RATE CHANGES OF THE MITOTIC CYCLE

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This is to certify that the

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of the requirements for

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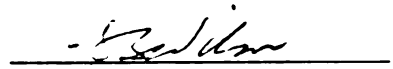

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ABSTRACT

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The purpose of this investigation was to develop and utilize a relatively simple technique for studying the effects of physiologically active chemicals on the mitotic cycle of proliferating cells in a complex tissue. The experimental tools used in achieving the goal proposed were very young pea seedlings, colchicine, and respiratory poisons. The general methodology involved treating the primary root meristem of pea seedlings for a period of 30 minutes with an appropriate concentration of colchicine. The cytological effect of colchicine is that of clumping or scattering of metaphase chromosomes and the prevention of cytokinesis by disruption of spindle organization and orientation. The final result of a short time treatment with colchicine is the production of a small, synchronously dividing, population of tetraploid cells. This population was subjected to a 15 minute treatment with 2, 4-dinitrophenol, 2, 4-dichlorophenol, potassium cyanide, and potassium fluoride. By treating the population when it was in early interphase of the mitotic cycle, it was noted that both 2, 4-dinitrophenol and 2, 4-dichlorophenol improved the synchrony and delayed the appearance in division of the tetraploid cells, whereas

a great deal of the synchrony was destroyed when cells were treated by these two chemicals at late interphase. Further, when the population was treated with cyanide at either early or late interphase, no great difference in response was observed.

When the tetraploid cells were exposed to 2, 4-dinitrophenol, 2, 4-dichlorophenol or cyanide at the fifth hour after colchicine treatment, the second division of the population occurred precociously, thus indicating that treatment at this hour caused a decrease in the time required to attain mitotic competence. The nature of the system or systems responsible for the accelerated mitotic cycle appeared to be dependent on an increased rate of anaerobic glycolysis, since a combination of a cytologically ineffective concentration of fluoride with either 2, 4-dinitrophenol or cyanide prevented to a greater or lesser degree the precocious second division of the marked population.

RATE CHANGES OF THE MITOTIC CYCLE

by

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INTRODUCTION

Ever since the discovery of the cell by Robert Hooke in 1664 and Nehemiah Grew in 1682 and the statement of the Cell Theory by both Schleiden and Schwann in the 1830's, cytologists have been studying the phenomenon of cell reproduction. At first most of the attention was directed toward the process of mitosis with special emphasis on the morphological changes observed during division. Such studies are continuing, for the process of mitosis still remains little understood. An important phase of cell reproduction that has not received much attention until recently is the period between successive mitoses, called interphase. Interphase has not been investigated in the past for at least two reasons: (1) there are few morphological changes when the nucleus is observed with the light microscope and, (2) until recently few suitable techniques have been available. A factor that can be ruled out with certainty is ignorance, for Virchow a century ago stated that cells originated only from pre-existing cells and thus implied the presence of a mitotic cycle.

The mitotic cycle has been defined by Wilson and Morrison (1959) as those events which occur between the onset of two consecutive mitoses. This cycle is a very complex sequence of events. It includes all the synchronized changes which occur in mitosis (Wilson and Hyppio 1955) and all the biochemical syntheses which are necessary for cellular growth and cell reproduction.

Much information about the mitotic cycle has been obtained by studying the biochemistry and physiology of synchronized populations of micro-organisms, cleaving eggs, and by observing single cells. Study of the mitotic cycle of dividing cells in tissues of higher organisms has proven to be more difficult. This difficulty probably arises largely from the interrelationship that exists between dividing cells and the physiological and morphological condition of the tissue as a whole.

Nevertheless, it seems logical that if unicellular organisms, cleaving eggs, and single cells show biochemical and physiological shifts during interphase, similar shifts should be observed in dividing cells of a complex tissue. However, in order to demonstrate such changes, a technique is needed that is appropriate for the study of the mitotic cycle and cell proliferation in general. The purposes of these investigations were to develop such a technique and to study the effect of certain physiologically active chemicals on proliferating cells that are in different time segments of interphase.

LITERATURE REVIEW

Laughlin in 1917 described the mitotic cycle as being the transition of a single living cell through a stage of high metabolic activity and of divisional stability to a stage of low metabolic activity and of divisional instability. He believed that mitosis is initiated following the establishment of the latter stage. It is evident from his description of the mitotic cycle that Laughlin realized that the interphase nucleus is seldom, if ever, resting. Sharp (1935) also realized that a nucleus seldom "rests" and suggested that the term "resting nucleus" be replaced by the term "metabolic nucleus". Berrill and Huskins (1936) disagreed with the term "metabolic nucleus", for they felt it implied that the nucleus was primarily concerned with cellular nutrition. The term they proposed was "energic nucleus" which, according to them, connotates the idea of a stationary working unit which is concerned with the maintenance of all cytoplasmic structures and functions, and can be contrasted with the "kinetic nucleus". A "kinetic nucleus" in this case refers to a nucleus that is in the act of dividing. Berrill and Huskins however, did point out that the term "resting nucleus" is correct in a descriptive morphological sense though quite inaccurate in the physiological sense. Their concept of the mitotic cycle one of an alternation with time between the "kinetic nucleus" and the "energic nucleus".

Before the idea of a "metabolic" or "energic" nucleus

was firmly established, investigation of the mitotic cycle was for the most part confined to the fraction of the cycle involving mitosis. The early students of cytology were quickly attracted to this part of the mitotic cycle, for it is characterized by obvious morphological changes. An account of these changes has been given by Wilson and Hyppio (1955). They described mitosis of pea root cells as a series of synchronized cycles that include the changes that occur in chromosome morphology and chromosome movement; the orientation and disorientation of the spindle; the dissolution and reappearance of the nuclear boundary; the disappearance and appearance of the nucleolus; and the cleavage of the kinetochore.

In his book "Mitosis", Schrader (1953) has presented an excellent review of both mitosis and the theories proposed to explain it. Because the older explanations are either not open to experimentation or are unsatisfactory, new theories continually appear. Among the more promising of these is that of Sato (1960). Sato has been using electron microscopy to examine sub-cellular changes during meiosis in Lilium. He finds that spindle fiber formation begins with the appearance of small particles near the nuclear boundary. These particles then proceed to align themselves from the kinetochore poleward. When this alignment is complete, the interparticular membrane dissolves forming a long contractile fiber. Went (1960) using an antigen-antibody system somewhat corroborates Sato's morpho-

logical observations. Went has been able to illustrate the existence of at least two proteins during spindle formation in sea urchin eggs. He hypothesizes that these two proteins represent a particulate form and a fibroid form of spindle protein.

Because of the variety of cell types, it is not surprising that the requisites of all cells, though generally similar, may differ to a greater or lesser degree. For example, the cell cycle of a microorganism may be equivalent to its life cycle, i.e., those events that occur between sequential divisions include a period of differentiation that is comparable to adulthood (Wilson and Morrison 1959).

Another variable in the mitotic cycle is illustrated when the process of cellular differentiation is considered. It is clear that if cellular differentiation were not carried on, such systems as cleaving eggs, plant meristems, etc., would be nothing but a dividing mass of tissue. Therefore, to avoid this situation, a certain number of cells must differentiate during a certain length of time (Gray and Scholes, 1951). To illustrate this point, if the ratio $\frac{\text{rate of cell differentiation}}{\text{rate of cell division}}$ were plotted against time, the curve produced would not be similar for all dividing systems. For example, in the case of the microorganism, the resulting curve is exponential and would proceed to infinity, assuming the absence of limiting factors, one of which is "differentiation" (i.e., spore formation).

As for the cleaving egg, this plot forms a sigmoid curve that reaches a limit, after which it approaches some number that is less than one at the time of maturation. In the plant root meristem the curve is generally flat having a value near one indicating a steady state of growth (Gray and Scholes, 1951; Quastler, 1960). The graph below represents a plot of the curves just discussed.

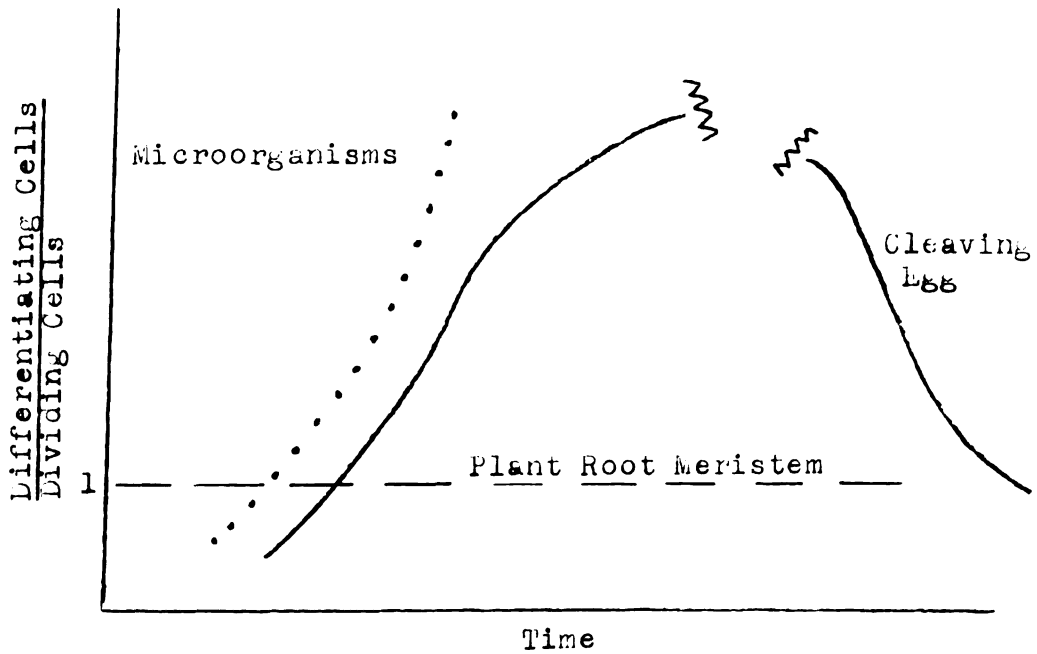


Figure 1. Theoretical curves of the ratio of differentiating cells to dividing cells versus time for different systems.

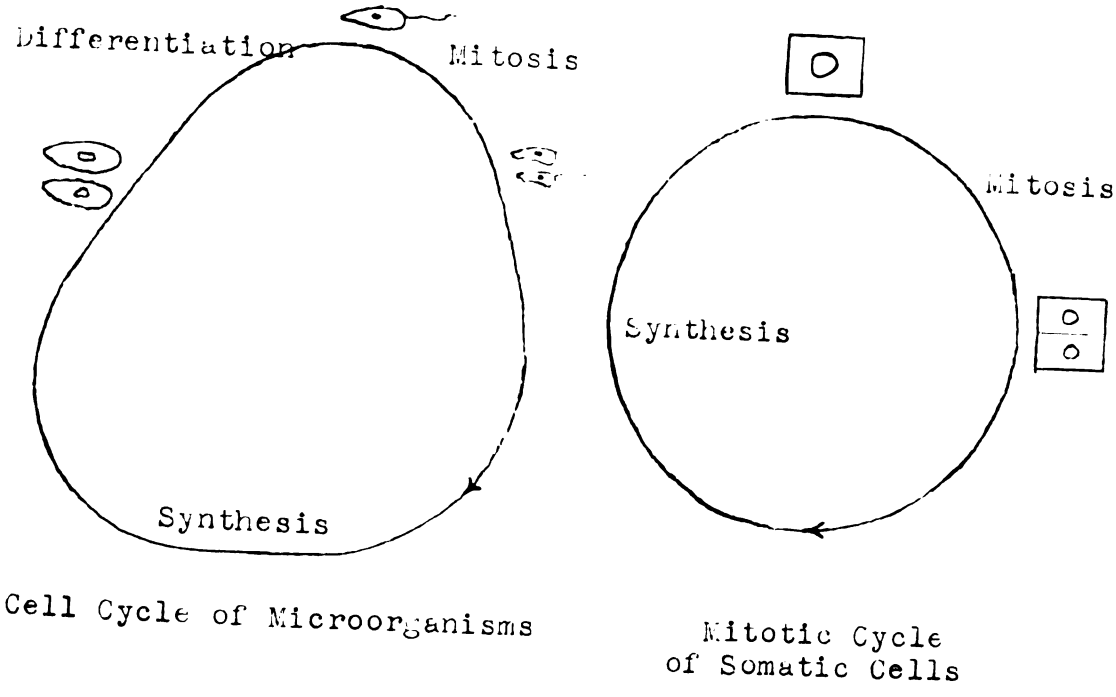
The meiotic cycle of germ cells is often used as a source of information concerning cell reproduction. The events that occur in the meiotic cycle however, are not all the same as those of the mitotic cycle. A few examples of differences that exist between these two cycles are listed below.

1) During prophase the chromosomes of dividing germ cells go through the complex manipulations involved in pairing and synapsis.
2) The germ cell usually proceeds through two divisions which result in the formation of four haploid cells.
3) No deoxyribonucleic acid (DNA) synthesis takes place between divisions I and II of meiosis (Swift, 1950).
4) All maturing primordial germ cells have a discontinuous cycle that is resumed generally by either fertilization or parthenogenesis.

