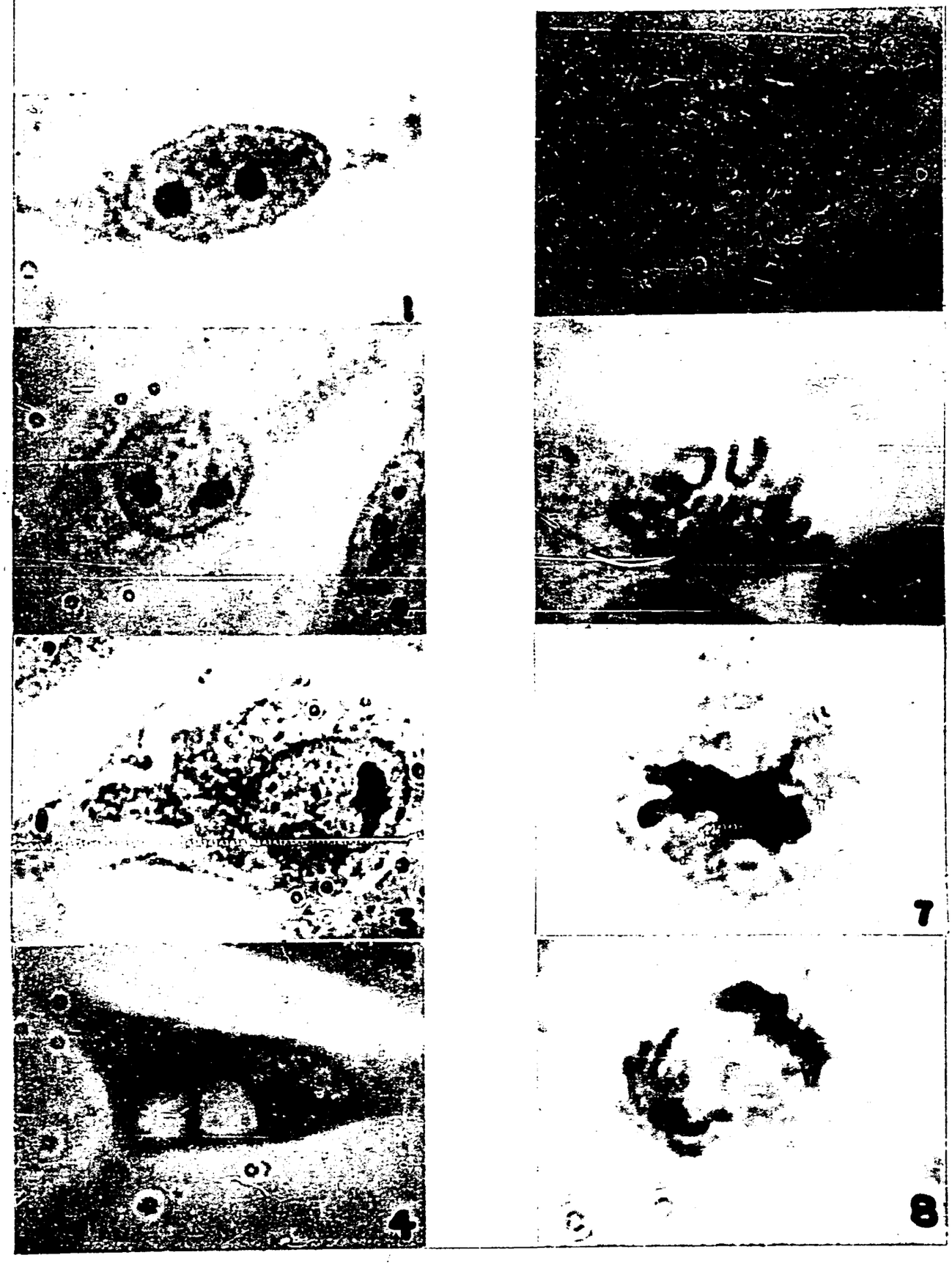
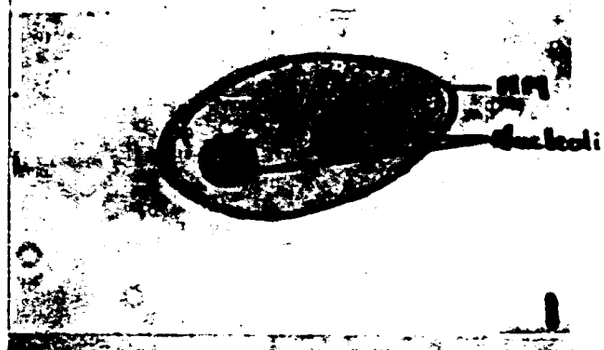
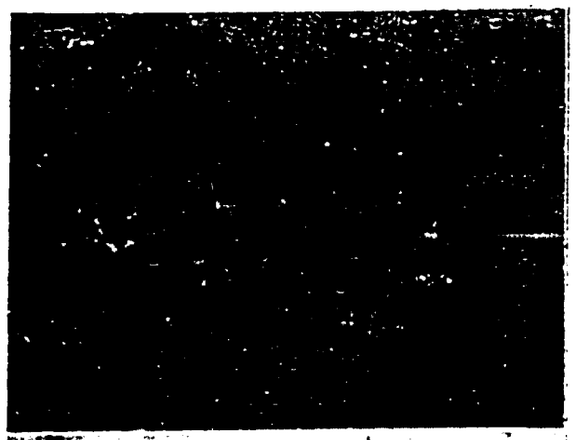
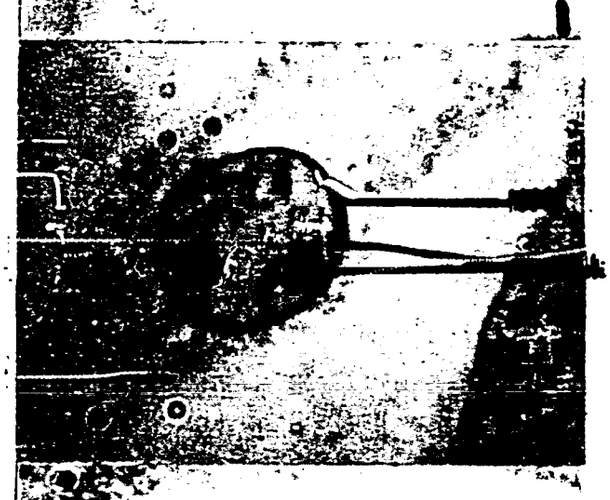