A schematic representation of the mitotic cycle, cell cycle, and the meiotic cycle are shown in Figure 2.

Between the beginning of one mitosis and the onset of the next, a number of syntheses must be carried out in order to prepare the cell for its division. It is of interest to the cytologist to know how long it takes a given cell type to attain mitotic competence and to know the sequence wherein these requirements are fulfilled.

Aside from those experiments performed with single cells, most of the information concerning the questions mentioned



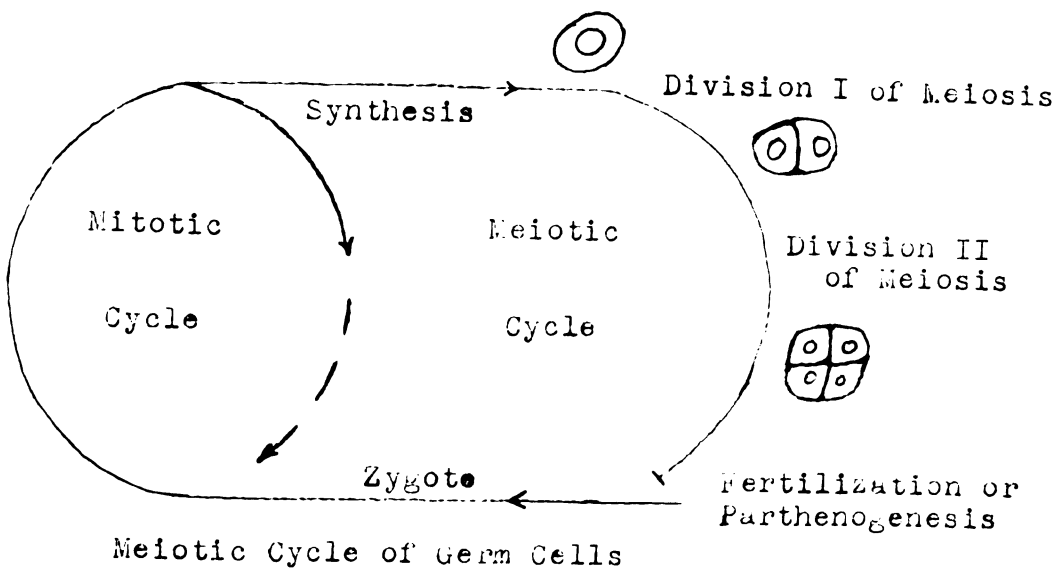


Figure 2. Diagrams representing the cell cycle of microorganisms, the mitotic cycle of somatic cells, and the meiotic cycle of germ cells.

above has come from the study of synchronous dividing cells. Therefore, before consideration is given to these questions, synchronous cell division will be discussed.

Some of the advantages of working with a synchronized population of dividing cells are that:

1) the duration of both the total mitotic cycle and of mitosis can be determined,
2) when performing an experiment, all the cells are more or less in the same segment of the mitotic cycle at any given time,
3) a synchronized population represents a mass of cells that are relatively homogeneous in their physiological state,
4) the experimenter's patience is not taxed as it is sometimes with unsynchronized cells (Hughes, 1952).

The experimental cytologist uses two types of synchronized cell division. The first of these is natural synchrony. Natural synchronized cell division occurs in the primary spermatocytes of animals and in the microspore mother cells of plants. Caspersson (1939) used grasshopper spermatocytes in his study of the role of deoxyribonucleic acids in cell division. Microspore mother cells were used by Sparrow and Sparrow (1949) in their experimentation. Natural synchrony also occurs in segmenting eggs of Echinoderms and amphibians during the first two or three cleavages. The advantages of using cleaving eggs are expressed by Heil-

brunn (1956) in his book, THE DYNAMICS OF LIVING PROTOPLASM. Another form of natural synchrony exists in tissue that is characterized by rapid growth and a relatively high rate of cell division. In such a tissue a certain portion of the dividing cells must be considered as being reasonably well synchronized through several divisions. These cells are also homogeneous with respect to their physiological state, as well as to their position in the mitotic cycle. Taking advantage of this homogeneity and making use of both a radioactive tracer and autoradiography, Howard and Pelc (1951) were able to label with P^{32} only those cells that were synthesizing DNA at the time of treatment and thereby marked a population of synchronized cells that could be followed through the subsequent division. The work of Howard and Pelc was one of the first in a series of experiments using autoradiography to study both cellular proliferation and cellular biochemistry.

The use of colchicine in studying cell reproduction is founded on the same arguments that apply to the use of radioactive tracers. Colchicine, because of its high specificity for cells that pass through metaphase (Levan 1939; Hadder and Wilson, 1958), will affect only those cells that are going through mitosis simultaneously at the time of treatment. Colchicine, therefore, affects only a relatively homogeneous group of cells that are dividing synchronously.

The second type of synchrony is produced when unicellular organisms are exposed to a series of short time physio-

logical shocks (Leuteen and Scherbaum, 1954). Synchrony produced in this manner is called induced or unnatural synchrony. Induced synchrony has been produced successfully in amoebae by James (1959, 1960) and in bacteria by Hunter-Szybalska et al (1956). In fact, this method has proven to be so successful that a number of systems have been developed since its origin. Campbell (1957) mentions eighteen systems that are in existence and many of these are discussed in a review by Scherbaum (1960).

In spite of its wide acceptance, induced synchronization does have its opponents. There is some disagreement as to whether or not the induced synchronized populations of unicellular organisms are equivalent to cells in the natural state (Mitchenson, 1957) since synchronized cells are larger (Scherbaum, 1960) and have twice the amount of DNA per cell than that contained in cells of a natural (unsynchronized) population (Scherbaum, 1957). The question, though well founded, is rather impractical, for the advantages of synchronization greatly outweigh the disadvantages.

The duration of the mitotic cycle in any particular case depends on a variety of factors: for example, Laughlin (1919) found the duration of anaphase in onion to be temperature dependent and Brown (1951) observed that the mitotic cycle time of pea root cells varied with temperature. Further, Frankland and Ward (1895) while measuring the time between fissions of a schizomycete, Bacillus ramosus, found that cell division and growth were influenced by light and

temperature.

The duration of the mitotic cycle of cleaving rabbit eggs was measured by Lewis and Gregory (1929) using photography. Pincus (1930) also followed the cleavage of rabbit eggs. Shumway (1941) has reported the cleavage time in frog eggs and Rugh (1948) cites the cleavage time of other amphibians. Zeuthen (1951) recorded cleavage time of sea urchin eggs in measuring fluctuations in oxygen consumption during the mitotic cycle.

Unlike cells of higher organisms, the cycle time of microorganisms usually is a matter of minutes rather than hours. This time can be determined by induced synchrony (Zeuthen and Scherbaum, 1954) or by measuring cell number increase during the log-phase growth period (Maaloe and Lark, 1954).

In studying the respiratory changes during mitosis, Stern and Kirk (1946) have been able to use anther size to determine the average stage of contained microspore mother cells. Time in this case is a rather poor indicator. Nevertheless, time in most instances is the more practical parameter.

Carlson and Hollander (1948) timed the mitotic cycle of the grasshopper neuroblast and found it to be three hours twenty-eight minutes. Interphase lasted only twenty-seven minutes and proved to be most sensitive to ultraviolet radiation. Fell and Hughes (1948) combined phase contrast micrography and cine-photomicrography to study the mitotic cy-

cle in mouse cells. They observed eighteen intermitotic times (interphase) and found them to range from 6 to 15 hours with the mode being at 9 hours. Microspectrophotometry, in addition to photometry, was used by Walker and Yates (1952) in their study of DNA synthesis. They found that the interphase of chick heart cells grown in tissue culture lasted for 10.7 hours and that the entire mitotic cycle time was 15 to 16 hours. Gray and Scholes (1951) calculated the mitotic cycle time of Vicia faba by delaying cells located in the x-ray sensitive segment of the mitotic cycle with x-radiation. Using a highly mathematical approach, they found the mitotic cycle to last from 18.4 to 25.4 hours, with mitosis being 2.6 hours and interphase being 16.8 to 22.8 hours. These values were corroborated by Howard and Pelc (1951) using the autoradiographic technique and also by Taylor, Woods, and Hughes (1956) who found octaploid cells after 36 hours of continuous colchicine treatment. In order to be octaploid, these cells had to complete two mitotic cycles, thus giving an average mitotic cycle time of 18 hours.

Colchicine was also used by Evans et al (1957) to determine the mitotic cycle time of Vicia faba. In this case it was reported to be about 24.5 hours with interphase being 20.7 of these 24.5 hours.

Tritiated thymidine has been used by Wimber (1960) who studied the mitotic cycle time of Tradescantia root tip cells, and by Quastler and Sherman (1959) in investigating the vari-

ous segments of interphase in the mitotic cycle of mouse. Radioactive isotopes have been used by Lornsey and Howard (1956) and Painter and Drew (1958) in similar studies. A review of the production and use of tritiated compounds by Taylor (1960) has recently been published.

The questions, what are the requirements for mitotic competence and in what order these requirements are fulfilled, may be considered simultaneously. The use of P^{32} and tritiated thymidine plus the autoradiographic technique has allowed the fractionation of interphase into G_1 (a period immediately following telophase wherein DNA synthesis does not take place), the S stage of interphase (wherein DNA synthesis does take place), and G_2 (a period following the S stage and just preceeding mitosis wherein no DNA synthesis takes place (Howard and Pelc, 1951; Hughes et al, 1956; Quastler and Sherman, 1959). The term "antephase" was proposed by Bullough (1952) to apply to the state of relatively low metabolism that exists in cells just before mitosis. It is probable that G_2 and "antephase" are synonyms.

The synthesis of chromosomal protein was reported to occur during interphase simultaneously with DNA synthesis by Howard and Pelc (1952). They used S^{35} and the autoradiograph technique on the cells of the root meristem of Vicia faba and found the S^{35} to be accumulated about the same time as P^{32} which was used to measure DNA synthesis.

More recently Prescott (1960) measured DNA synthesis, (nNA) ribonucleic acid synthesis, and protein synthesis in

synchronized populations of Tetrahymena and found DNA synthesis to be nonlinear and to occur first following division. RNA synthesis also occurred at a non-linear rate but did not reach significance until DNA synthesis was completed. Most of the RNA was synthesized just before division. Protein synthesis on the other hand proceeded at a slow linear rate during the entire interphase. Protein synthesis therefore, occurred simultaneously with DNA and RNA synthesis but never at the same rate.

The subjects of cellular energetics, carbohydrate metabolism, and respiration have been investigated for many years. The work done by Swann (1954) indicated a CO light reversible inhibition of cleavage of marine eggs. Swann discovered that there was a time before which CO would inhibit the cleavage immediately following treatment. If treatment were performed after this time, only the second cleavage following treatment could be inhibited. Swann interpreted these results as indicating the existence of an ATP (adenosine triphosphate) storage mechanism which had to be at a certain level before cleavage could proceed. He further hypothesized that this ATP storage mechanism would have to be replenished following each cleavage. This concept is supported by the work of Clowes and Krahl(1937) and Krahl and Clowes (1937) who observed that a high respiratory rate due to uncoupling resulted in the inhibition of cleavage in the sea urchin egg. They obtained similar results with both nitrated and halogenated phenols when these compounds were used

to increase oxygen uptake. Hamburger and Zeuthen (1959) also found that DNP delayed the division of a synchronized population of Tetrahymena. Similar results were observed by Immers and Rannstrom (1960) when they treated sea urchin eggs for only 30 minutes with a very low concentration of DNP. The importance of carbohydrate metabolism is emphasized by the work of Pollough (1952) and Wilson, Morrison and Knobloch (1959). These workers report that the addition of a carbon source to a competent system of dividing cells located in antephase will increase mitosis, thus suggesting that antephase is somewhat dependent on a carbon source for the entrance into mitosis.

A compilation of the observations reported concerning the mitotic cycle allows the construction of a model which represents most of what is known to occur during a single revolution of the cycle. Such a model is illustrated in Figure 3. It should be pointed out that this model is not meant to represent absolutely the relative time of occurence of a given synthesis or reaction.

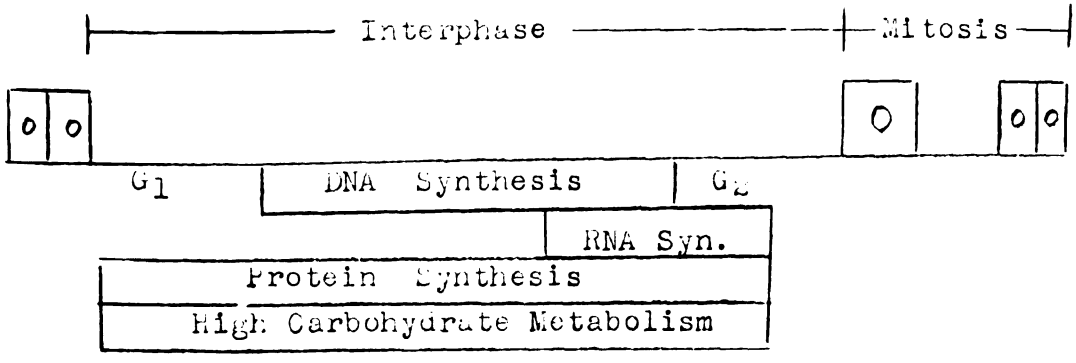


Figure 3. Model of the Mitotic Cycle

MATERIALS AND METHODS

GENERAL EXPERIMENTAL PROCEDURE

The tissue used in these investigations was the growing root tip of Pisum sativum var. Alaska. The peas were furnished by the Ferry-Morse Seed Company who took precautions to make sure the seeds were disease-free, of relative genetic homogeneity, and had not been treated with any anti-fungal agents.

The peas used for experimentation were soaked in distilled water for six hours at 25°C. They were then rolled in paper toweling and moistened. Next the peas, rolled in paper towels, were placed in an upright position in beakers containing about one inch of distilled water. A sheet of waxed paper was then wrapped about each rolled towel after which the peas were allowed to germinate in a germinator at 25°C. Germination continued until the pea seedlings had roots that were between two and one-half to three and one-half centimeters long. At this time the seedlings were collected for treatment.

Seedlings with roots of the desired length were suspended on waxed, one-quarter inch wire mesh above 600 ml. beakers that contained one-fourth strength Hoagland's nutrient solution. The contents of one-quarter strength Hoagland's solution are listed below in grams per liter.

$Ca(NO_3)_2 \cdot 4H_2O$ 0.0354

NH_4NO_3 ..... 0.033

$MgSO_4 \cdot 7H_2O$ 0.0492

KH_2PO_4 ..... 0.0342

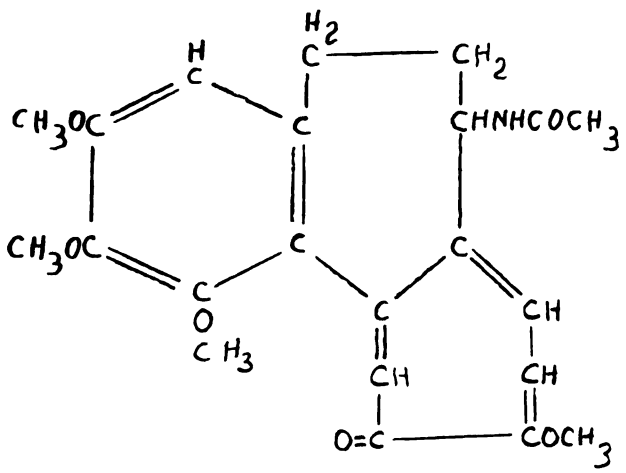
K_2HPO_4 ..... 0.00179

They remained in the nutrient solution for a minimum of four hours at 22.5°C. All solutions were aerated by a fine stream of bubbles of filtered air and this aeration was continued throughout the experiment.

Following the stay in nutrient solution, groups of seedlings were chemically treated by placing them, still suspended on the wire mesh, into beakers containing a solution of the chemical and nutrients. When the chemical treatment had been completed, the seedlings and wire mesh were washed with distilled water and returned to beakers containing only nutrient solution.

All solutions used had a pH range of 5.5 to 5.7. 1 N HCl and a 20% KOH solution were used in cases where pH adjustments were necessary.

The colchicine employed in these investigations was a 99.9 % pure preparation purchased from Light's Organic Chemicals Ltd. Its structural formula (Muldoon, 1950) has been determined to be:



All colchicine solutions were prepared five minutes before use to insure a minimum of chemical decay, since colchicine loses some of its effectiveness when allowed to stand in solution too long. The duration of treatment with these solutions was 30 minutes or less.

Sampling was done at two hour intervals unless specified otherwise. In all cases five samples were taken at the designated time.

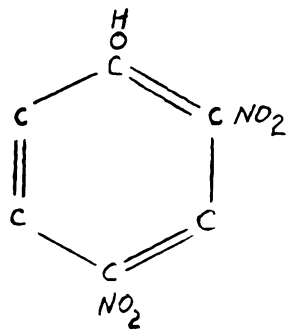
The collected root tips were immediately immersed in a fixative made of three parts ethyl alcohol and one part glacial acetic acid and placed in a vacuum for about 10 minutes and then stored in a refrigerator.

The fixed root tips were hydrolyzed 18 to 20 minutes in 2.7 N HCl at 60°C. The HCl was then decanted and leucobasic fuchsin (Schiff's reagent) poured into the vial containing the root tips.

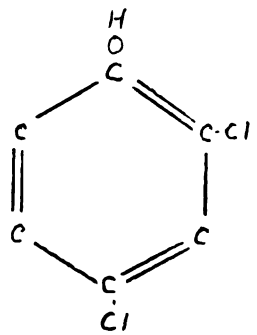
After the root tips were stained a very dark purple by the Schiff's reagent, the highly stained meristematic region was excised from the root and placed in a drop of a 0.1% Fast Green in 45% acetic acid solution. The tissue was then thoroughly macerated with the flat end of a solid glass rod after which the whole mass was stirred to ensure as even a distribution of cells as possible. A coverslip was placed on the slide and then they were heated gently over a low flame. Excess material was removed by firmly pressing the preparation between paper towels. The slide was then placed in an alcohol solution of 90% tertiary butyl alcohol and 10%

ethyl alcohol to dehydrate. Following dehydration the slide was made permanent with diaphane.

Treatment solutions were made from the following chemicals:



2,4-dinitrophenol,



2,4-dichlorophenol,

potassium fluoride (KF), and potassium cyanide (KCN). The source of DNP was Eastman Chemical Company; DCP was purchased from Light's Organic Chemical Company; KF and KCN were obtained from the Baker Chemical Company.

The DNP solutions were prepared by pouring heated distilled water into a flask containing a small amount of DNP. From this solution the desired concentrations were made by diluting with distilled water. In all the experiments utilizing DNP, the concentration was 4.3×10^{-5} M.

Solutions of DCP were made by adding the weighed amount of DCP to a flask containing distilled water. This solution was then placed on a magnetic stirrer to assure complete dissolution of the DCP. Treatment solutions of DCP were prepared in the same manner as those of DNP, the one exception being that the treatment concentration for DCP was 4.3×10^{-4} M.

KF and KCN solutions were made in much the same manner as those of DNP and DCP. The use of KCN necessitated the use of the hood for all its handling including the experimentation. When experiments were being carried out in the hood, environmental conditions were equivalent to those of experiments not conducted in the hood.

OBSERVATIONS I

Within the meristem of a growing pea root, there is a fraction of the cell population that is revolving in the mitotic cycle. In the presence of an effective amount of colchicine these cells as they pass through mitosis will have clumped and/or scattered metaphases (Hauber and Wilson, 1958). Some of the cells having aberrant metaphases will in the following mitosis be tetraploid and therefore distinguishable from the cells not affected by the colchicine which remain diploid. In the pea root, diploid cells and tetraploid cells contain 14 and 28 chromosomes respectively.

These facts present the possibility of producing a tetraploid population of cells by treating the meristem with colchicine for a short period of time. The tetraploid or marked cells should be only those that passed through metaphase during the time of treatment. These cells should also be relatively homogeneous with respect to position in the mitotic cycle and consequently divide somewhat synchronously in subsequent mitoses.

To test these hypotheses, three experiments were performed. The first is composed of two parts. Part one was carried out to determine what relationship existed between the concentration of colchicine and the degree and duration of effect. Figure 4A shows graphically the rates of appearance and disappearance of clumped and scattered metaphases following a 30 minute treatment with colchicine concentration of 6.26×10^{-4} M (curve A), 5.63×10^{-4} M (curve B), 5.0 x

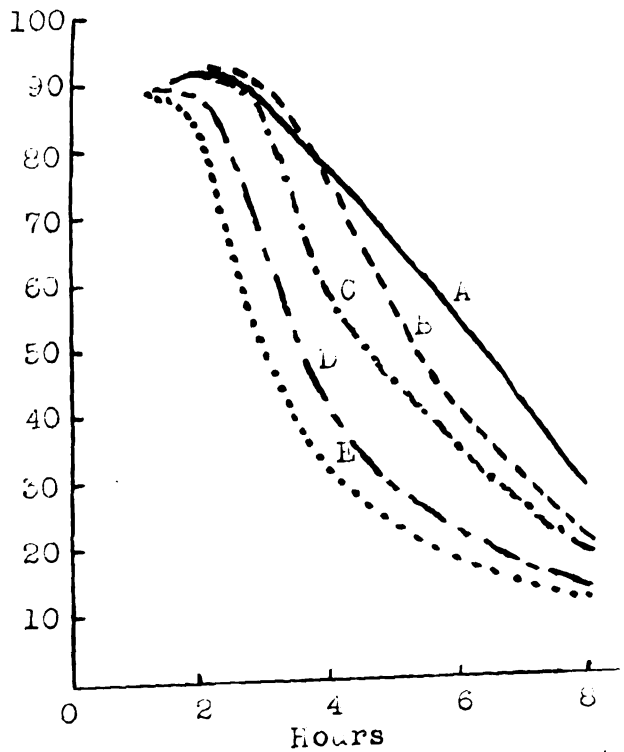


Figure 4A. Change in percent of effect produced by a 30 minute treatment with various concentrations of colchicine.

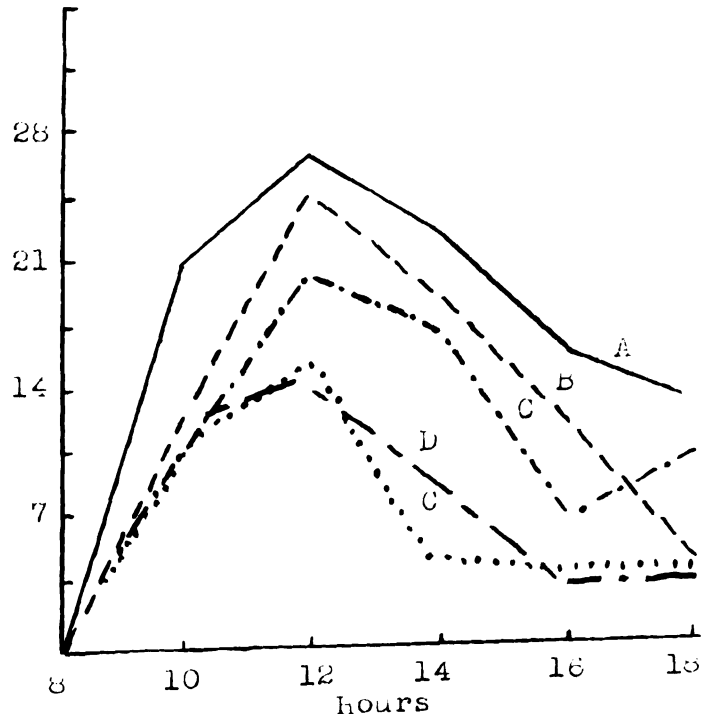


Figure 4B. Tetraploid populations produced by various concentrations of colchicine.

A (———) 6.26×10^{-4} M colchicine
B (- - - -) 5.63×10^{-4} M colchicine
C (·—·—·) 5.0×10^{-4} M colchicine
D (- ——— -) 4.38×10^{-4} M colchicine
E (·····) 3.76×10^{-4} M colchicine

10^{-4} M (curve C), 4.38 X 10^{-4} M (curve D), and 3.76 X 10^{-4} M (curve E).

Since the rate of appearance of aberrant metaphases is very similar for all concentrations used, little attention will be given to this part of the data. On the other hand, the recovery curves or the rates of disappearance of aberrant metaphases show a great deal of difference. Between one and two hours the curves begin to reflect the different amounts they represent. The number of abnormal divisions is already decreasing at this time for 4.38 X 10^{-4} M (curve D) and 3.76 X 10^{-4} M (curve E), while the three higher molarities, though not decreasing, show no net gain in clump and/or scattered metaphases. At three hours all five concentrations show either a decrease or no net increase in effect. From the general pattern of these recovery curves it can be said that the rate of recovery from a 30 minute treatment with colchicine is more or less inversely proportional to the concentration.

It should be mentioned that the curves as shown in Figure 4A and 4B have not been subjected to statistical treatment to demonstrate a significant difference between them, since the existence of a statistical difference is irrelavent to the more practical purpose of the experiment. In addition to testing the two hypotheses mentioned previously, the experiment was also performed to determine what concentration of colchicine would produce a tetraploid population of a usable size and a minimal distribution in the

mitotic cycle. A population having these characteristics should be easily distinguished because of its pronounced maximum and minima. Further, colchicine has a cytological effect that changes both qualitatively and quantitatively with dose. These changes have been taken into account by Hadder and Wilson (1955) in their analysis of the colchicine effect on pea root cells and by Hyppio (1954) in his observations on the residual cytological effect of colchicine after short time treatments using the same material. The cytological effect of colchicine, as indicated by these studies is statistically analyzable during continuous treatment, however the recovery curves observed after a short time treatment are not mathematically intelligable if both the qualitative and quantitative changes are taken into account. Figure 4A represents only the quantitative change after treatment and therefore does not show entirely what is happening. On the other hand, a plot of the percent of clumps and scatters against time appears to be the only logical way to illustrate a correlation between the number of cells affected by colchicine and the tetraploid cells originating from the affected cells.