PLATE I













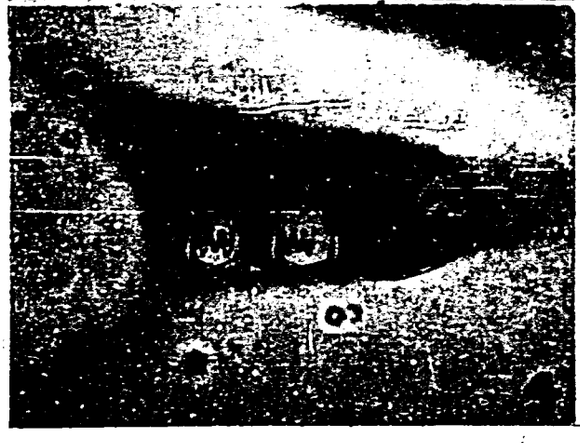




PLATE II

Photograph 9. Nucleotoxic effect produced by 3-methyl-16-keto-estradiol 1:100,000. Nuclei show excessive granulation of chromatin (GC). Aceto-orcein stain. Magnification x100.

Photograph 10. Nuclei with confluence of chromosomes (CG). 3-Methyl-16-keto-estradiol 1:100,000. Aceto-orcein stain. Magnification x100.

Photograph 11. Numerous metaphase arrests (MA) as indicated by darker staining cells. The ratio of mitoses to interphase cells is approximately 1 to 15. Diethylstilbestrol 1:10,000. Aceto-orcein stain. Magnification x100.

Photograph 12. Nuclei with confluence of chromatin (CC). Three pyknotic nuclei (P). Mitotic figure to the left shows clubbing of chromosomes. Diethylstilbestrol 1:10,000. Aceto-orcein stain. Magnification x100.

Photograph 13. Prophase nuclei with contracted pyknotic chromosomes (PC). 3-Methyl-16-keto-estradiol 1:100,000. Magnification x500. Aceto-orcein stain.