The second part of the first experiment concerns the measurement of the number and distribution of tetraploid cells produced by the 30 minute treatment with the concentrations mentioned above. Figure 4B represents the results of these measurements.

When considering the general pattern of the curves in

Figure 4B the following characteristics are apparent.

1) All the curves have a maximum at twelve hours.
2) The curves are more alike from eight to twelve hours than from twelve to eighteen hours.
3) The distance between the first appearance of tetraploids at eight hours and the first minimum is approximately proportional to the molarity used.
4) The area under the curves between eight hours and the first minimum is also somewhat proportional to the molarity used.

Moreover, a comparison between the curves of Figure 4A and the curves of Figure 4B show that the number of tetraploids is proportional to the number of aberrant metaphases and that the distribution of the tetraploid population reflects the rate of effect and the rate of recovery from a treatment with a given colchicine concentration. The slower the rate of recovery, the greater the distribution of tetraploids.

To determine more accurately the nature of the relationship between the number of abnormal metaphases and the number of tetraploids, the data shown in Figure 4A were expressed as the number of affected cells per thousand cells. The conversion was done in this manner: the average mitotic index (the number of dividing cells per thousand cells) in the pea root was found to be about 70. One-half of the 70 dividing cells were post-prophases; therefore, 100% on the ordinate of Figure 4A is equivalent to 35 aberrant metaphases.

The use of the proportion, $\frac{100}{\Sigma t}$ as $\frac{Y}{\lambda}$, where Y is the percentage as read form Figure 4a and λ is the number of affected cells per thousand, completes the conversion.

Further, since the polyploid index is expressed as the number of tetraploid cells per thousand cells, consideration must be given to the relative contribution of dividing cells to the thousand cells after one complete revolution of the mitotic cycle. The affected cells do not undergo cytokinesis, hence they contribute but <u>one cell</u> to the total population of cells in the meristem. The unaffected cells however, do undergo cytokinesis and therefore each adds <u>two cells</u> to those of the total meristem. The ratio of cells contributed by affected and unaffected cells is then $\frac{1}{2}$ or 0.5. The net result of this difference is a dilution of the tetraploid population by factor of 0.5. Taking into account this dilution factor, the following relationship exists between the affected cells and the tetraploid cells.

$$(P)\left(\frac{N_a}{D_m}\right) \quad (0.5) = (P)\left(\frac{N_t}{D_m}\right)$$

Where P is the probability of a cell completing a revolution of the mitotic cycle,

N_a is the number of affected cells per thousand cells observed from time t_o to time t_1,

N_t is the number of tetraploid cells per thousand cells counted between eight hours and the first minimum as shown in Figure 4b,

D_m is the length of time a given cell is observed in mitosis,

t_o is the time the experiment was begun, and

t_1 is the time the recovery curve has a value of ten affected cells per thousand cells.

D_m for the affected cells is approximately 1.5 hours, while D_m for the tetraploid cells is three hours. The different values for D_m are due to the fact that colchicine affects only post-prophase cells. Therefore, the amount of time a given clumped and/or scattered metaphase can be observed is 1.5 hours. On the other hand, tetraploids are counted in all stages of mitosis and thus are observed throughout the three hour mitotic period (Hyppio, 1954).

Calculations for five molarities of colchicine are given in Table I.

Table I. The Relationship between Colchicine Concentration and N_a, $\frac{N_a}{2}$, and N_t

CONCENTRATION	N_a	$\frac{N_a}{2}$	N_t
6.26×10^{-4} M	600	300	293
5.63×10^{-4} M	534	267	250
5.0×10^{-4} M	467	233	163
4.38×10^{-4} M	283	141	125
3.76×10^{-4} M	280	140	116

The data listed in Table I suggest that a one to one relationship exists between the number of affected cells and the number of tetraploid cells. At higher concentrations this relationship is excellent, however at lower concentra-

tions there is less agreement between the data. This lack of agreement is probably due to the fact that a greater proportion of affected cells are scattered metaphases when lower colchicine doses are used (Hadder and Wilson, 1953), and the probability of a scattered metaphase becoming a tetraploid cell is much less than one. (A scattered metaphase is less likely to include all of the chromosomes in the reconstituted nucleus because they are distributed throughout the cell. If a reconstituted nucleus does not include all the chromosomes it probably will not divide again). On the other hand, the probability of a clumped metaphase becoming a tetraploid cell is almost one (Hyppio, 1954).

The second experiment performed to test the hypotheses was to treat pea seedlings with the same concentration of colchicine for various lengths of time. The molarity used was 5.0×10^{-4} M and the treatment times were 10, 15, 20, and 25 minutes. A secondary purpose of this experiment was to determine whether or not an improvement could be made on the rate of recovery as well as the production of a tetraploid population of appreciable size and limited distribution in time. Figures 5A and 5B show the results of this experiment. Figure 5A indicates that the percent of affected cells is increased with treatment time and that the rate of recovery is approximately inversely proportional to the duration of treatment.

Figure 5B indicates the possible existence of a threshold

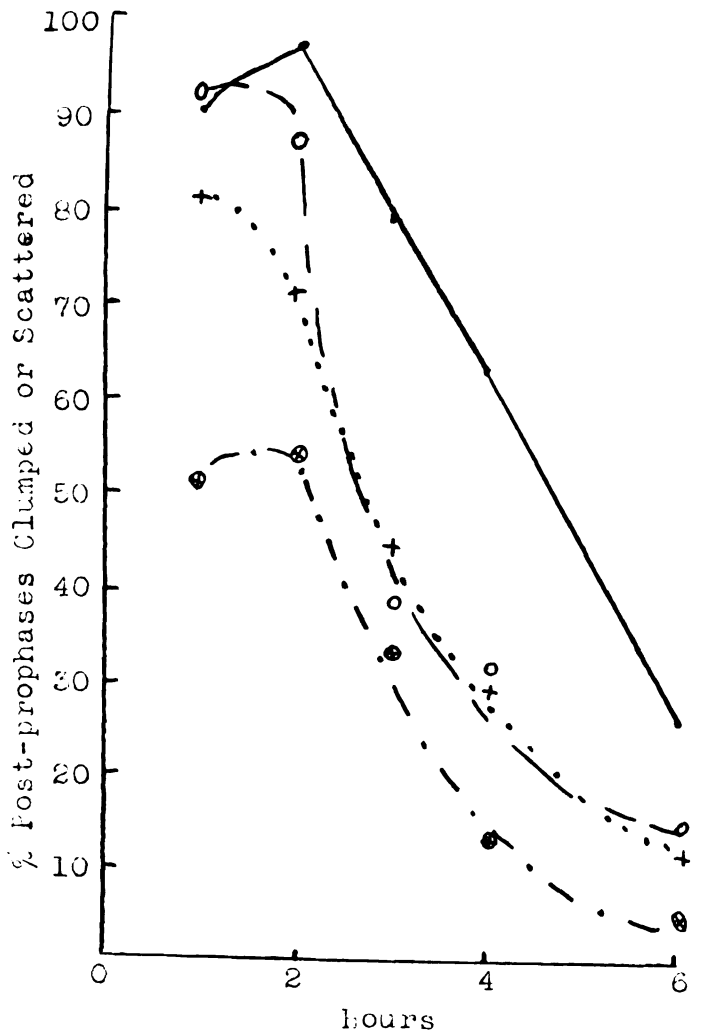


Figure 5A. Variation of effect by 5.0×10^{-4} M colchicine. (⊕•——•) 10, (x····) 15, (o——) 20, and (•——•) 25 minute treatment.

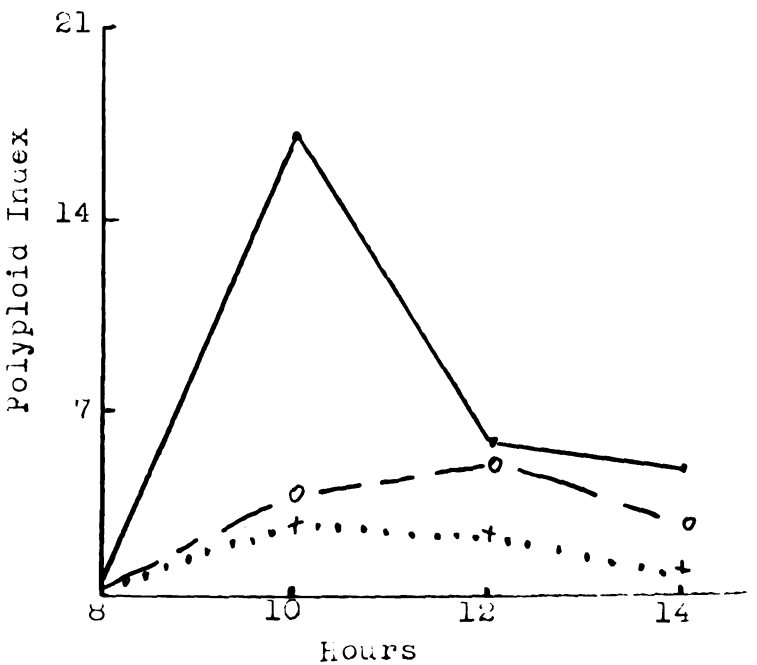


Figure 5B. Tetraploid population produced by a (x····) 15, (o——) 20, and (•——•) 25 minute treatment with 5.0×10^{-4} M colchicine.

that must be exceeded in order to produce a population of any notable size. The threshold in this case was exceeded only by the 25 minute treatment.

The third experiment was designed to determine whether or not a tetraploid population would divide somewhat synchronously for three revolutions of the mitotic cycle. Figure 6 shows the experimental results. The population illustrated in Figure 6 was formed by a 30 minute treatment with 3.76×10^{-4} M colchicine at zero hour. This population divided synchronously for at least three revolutions of the mitotic cycle. The synchrony, however decreased with each revolution as indicated by both the lowering of the maxima and the increased dispersion with time of the tetraploid cells. Indeed, if a curve is drawn from maximum to maximum and extrapolated to include at least three more mitotic cycle revolutions, it appears as though no synchrony remains after this time. At this point the marked population would be equally distributed throughout the mitotic cycle.

The appearance of two maxima during the third (III) division is another interesting observation. Evidently there is beginning at this time a separation between cells that are revolving about the cycle at different speeds. The first maximum being the faster cells; the second being the slower cells.

Figure 6 yields another very important piece of information. The distance between the maxima of divisions I, II, and III averages twelve hours and since each division

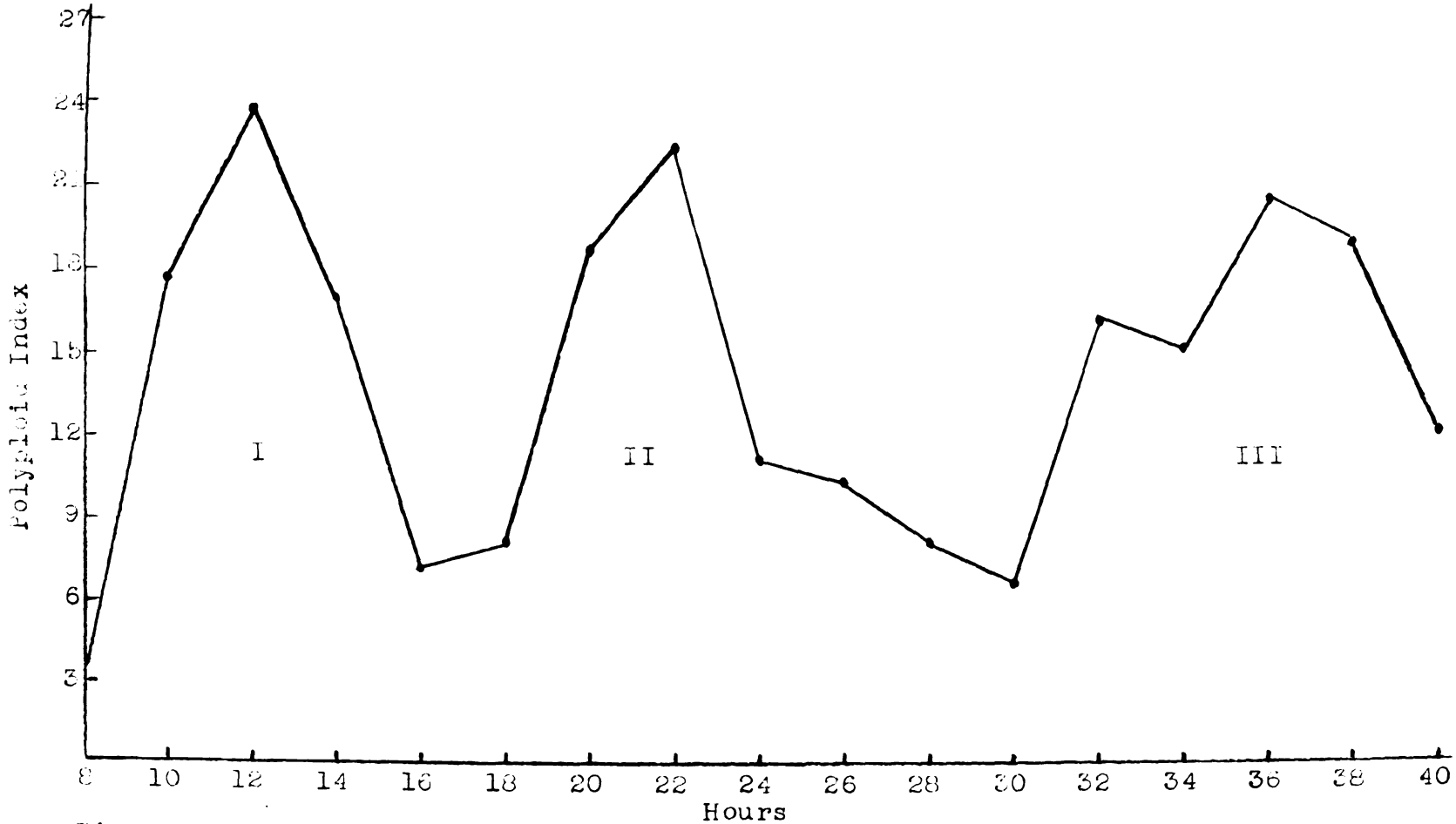


Figure 8. Three divisions of a tetraploid population produced by a 30 minute treatment with 3.76×10^{-4} M colchicine.

represents a complete revolution of the mitotic cycle the twelve hour average is the total cycle time of the meristematic cells in the growing pea root at 22.5°C, which is in agreement with Brown's (1951) estimation of the cycle time in the same material.

SUMMARY OF OBSERVATIONS I

1) Doses of colchicine exceeding a threshold will produce a tetraploid population.
2) The size and distribution of the marked population is determined primarily by the rate of recovery from a given dose.
3) The rate of recovery from a treatment of colchicine is approximately inversely proportional to both the concentration and the duration of treatment.
4) A marked population of cells will divide synchronously for at least three revolutions of the mitotic cycle.
5) The mitotic cycle time of pea root meristem cells is approximately twelve hours at 22.5°C.

DISCUSSION I

Originally two hypotheses were made concerning the use of colchicine in marking a naturally synchronized population of cells. These hypotheses were: (1) short time exposure to an effective concentration of colchicine should tag a group of cells that pass through mitosis during the time of treatment, the distinguishing mark being a doubling of the number of chromosomes and (2) the marked population should divide somewhat synchronously during the successive mitoses.

The experiments performed to test these hypotheses indicated that they are reasonably correct. In fact, the data presented in Figure 6 approaches the ideal curves of Quastler (1960) for systems having a constant speed of proliferation. This is probably due to the fact that colchicine affects only a small portion of the mitotic cycle of a cell. In pea the duration of metaphase is between 24 and 54 minutes (Brown, 1951; Hyppio, 1954) therefore, the colchicine is effective during 1/30 to 1/13 of the cycle time. This might be compared with the use of the deoxyribonucleic acid (DNA) labeled with P^{32} or H^3-thymidine. In mouse intestine cells the DNA synthetic period is about 7.5 hours and the total cycle time is approximately 19 hours (Quastler, 1960). The fraction of cells labeled would then be 7.5/19. In Tradescantia root cells this fraction is 10.8/20 (Wimber, 1960) and in mouse Ehrlich ascites tumor cells it is 12/18 (Hornsey and Howard, 1956). It is suspected that the small fraction of cells labeled by colchicine accounts for its approach to ideality.

The loss of synchrony and the continual randomization of cells in the mitotic cycle is also illustrated in Figure 6. These two phenomena are by necessity inversely proportional to each other. For example, if continual randomization did not take place with time the distribution of the marked population would be similar to the distribution of the affected cells (Figure 4A) for all successive mitoses. Obviously this is not the case as indicated by Figures 4A and 4B and Figure 6.

Randomization is primarily due to cells that are revolving in the mitotic cycle at different speeds. This difference in total cycle time may be due to an over all slowing down of all the processes of the cycle or it may be due to a delay in some particular segment of the cycle (Quastler, 1960).

In any event, randomization does take place in the pea root and one of the factors aiding randomization is a difference between cells revolving in the cycle at different speeds. This is evident from the fact that the population shown in Figure 6 has a lower maximum with each successive mitoses and therefore a wider base between minima as well as a general separation of fast and slow revolving cells as shown by the two maxima in division III.

In spite of the effect of randomization, the technique developed by these investigations does present an excellent method for studying the different stages of interphase since synchronization lasts for at least three division cycles

(Figure 6).

Measurements illustrating the differences that occur during interphase are significant only in terms of their relationship to the controls. These differences however, are real and repeatable as will be seen in the following discussions.

OBSERVATIONS II

The physiologically active chemicals used to investigate the hypothesis that differences may exist in cells which are in different segments of the mitotic cycle were those that may be classified as respiratory poisons. The first of these was 2, 4-dinitrophenol (DNP).

DNP was used for a number of reasons, some of which are:

1) The cytological effects of DNP had been well described by Muhling et al, (1960), for the pea root cell. These effects are visible and measurable with the aid of a light microscope and are represented by an unusual accumulation of late-prophases.
2) The cytological effects of DNP are reversible at non-toxic concentrations (Muhling et al, 1960).
3) The physiological and biochemical effects of DNP are relatively well known (Simon, 1953).
4) DNP affects probably one of the most basic processes of living protoplasm, namely, the process of bioenergetics. Therefore, in selecting a chemical capable of showing the existence of physiological differences in the various cycle segments, DNP seemed to be the logical choice.

The first experiment utilizing DNP was carried out in

the following manner: a tetraploid population was formed by treating pea seedlings for 30 minutes with 3.75 X 10^{-4} M colchicine. Four hours later, by which time nearly all the marked population was in early interphase, a single group of seedlings containing a marked population was exposed to 4.3 X 10^{-5} M DNP for 15 minutes. A similar group was treated five hours after marking, still another at six hours, and etc., up to nine hours after colchicine treatment. By nine hours the tetraploid population had passed through the interphase segment of the cycle and was already entering mitosis.

Figure 7 shows the time of appearance and the distribution of the untreated control population, the population that was treated at four hours (early interphase), and the population that was treated at 8 hours (late interphase). These curves indicate that a difference does exist between early and late interphase. The tetraploid cells that were treated at the 4th hour show both a two hour delay in appearance in mitosis and a decrease in distribution of the marked cells. On the other hand, even though the eight hour curve also shows a two hour delay, the population has an increased distribution. In addition to these observations, Figure 7 suggests that in order to carry out investigations involving two cycles, only early interphase treatments would be practical, for too much synchrony is lost if DNP treatment is given when the population is in late interphase.

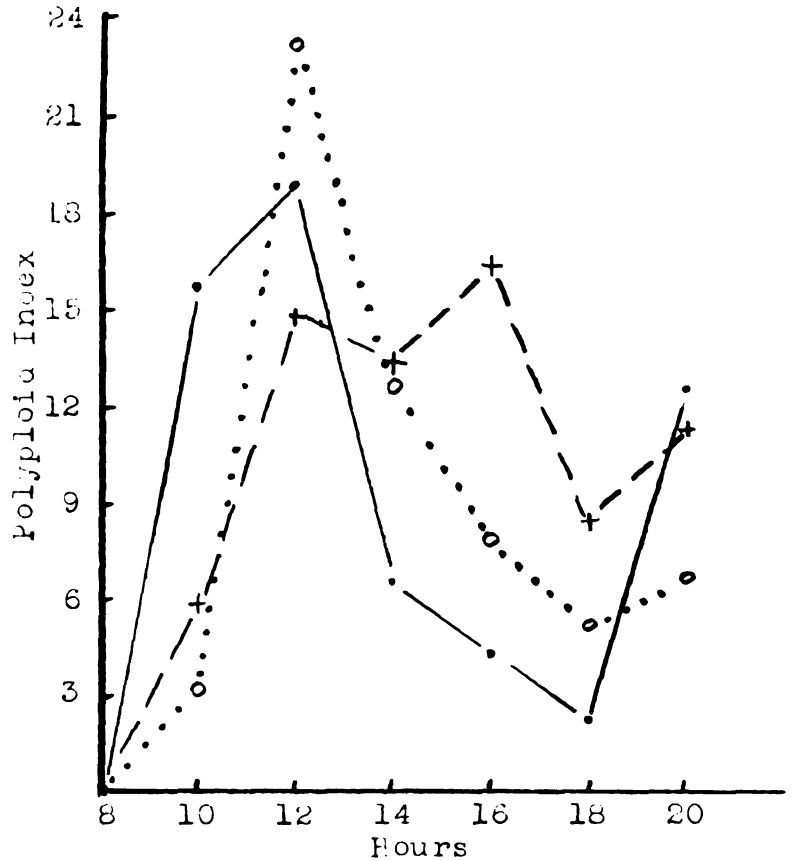


Figure 7. The first division of tetraploid populations treated in early or late interphase with DNP. (o·····o) population treated four hours after marking. (×– – –×) population treated eight hours after marking. (•———•) control.

Because it was noted that a 15 minute treatment with DNP at 4 hours resulted in a two hour delay in the appearance of the marked population, the question arose as to whether or not a series of 15 minute treatments at two hour intervals would cause the tetraploids to be delayed longer than two hours. To answer this question the following experiment was designed. Four groups of seedlings were used. The first was the control, the second was treated at 4 hours after marking a population, the third was treated at 4 and 6 hours, and the fourth was treated at 4, 6, and 8 hours after colchicine treatment.

The experimental results are illustrated in Figure 8. These again show a delayed appearance of the treated population as compared to the control. (Figure 8 does not contain the results of the 4, 6, and 8 hour treated population because, as stated previously, a population treated in late interphase is too dispersed for any useful measurement to be made during the second division). Again the distribution was decreased by treatment. There are, however, two additional observations that are of major importance. These are (1) the delay in the first division is no greater when a population is exposed twice to DNP and (2) the population that was treated twice with DNP appears on the average two hours earlier in the second division than the single treated population. Moreover, the cycle time for the 4 and 6 hour treated tetraploids is of the order of 8 hours whereas, the cycle time for the control and the 4 hour treated cells

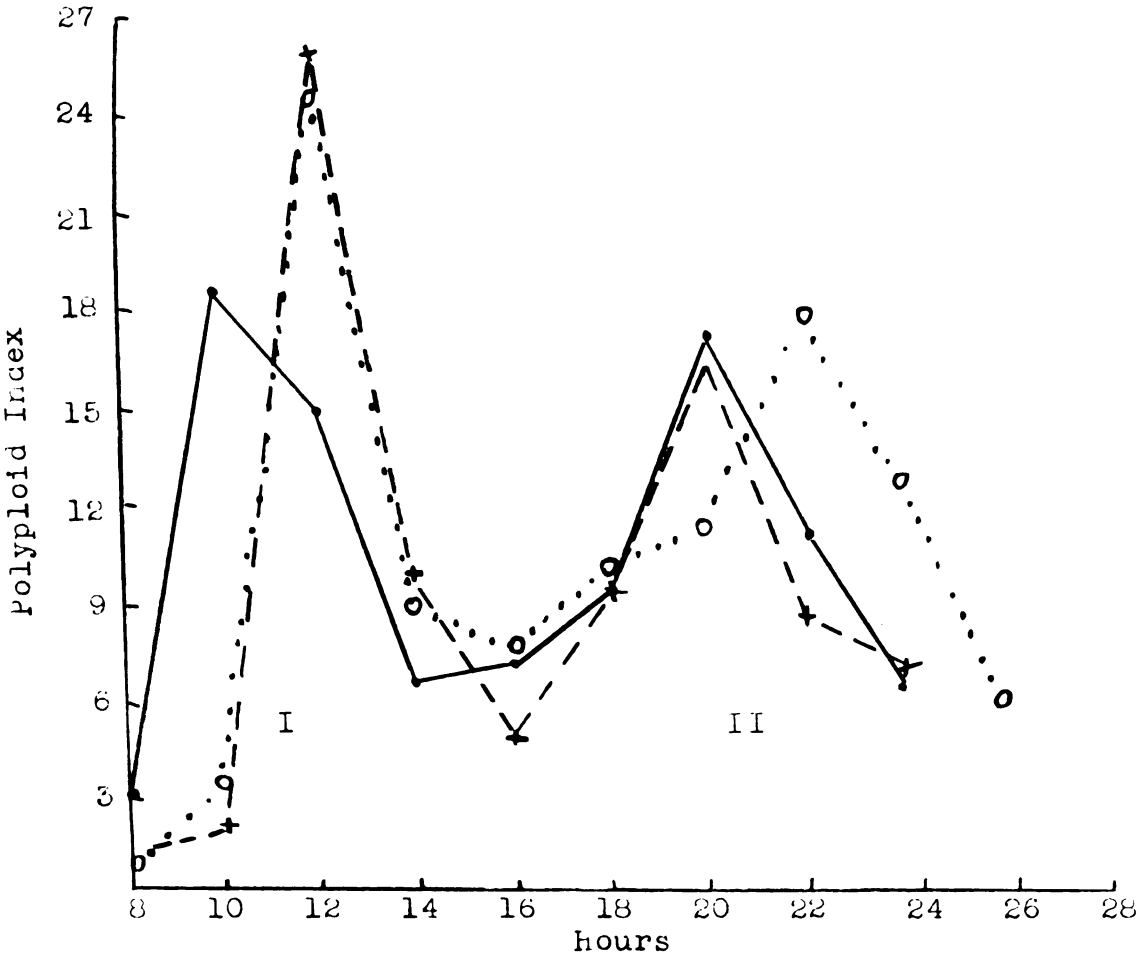


Figure 8. First and second divisions of tetraploid populations. (●——●) control. (o·····o) treated with DNP 4 hours after marking. (x– – –x) treated with DNP at 4 and 6 hours after marking.

is about 10 hours. Therefore, it may be stated that the duration of the mitotic cycle between divisions I and II was decreased by the double treatment with DNP.