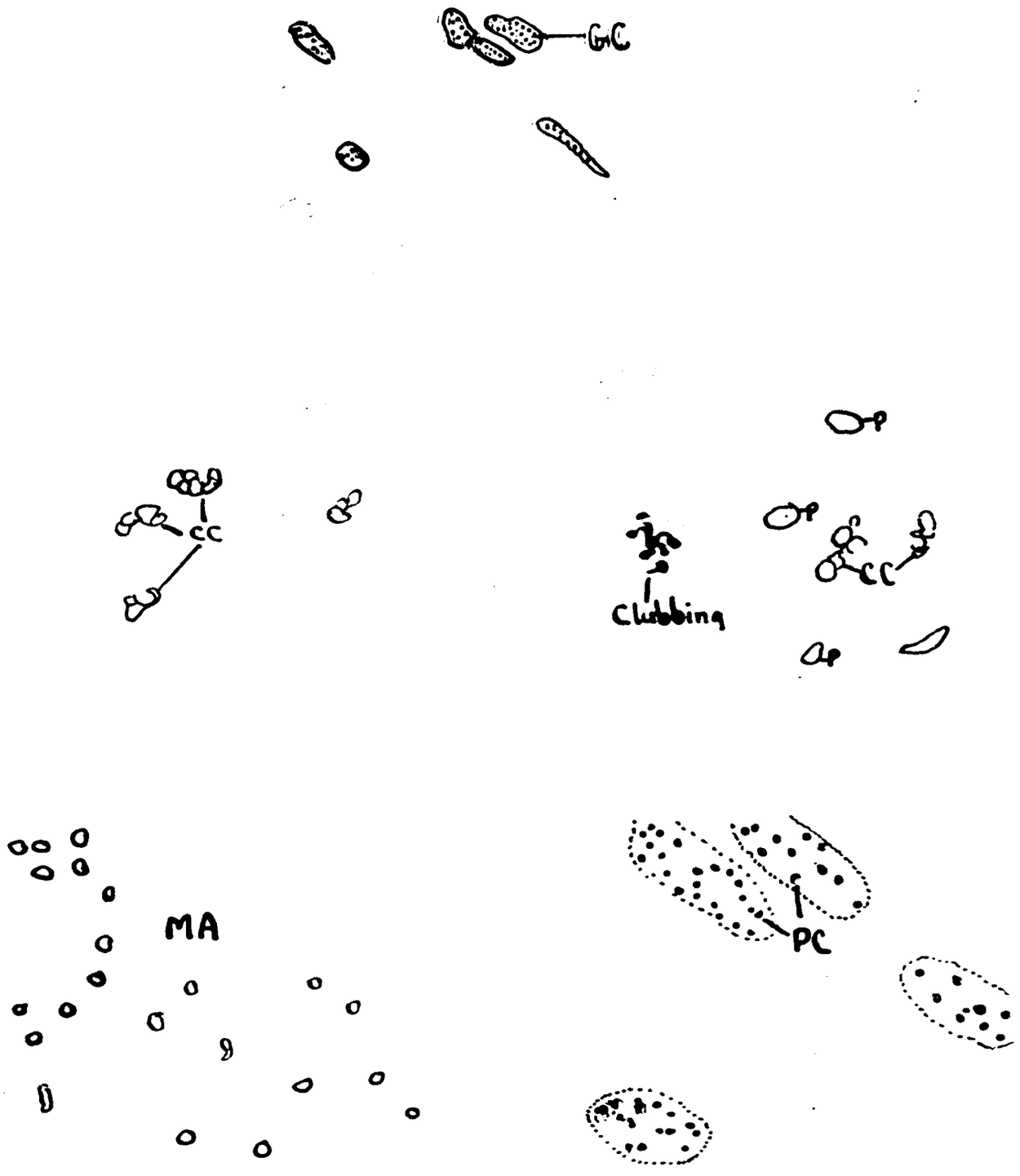
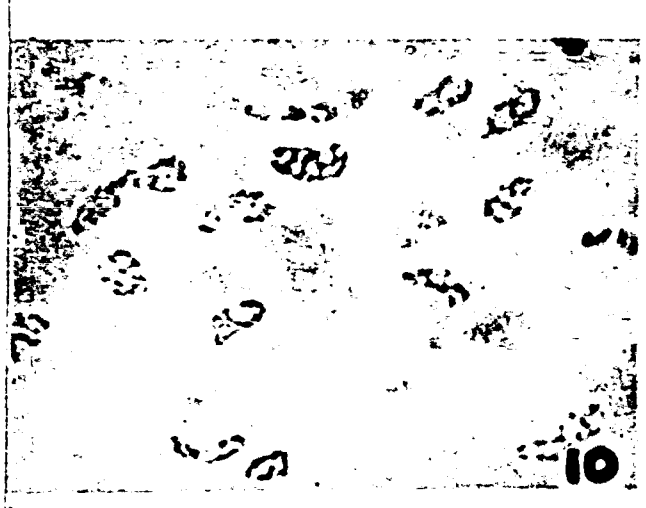

GC
CC
Clubbing
CC
MA
PC

PLATE II







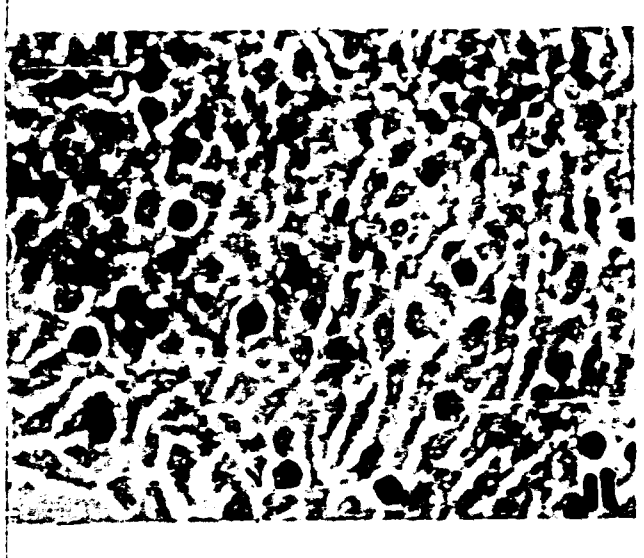




PLATE II









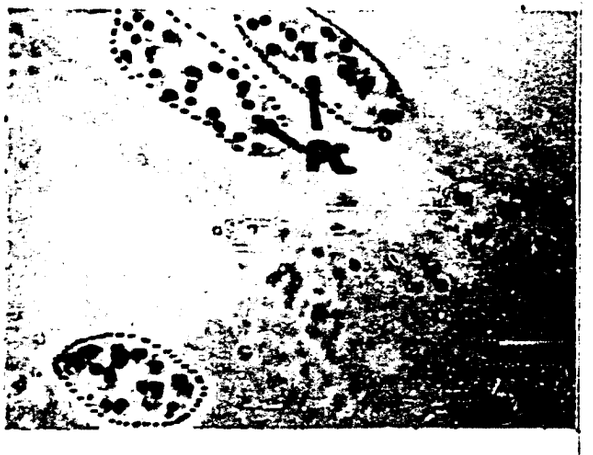


PLATE III

Photograph 14. Clubbed appearance of chromosomes (Cl) caused by shrinkage. The somewhat triangular orientation of the chromosomes may be a prelude to a triple division. 16-Keto-estrone 1:1,000,000. Aceto-orcein stain. Magnification x2500.

Photograph 15. Clubbed chromosomes (Cl). Some chromosomes appear to branch, indicating that they are sticking together (St) or agglutinating with sister chromosomes. 16-Keto-estradiol 1:1,000,000. Aceto-orcein stain. Magnification x2500.

Photograph 16. Confluence of chromosomes (CC)demonstrating the adhesive nature of their surface. 3-Methyl-16-keto-estradiol 1:100,000. Aceto-orcein stain. Magnification x1500.

Photograph 17. Confluence of chromosomes (CC). 3-Methyl-16-keto-estradiol 1:100,000. Aceto-orcein stain. Magnification x2500.

Photograph 18. Failure to form a metaphase plate. Some chromosomes have clubbed appearance (Cl). Others have left viscous strands (S) coursing through vacuole (V). 3-Methyl-16-keto-estradiol 1:100,000. Aceto-orcein stain. Magnification x1500.

Photograph 19. Lagging chromosome (LC) visible to the right of the metaphase plate. This chromosome had either become detached from the spindle fiber or the fiber lacked the capacity to pull it at the same rate as the others. 3-Ethyl-16-keto-estradiol 1:100,000. Aceto-orcein stain. Magnification x1000.

Cl.

CC

Cl.

S
Cl.

CC

PLATE III







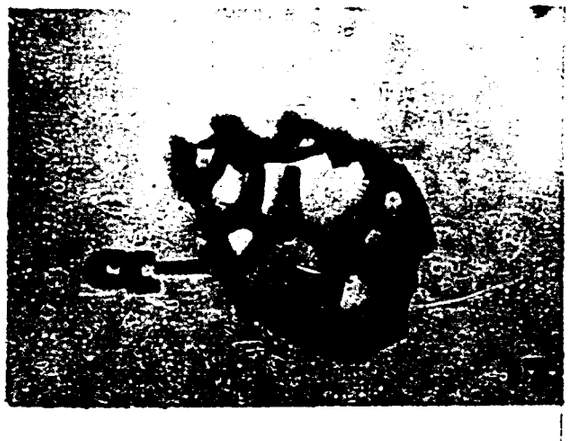






PLATE III





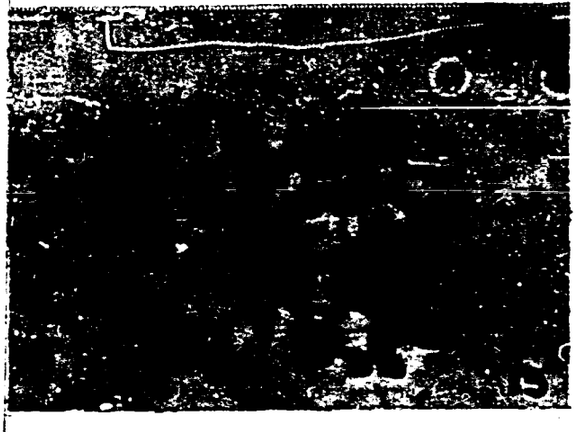
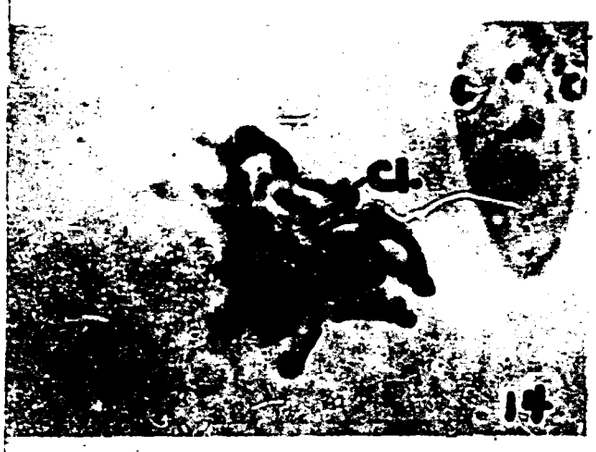








PLATE III



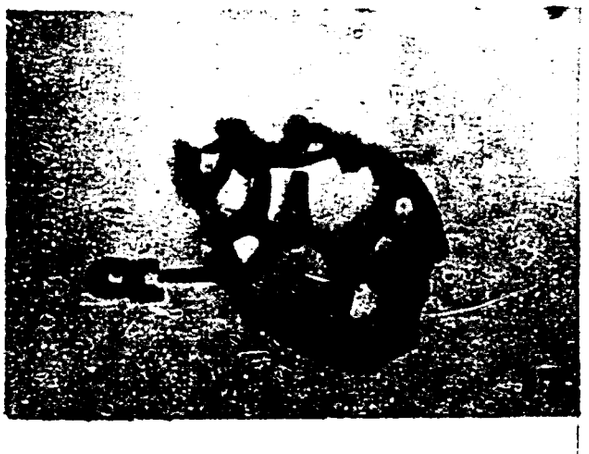










PLATE IV

Photograph 20. Chromosomes have retracted due to spindle disruption and are crowding together in the metaphase plate. 16-Keto-estrone 1:1,000,000. Aceto-orcein stain. Magnification x2500.