Naturally, a number of questions arose concerning the cause of the effect exhibited by the double treatment. One of these involved the necessity of a double exposure in early interphase. The accelerated cycle was possibly the result of treating a group of cells located in a particular part of interphase that could respond differently than other interphase cells. The argument seemed probable, for cells that were exposed twice may have resumed revolving in the cycle after the 4 hour treatment, and thus occupied a different segment of interphase when the 6 hour treatment was given.

To answer these questions, an experiment was designed that involved the treatment of different populations at 4, 5, and 6 hours. The experimental results are shown in Figure 9A, 9B, and 9C. The results of a similar experiment are illustrated in Figures 10A, 10B, and 10C. In each case, the experiment was carried through two revolutions of the mitotic cycle.

It is evident from these data that only a single treatment with DNP is necessary to decrease the cycle time and further, it is apparent that the cells in the fifth hour segment of the cycle are the only cells that show an accelerated second cycle.

The effects of a short time treatment with DNP on cells

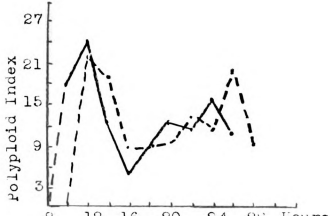


Figure 9A

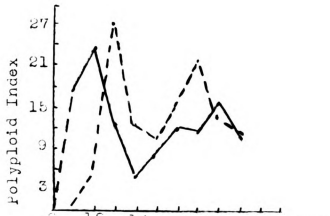
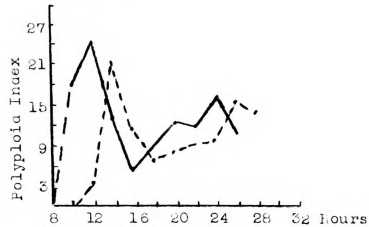


Figure 9B



The effect of a 15 minute treatment with 4.3×10^{-5} M IAP at 4 hours (Figure 9A), 5 hours (Figure 9B), and 6 hours (Figure 10C) after marking with colchicine.

(———•) Control
(- - - - -•) Treated

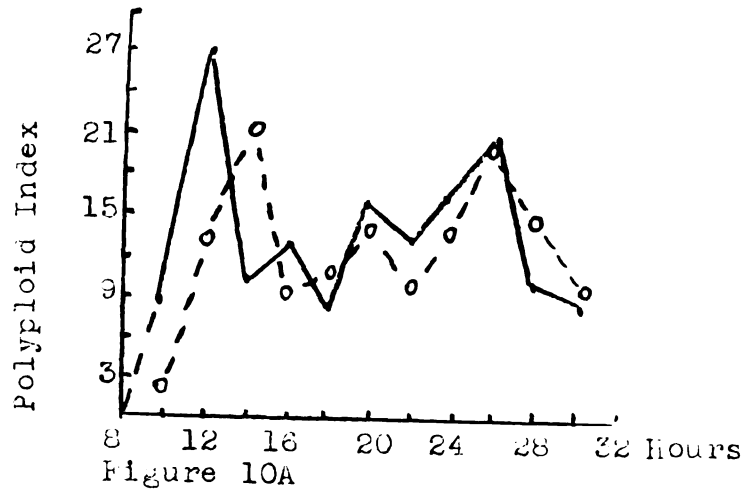


Figure 10A

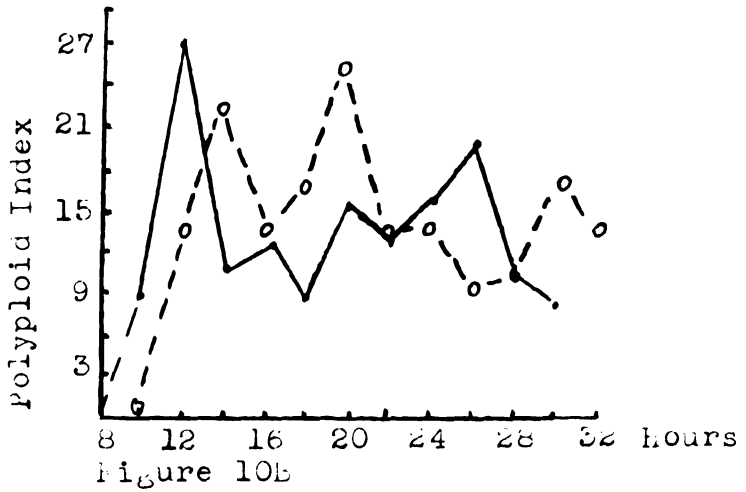


Figure 10B

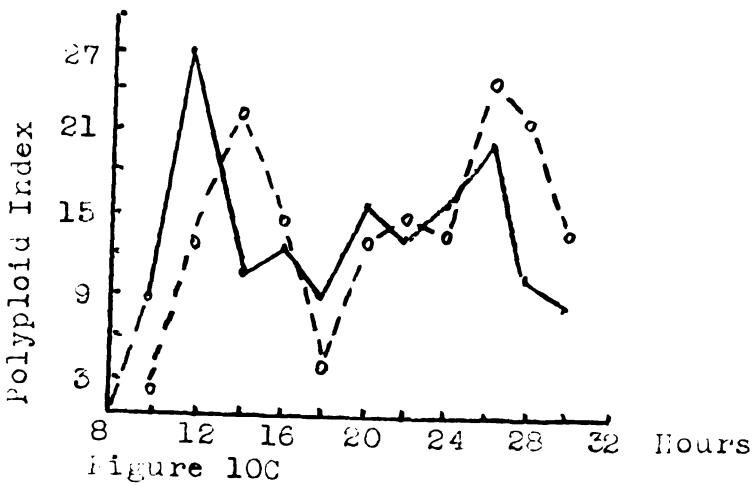


Figure 10C

The effect of a 15 minute treatment with 4.3×10^{-5} M LNP at 4 hours (Figure 10A), 5 hours (Figure 10B), and 6 hours (Figure 10C) after marking with colchicine.

(•——•) Control
(o- - - -o) Treated

in different segments of the mitotic cycle may be summarized as follows:

1) Cells in the fourth, fifth, and sixth hour segments of interphase are, as compared to control,
   a) usually delayed two hours before entering into division and
   b) generally less dispersed and therefore better synchronized.
2) Cells in late interphase (eighth and ninth hour segments) divide less synchronously as compared with the control while,
3) cells of the fifth hour segment divide earlier in the second division than either the control, the fourth hour treated or the sixth hour treated populations.

DNP has been classified for quite some time as an oxidation-phosphorylation uncoupler, that is, when living tissue is exposed to DNP, oxygen consumption usually increases markedly while the production of phosphorylated compounds show no parallel increase (Loomis and Lipman, 1948). DNP might be called the classical uncoupler although there are many other similarly acting compounds. One of these is 2, 4-dichlorophenol (DCP). DCP has been shown by Krahl and Clowes (1937) to affect sea urchin eggs in a way similar to DNP (Clowes and Krahl, 1937). DCP has also been reported as having the same cytological effects as DNP only at greater concentrations (Muhling et al, 1960). Furthermore, DCP has

been shown to exhibit the same physiological effects as DNP on plant tissue (Gaur and Beevers, 1959). Therefore, it seemed logical to determine whether or not DCP would have the same effects as DNP on cells in different segments of the mitotic cycle. The results of an experiment where cells were treated at either 4, 5, or 6 hours after marking are shown in Figures 11A, 11B, and 11C. It is evident that the experiment with DCP corroborates those of DNP and that the summary of the effects of DNP also apply to DCP.

Verification of the DNP results by DCP stimulated some speculation as to the nature or cause of the acceleration of the mitotic cycle. Due to the interest shown in the biochemical activity of DNP a large number of reports have been published dealing with this subject. In considering these, it appears that perhaps the most general effect of DNP *in vivo* is the activation of the enzyme adenosine triphosphatase (ATPase) (Cooper and Lehninger, 1957; Kendall, 1960; Pullman *et al*, 1960; Simon , 1953). This activation could result in an increase in the intracellular concentration of ADP (adenosine diphosphate) and P_i (inorganic phosphate) since ATPase catalyzes the following reaction:

$$ATP \xrightarrow{ATPase} ADP \quad P_i$$

The characteristics of the DNP-stimulated ATPase reaction have been reasonably well described by Pullman *et al*, 1960).

Further, since the rate of anaerobic glycolysis has been reported to be dependent on the concentration of ADP

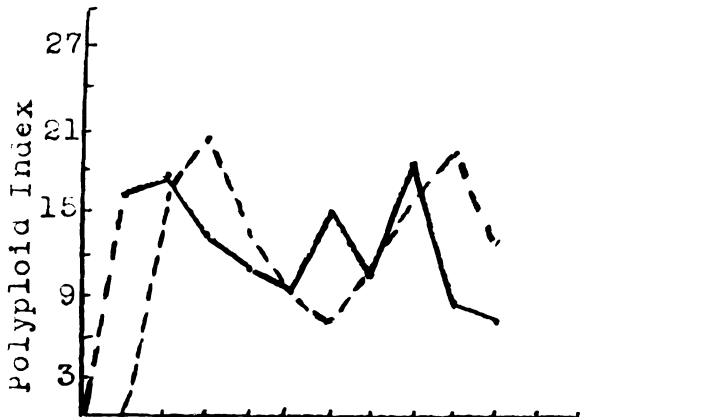


Figure 11A

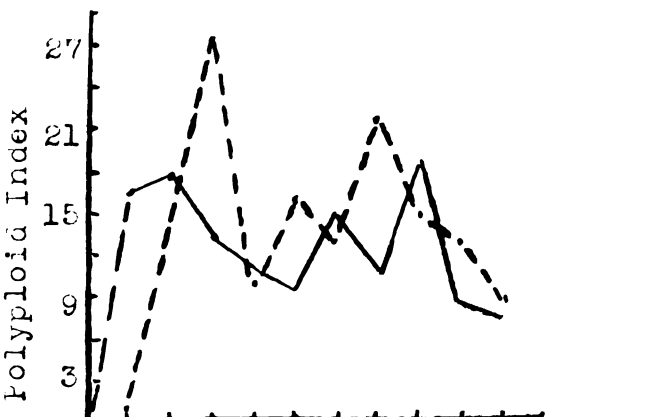


Figure 11B

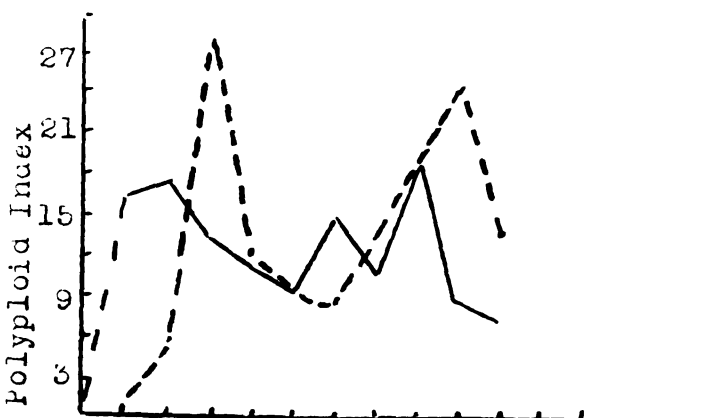


Figure 11C

The effect of a 15 minute treatment with 4.3 X 10^{-4} M DCP at 4 hours (Figure 11A), 5 hours (Figure 11B), and 6 hours (Figure 11C) after marking with colchicine.