Photograph 21. More advanced crowding of pyknotic chromosomes (PC) into a smaller space. The clear halo about the chromosome mass is believed to be an aggregate of spindle sap (Sp. S.) which collected after the spindle was destroyed. 16-Keto-estrone 1:1,000,000. Aceto-orcein stain. Magnification x2500.

Photograph 22. Final coalescence of chromosomes into a pyknotic nucleus (PN) in which the spaces between the chromosomes have been almost completely obliterated. Diethylstilbestrol 1:100,000. Aceto-orcein stain. Magnification x2500.

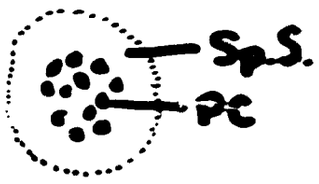
Photograph 23. A metaphase plate at the instant of entry into anaphase. Separation of the daughter chromosomes is being initiated. Two deleted chromosomes or chromosomal fragments (CF) are located to the left of the plate. 3-Ethyl-16-keto-estradiol 1:100,000. Aceto-orcein stain. Magnification x2500.

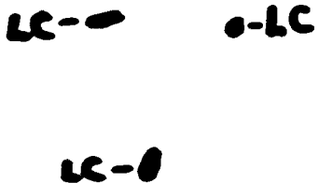
Photograph 24. A metaphase plate and three lagging chromosomes. 3-Methyl-16-keto-estradiol 1:100,000. Aceto-orcein stain. Magnification x2500.

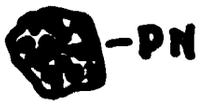
Photograph 25. A metaphase plate shifted toward a polar position with one deleted chromosome (Del. C.) and several chromosome fragments (CF). Colchicine 1:1,000,000. Iron hematoxylin stain. Magnification x1500.




CF-0


Sp.S.
PC


LC-
0-LC
LC-0


-PN

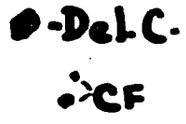

0-DeLC.
CF

PLATE IV



20



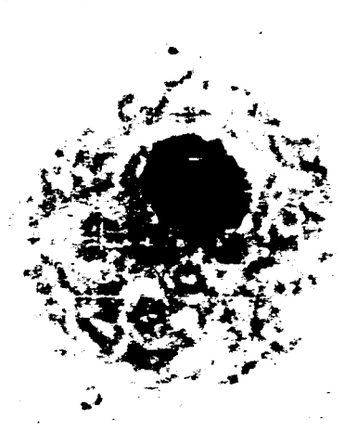
23



21



24

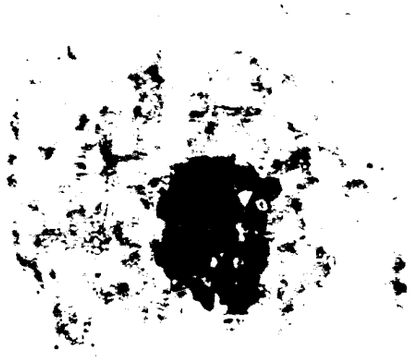


22



5

PLATE IV



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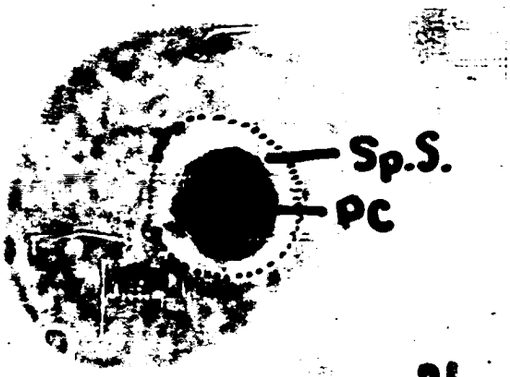

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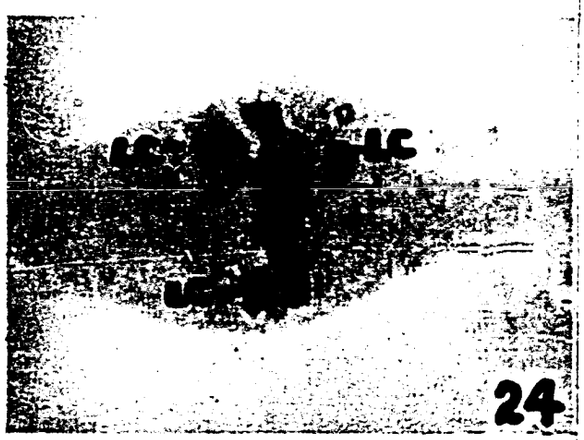

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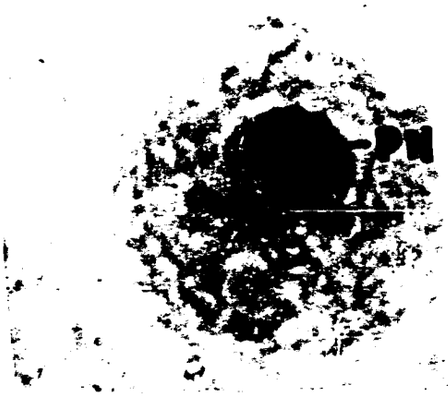


22



PLATE V

Photograph 26. Metaphase in a cell that appears to be entering tripolar division. Chromosomes are shortened and stubby (St. C.). 16-Keto-estrone 1:100,000. Aceto-orcein stain. Magnification x2500.

Photograph 27. Metaphase with two displaced chromosomes (DC). 16-Keto-estradiol 1:10,000. Aceto-orcein stain. Magnification x2500.

Photograph 28. A star metaphase. Chromosomes fused at extremities (FC). 3-Ethyl-16-keto-estradiol 1:1,000,000. Aceto-orcein stain. Magnification x1000.

Photograph 29. Metaphase plate (MP) displaced to a polar position. Chromosomes are pyknotic (PC). Cytoplasm is vacuolated (V). 3-Ethyl-16-keto-estradiol 1:100,000. Aceto-orcein stain. Magnification x2500.

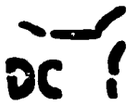
Photograph 30. Late metaphase showing stubbing of ends of chromosomes (St. C.) as they are shrinking. Spindle fibers (Sp. f.) are visible. 16-Keto-estradiol 1:10,000. Kedrovsky stain. Magnification x2500.

Photograph 31. Early metaphase showing disorganization of chromosomes and unequal distribution and density of chromatin. 16-Keto-estrone 1:100,000. Magnification x2500.