(———•) Control
(•- - - -•) Treated

Chance and Hess, 1956; Ammlot and Bos, 1959; Hess and Chance, 1961; Johnson, 1941; Lardy and Parks, 1956; Lehninger, 1957), it is quite possible that the accelerated cycle may be the result of a temporary stimulation of the Embden-Meyerhof pathway due to a momentary increase in intracellular ADP and P_i. If this were so, then a combination of an anaerobic glycolytic inhibitor and DNP should show a different response than either the glycolytic inhibitor or DNP used singly, Figures 12A, 12B, 12C, and 12D and 12E show the results of such an experiment. In this case the inhibitor fluoride was used. The use of fluoride was based on its ability to form a complex with magnesium which is a necessary cofactor for enolase. Figure 12A represents graphically the effect of a 15 minute treatment at the fifth hour with 0.01 M fluoride. The data indicate that the fluoride had little influence on the tetraploid population and its appearance in division II.

Figure 12B however, shows that a similar treatment with 0.05 M fluoride does delay the appearance of the marked cells in division I but does not decrease the cycle time between divisions I and II.

Figure 12C supports previous observations in that a 15 minute treatment at the fifth hour with DNP both delays division I and accelerates the cycle leading to division II.

Figure 12D shows the results obtained by a combined treatment with 4.32×10^{-5} M DNP and 0.01 M fluoride at the fifth hour. The combined treatment obviously delayed

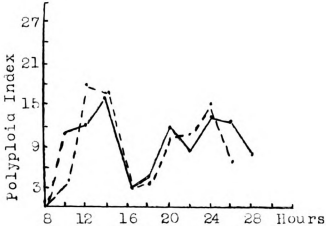


Figure 12A

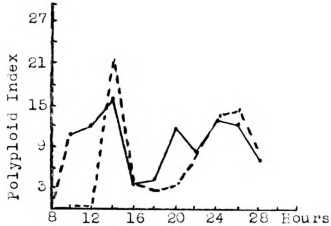


Figure 12B

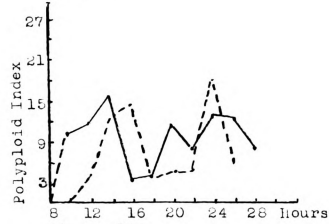


Figure 12C

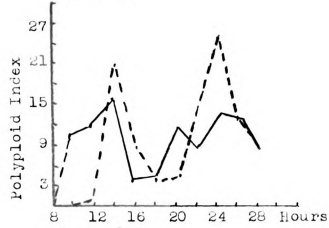


Figure 12D

The effect of a 15 minute treatment with 0.01 M fluoride (Figure 12A), 0.05 M fluoride (Figure 12B), 4.5×10^{-5} M DNP (Figure 12C), and 4.5×10^{-5} M DNP plus 0.01 fluoride (Figure 12D) at the fifth hour after marking with colchicine. (•————•) Control; (•– – – –•) Treated

the population and caused an increase in synchrony. More important however, the accelerating effect of DNP appears to be somewhat dampened by the fluoride. A comparison between Figures 12C and 12D certainly suggests that an inhibition has occurred.

The inhibitory effect of fluoride is rather pronounced when DNP and 0.05 M fluoride are combined. Figure 12E shows a very great delay in the first division of the marked population. This lengthy delay is probably the result of both DNP and the fluoride, since DNP itself delays and, as Figure 12D indicates, the 0.05 M fluoride also produces a delaying effect.

The observations made on the fifth hour segment of interphase with these experiments may be summarized as:

1) 0.01 M fluoride appears to have no effect on the population in any way,
2) 0.05 M fluoride produces a delay in the appearance of the marked cells in division I but does not decrease the duration of the cycle between divisions I and II,
3) fluoride when combined with DNP tends to prevent the decrease in cycle time between the first and second division after treatment.

These observations support the hypothesis that conditions favoring anaerobic glycolysis at the fifth hour segment tend to accelerate the second mitotic cycle following treatment. There is, however, some question concerning

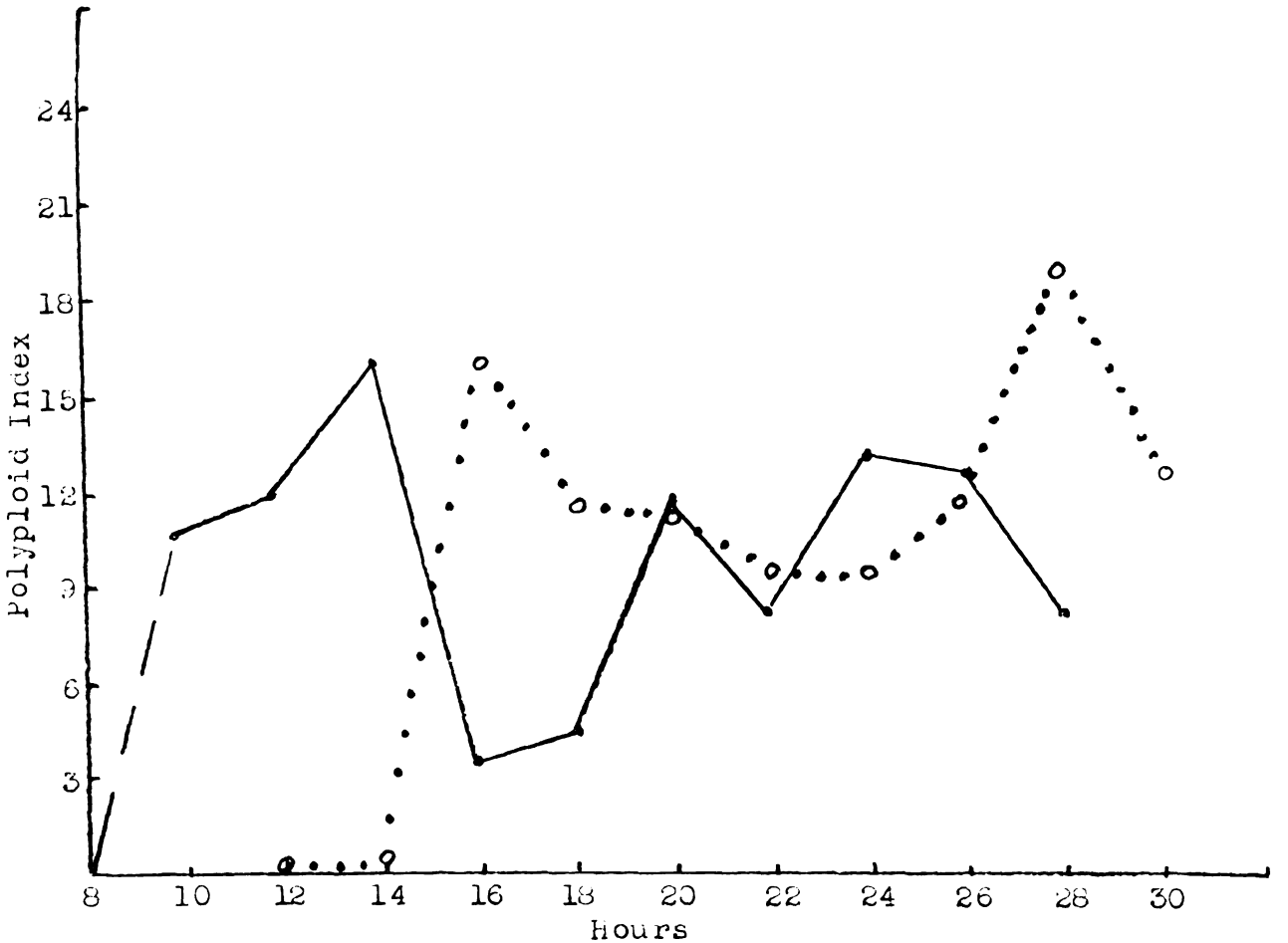


Figure 12E. The effect of a 15 minute treatment with 4.3×10^{-5} M DNP plus 0.05 M fluoride at the fifth hour after marking with colchicine. (●——●) Control; (o····o) Treated

the specificity of the inhibition by fluoride. Pullman et al, (1960) have shown that potassium fluoride will inhibit the activation of ATPase by DNP to a degree. This inhibition occurs because magnesium is a cofactor in the reaction catalyzed by ATPase. The effect of fluoride therefore, may not be the result of enolase inhibition but rather the result of the inhibition of ATPase activation by DNP. Hence some doubt remained as to whether or not anaerobic glycolysis was favored by a short time treatment with DNP. It seemed quite possible that both ATPase activation and anaerobic glycolysis were effected by the fluoride.

Because of this uncertainty another series of experiments involving the use of potassium cyanide was designed. Potassium cyanide was chosen because its use in a short time treatment would tend to increase the rate of anaerobic glycolysis. This would presumably come about by increasing the amount of intracellular ADP and P_i. As pointed out earlier, the increased availability of ADP and P_i should increase the rate of the Embden-Meyerhof pathway. Potassium cyanide, moreover, differs greatly from DNP in the mode of inhibition. Cyanide does not activate ATPase nor does it increase oxygen consumption in the treated tissue. Cyanide does however, inhibit oxygen consumption and prevents the operation of the cytochrome system by combining with enzymes having metalloporphyrin units.

The first series of experiments with cyanide was de-

signed to find out if it had the same effect as DNP on cells that were in late interphase. The concentration used in these experiments was 4×10^{-5} M. This concentration was shown by Eichenberger and Thimann (1957) to inhibit respiration in the pea. Figure 14 shows that a 15 minute treatment at 7, 8, or 9 hours delays the cells a little but does not approach the degree of delay produced by DNP.

Figure 14 A indicates that a similar treatment with cyanide at the fifth hour does not improve the synchrony of the population even though it is delayed. It is important however, to notice that the second appearance of the population occurs before that of the control. Therefore, a fifth hour treatment with cyanide produces the same result as DNP with regard to the time of onset of the second division after treatment (Figure 14 B). When a comparison is made between Figures 14 A and 14 B, the difference and the similarity of these two chemicals are well illustrated. It is also interesting that when the population was treated at the fifth hour by DNP and cyanide combined, the effect produced was neither complementary nor antagonistic (Figure 14 C).

The next series of experiments involving potassium cyanide was designed to detect differences, if they existed, between the fourth, fifth, and sixth hour segments with respect to the production of an accelerated second cycle. The duration of treatment and the concentration of cyanide used in these experiments were the same as those mentioned earli-

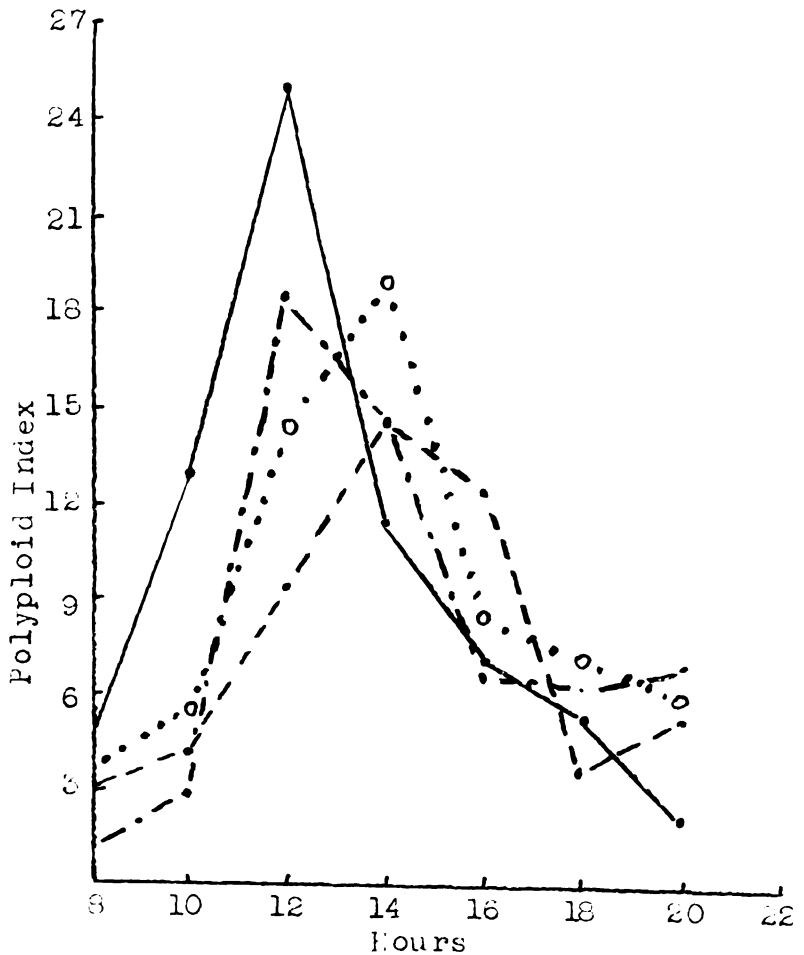


Figure 13. The effect of a 15 minute treatment with 4 x 10^{-5} M cyanide at 7 hours (•— — —), 8 hours (•—•—•), and 9 hours (o•••• o) after marking with colchicine on the first division of the tetraploid population. (•———•) Control.