SrC


V
FC
MP

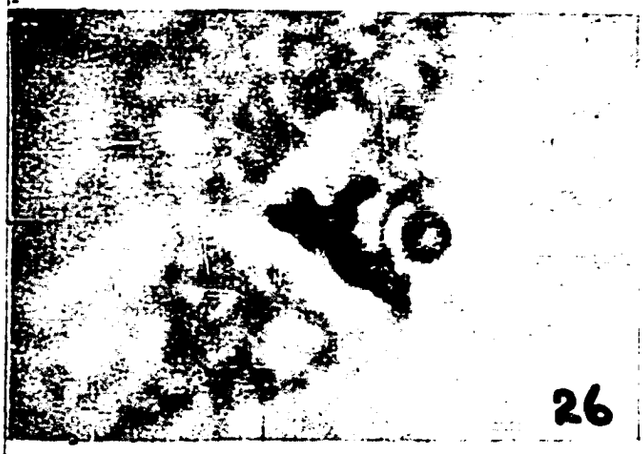

SrC
SpF

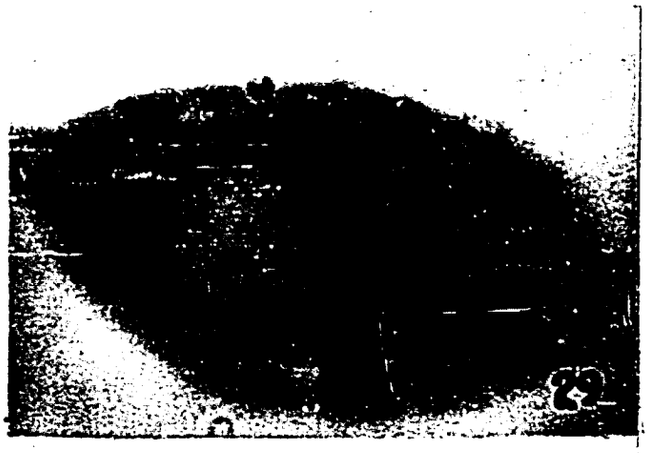

DC

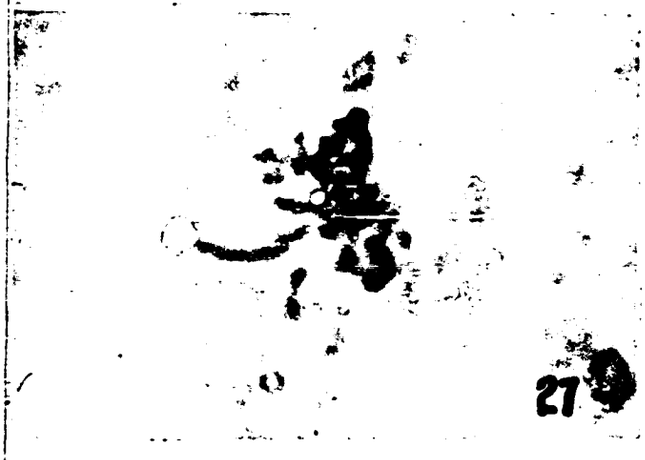

FC



PLATE V









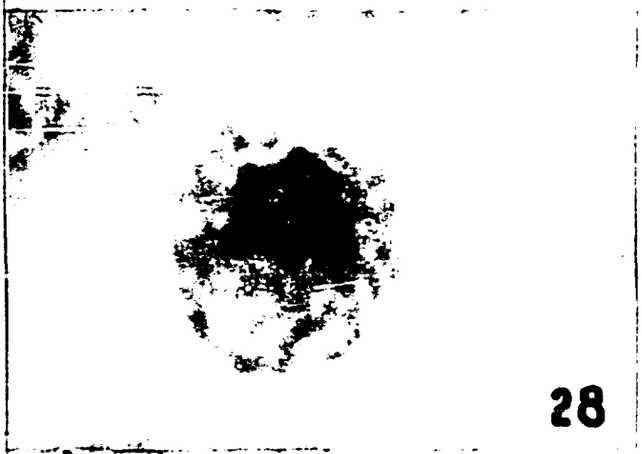




PLATE V









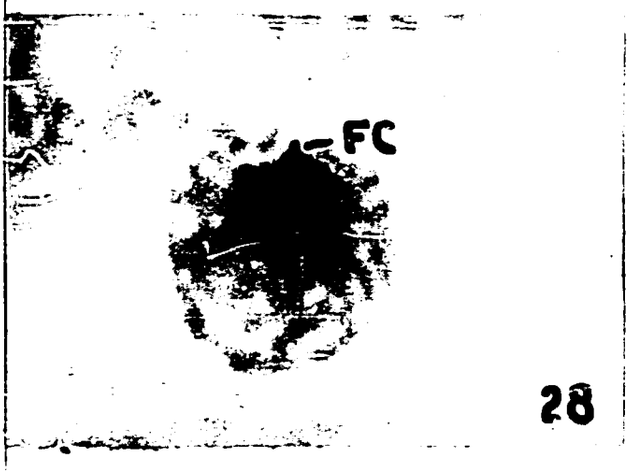




PLATE VI

Photograph 32. Prophase with numerous pyknotic chromosomes (PC). The three-lobed appearance of the nuclear outline may be indicative of a tripolar division. 3-Ethyl-16-keto-estradiol 1:100,000. Aceto-orcein stain. Magnification x2500.

Photograph 33. Metaphase division with spindle fibers visible. Two chromosomes to the right of the metaphase plate show disjunction (DC) due to spindle fiber breakage. Chromosome at the left side of plate shows separation of the two arms at the kinetochore (SC). 3-Ethyl-16-keto-estradiol 1:100,000. Aceto-orcein stain. Magnification x2500.

Photograph 34. Multipolar division with approximately six attraction centers (1, 2, 3, 4, 5, 6). 3-Ethyl-16-keto-estradiol. 1:100,000. Aceto-orcein stain. Magnification x2500.

Photograph 35. Disjunction of chromosomes (DC) in metaphase. 3-Ethyl-16-keto-estradiol I:100,000. Aceto-orcein stain. Magnification x2500.

Photograph 36. Pyknotic metaphase plate (PM) with some displaced (DC) and fragmented chromosomes (FC). 3-Ethyl -16-keto-estradiol 1:100,000. Aceto-orcein stain. Magnification x2500.

Photograph 37. Metaphase with displaced chromosomes (DC). 3-Ethyl-16-keto-estradiol 1:100,000. Aceto-orcein stain. Magnification x2500.

PC
DC
DC
SC
DC
DC
DC

PLATE VI













PLATE VII

Photograph 38. Anaphase with beaded chromosomes (BC) indicating zones of shrinkage. 3-Ethyl-16-keto-estradiol 1:100,000. Aceto-orcein stain. Magnification x2500.

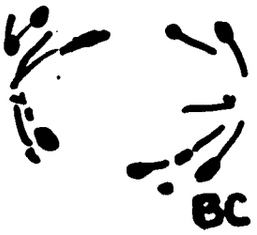
Photograph 39. Laterally displaced metaphase plate composed of short stubby chromosomes (St. C.). Cytoplasm of cell is vacuolated (V). 3-Methyl-16-keto-estradiol 1:1,000,000. Aceto-orcein stain. Magnification x2500.

Photograph 40. Pyknotic metaphase plate and dissociated chromosomes (DC). Diethylstilbestrol 1:1,000,000. Aceto-orcein stain. Magnification x2500.

Photograph 41. Pyknotic metaphase plate (PM) of uniform thickness and numerous detached chromosomes (DC). 16-Keto-estrone 1:100,000. Aceto-orcein stain. Magnification x2500.

Photograph 42. Pyknotic metaphase plate and many detached chromosomes (DC). 16-Keto-estrone 1:100,000. Aceto-orcein stain. Magnification x2500.

Photograph 43. Pyknotic metaphase plate (PM) with detached chromosomes (DC). 3-Methyl-16-keto-estradiol 1:100,000. Aceto-orcein stain. Magnification x2500.


BC


DC
DC
PM


StC
V


DC
DC


DC

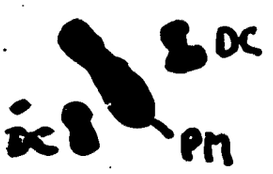
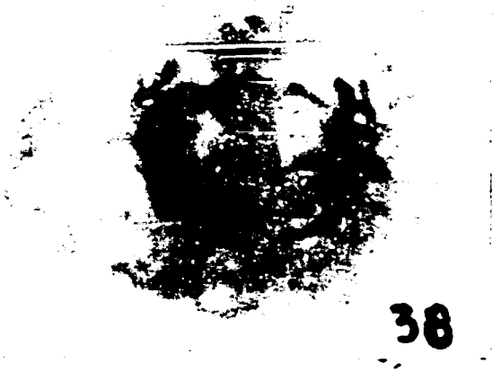

DC
DC
PM

PLATE VII









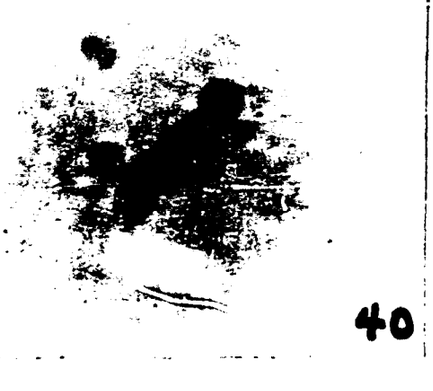




PLATE VII





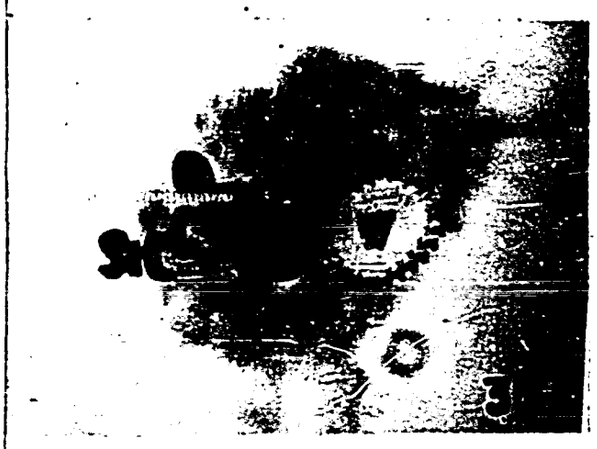








PLATE VIII

Photograph 44. Anaphase with tilted chromosome groups (TA) on right side indicating spindle fiber disruption on one side. 3-Ethyl-16-keto-estradiol 1:100,000. Aceto-orcein stain. Magnification x2500.

Photograph 45. Pyknosis of chromosome groups (PC) during anaphase and a dissociated group of chromosomes (DC) on right. Diethylstilbestrol 1:100,000. Aceto-orcein stain. Magnification x2500.

Photograph 46. Pyknotic chromosome groups (PC) in anaphase. Confluence is more accentuated. On right is a dissociated chromosome (DC). Diethylstilbestrol 1:10,000. Aceto-orcein stain. Magnification x2500.

Photograph 47. Binucleated cell indicating failure of cytoplasmic division. One nucleus is successfully reconstructed (RN) while the other is pyknotic (PN). 3-Methyl-16-keto-estradiol 1:100,000. Aceto-orcein stain. Magnification x2500.

Photograph 48. Reduced cell volume and clumping of chromatin (CC) in late telophase. Diethylstilbestrol 1:10,000. Aceto-orcein stain. Magnification x2500.

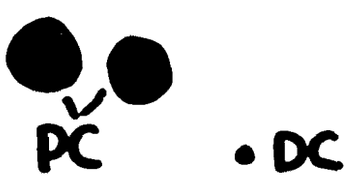
Photograph 49. Reduced cell volume and clumping of chromatin (CC) in late telophase. Colchicine 1:100,000. Aceto-orcein stain. Magnification x2500.






DC
IRC


CC
CC


PC
DC


CC

PLATE VIII







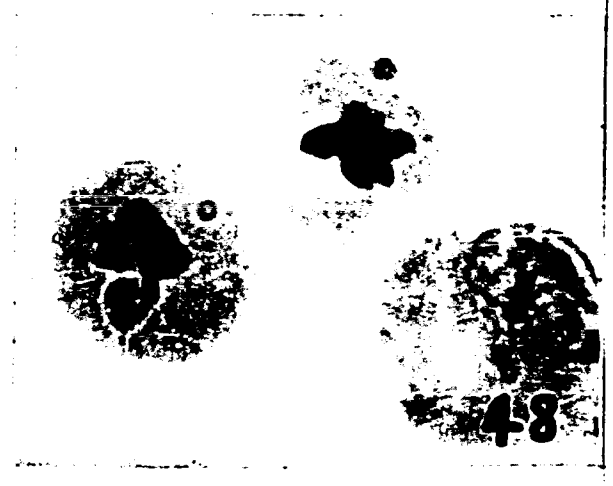






PLATE VIII