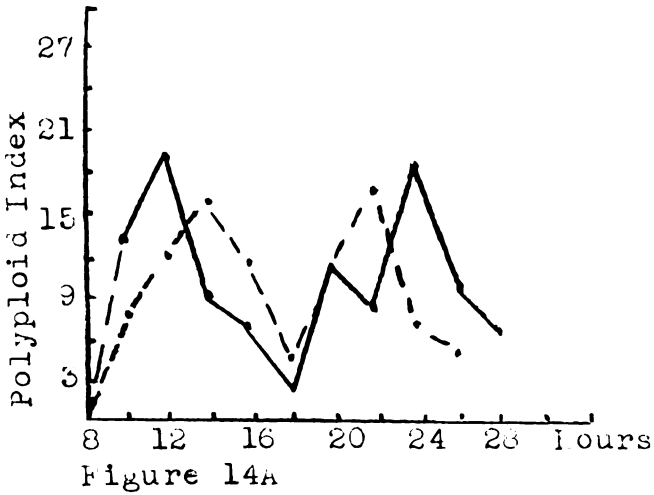


Figure 14A

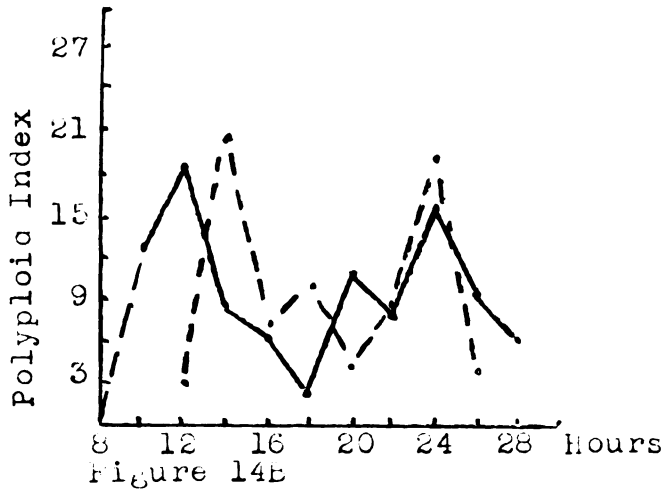


Figure 14B

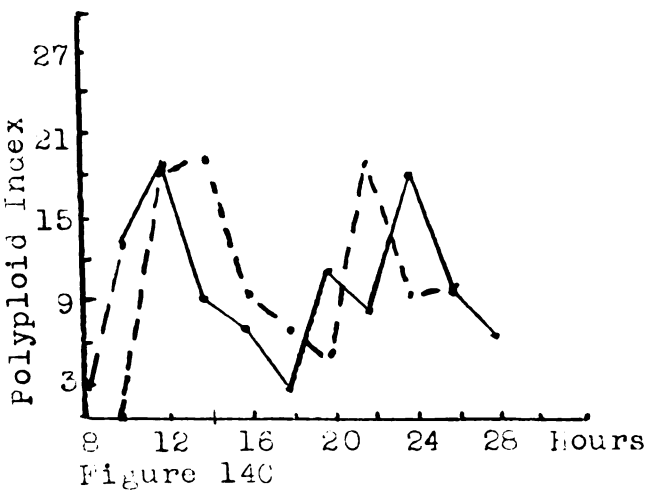


Figure 14C

The effect of a 15 minute treatment with 4 X 10^{-5} M cyanide (Figure 14A), 4.3 X 10^{-5} M DNP (Figure 14B), and 4 X 10^{-5} M cyanide plus 4.3 X 10^{-5} M DNP (Figure 14C) at the fifth hour after marking with colchicine on the first and second division of the tetraploid population.

(———•) Control
(– – –•) Treated

er.

By comparing the general pattern of the curves shown in Figures 15 A, 15 B, and 15 C it is evident that treatment with cyanide at early interphase does not produce a delay in the appearance of the tetraploid population and only the fifth hour treatment brings about an acceleration of the second cycle following treatment. Furthermore, Figure 15 D shows that the accelerated second cycle can be prevented somewhat by 0.01 M fluoride.

These results suggest further that conditions resulting in an increase of anaerobic glycolysis during the fifth hour segment of the mitotic cycle results in a decrease of the cycle time of the second division following treatment.

A summary of the observations made on the cyanide experiments is outlined below.

1) Cyanide does not improve or decrease the synchrony of populations treated in either late or early interphase.
2) The delay produced by cyanide appears to be the result of a general effect on all cells in the population.
3) Only the fifth hour segment of interphase responds to cyanide treatment in the production of an accelerated second cycle.
4) 0.01 M fluoride prevented the acceleration of the second cycle produced by cyanide treatment at the fifth hour.

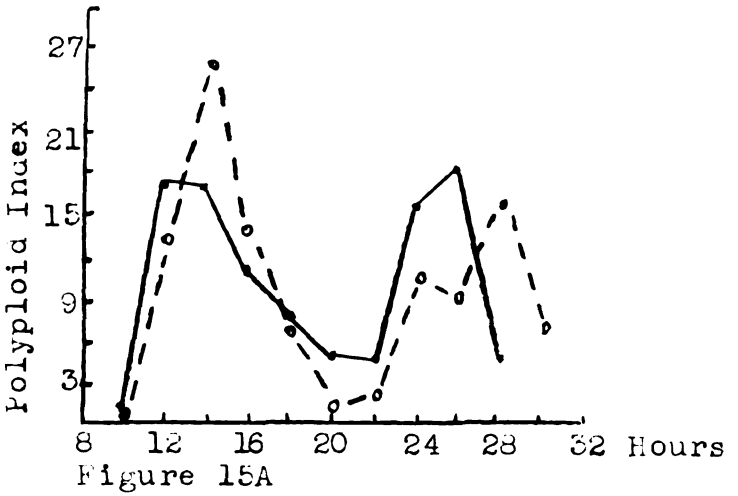


Figure 15A

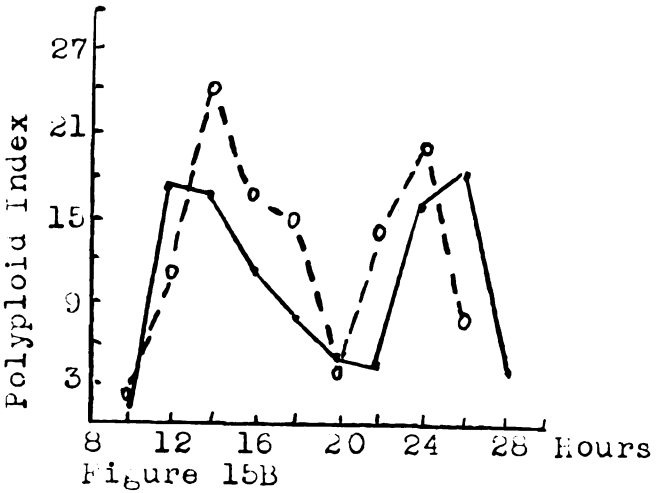


Figure 15B

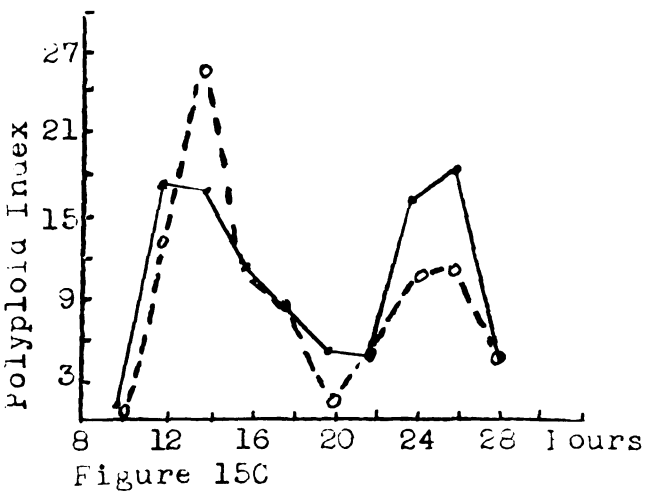


Figure 15C

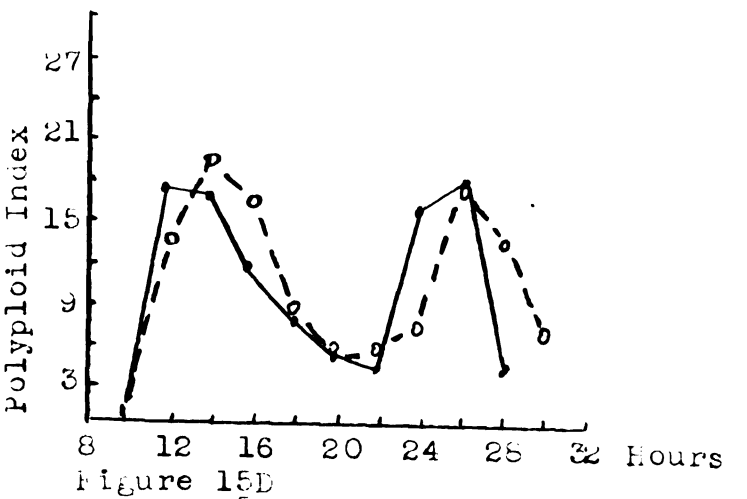


Figure 15D

The effect of a 15 minute treatment with 4 X 10^{-5} M cyanide at 4 hours (Figure 15A), 5 hours (Figure 15B), 6 hours (Figure 15C) and of 4 X 10^{-5} M cyanide plus 0.01 M fluoride (Figure 15D) at 5 hours after marking with colchicine on the first and second division of the tetraploid population.
(———) Control; (– – – –) Treated

5) Cyanide and DNP, when used in combination to treat cells in the fifth hour segment of interphase, are neither complementary nor antagonistic with respect to the production of an accelerated second cycle.

DISCUSSION II

Bullough in 1950, after studying the mitogenic actions of starch and oestrone on ear epidermal cells of a mouse, was led to conclude that cell metabolism alters both quantitatively and qualitatively during the mitotic cycle. He pointed out that this conclusion is not unexpected since cells of early interphase must presumably accomplish a number of syntheses before attaining mitotic competence. Cells of late interphase, on the other hand, are either mitotically competent or nearly so. Since these syntheses are directly or indirectly dependent on the availability of ATP, it is not surprising that cells in different time segments of interphase should respond to DNP treatment as they do. Therefore, the delay and increase in synchrony exhibited by cells when treated with DNP in early interphase could be explained on the basis of a momentary deficiency of ATP. The improvement of synchrony could be brought about in an interesting way. Apparently the first cells affected by DNP are the leading cells of the population. These cells are blocked first and the trailing cells proceed to the point of blockage. This situation is somewhat analogous to the accumulation of automobiles at a red traffic light. When the cause of delay is removed or overcome the cells continue revolving in the cycle just as the automobiles begin to move when the traffic light changes to green.

This explanation is substantiated by the lack of a noticeable increase in synchrony when the tetraploid cells

are treated with cyanide. Cyanide, even though its overall effect is a decrease in aerobic ATP production, does not disrupt respiration in the same manner as DNP. All the cells of the population are affected by cyanide simultaneously, therefore no accumulation of cells occurs and no great improvement of synchrony is produced. This difference of effect by DNP and cyanide is most likely due to the lack of ATPase activation by cyanide.

The loss of synchrony by a population of cells when treated with DNP at late interphase may or may not be due to a decrease in available energy. As Bullough (1951) pointed out, this part of the cycle is characterized by a decrease in metabolism and a more or less successful completion of the syntheses necessary for mitotic competence. Therefore, it is possible that a loss of synchrony may be due to the physiological heterogenity of these cells, since all syntheses should not be expected to be completed at the same time. Further, late interphase cells would have had more time to become randomized (Quastler, 1960).

Even though increased heterogenity may be a factor in the loss of synchrony, changes in cell energetics remain the more likely explanation, for Wilson, Morrison, and Knobloch (1959) have shown that pea root cells in antephase were encouraged to divide by the addition of proper carbohydrates. It is unlikely that some source of energy was not expended in the uptake of these compounds. Moreover, as cells approach prophase they increase in volume (Brachet, 1957; Hughes,

1952; Leak and Wilson, 1960) and again it is not improbable that such a change does require a certain amount of energy. Therefore, the effect of DNP on late interphase cells can be explained in terms of a decrease in usable energy.

The lack of effect of 0.01 M fluoride on the first division of the tetraploid population emphasizes the importance of ATP during early interphase. Assuming the fluoride momentarily decreased anaerobic glycolysis, the absence of a delay is not unexpected for a decrease in ATP produced by this pathway is almost inconsequential as compared to the amount produced by aerobic glycolysis. Nevertheless, the delay produced by 0.05 M fluoride shows that a more complete inhibition of the Embden-Meyerhof pathway also reduces the amount of substrate for aerobic glycolysis and therefore the total ATP production.

Finally, the effect on the first division produced by a combination of either DNP and cyanide, or DNP and fluoride, show that each chemical acted somewhat independently of the other and that no combination produced an effect greater than that observed when either DNP or cyanide was used singly.

In general it may be said that these observations agree with those of Bullough and Johnson (1951) using mouse ear epidermis, with those of Barnett (1955) on dividing Arbacia eggs, with those of Hamburger and Zeuthen (1957) on synchronously dividing Tetrahymena, and with those of Stich (1954) on polychaete eggs.

Consideration of the precocious second division of the tetraploid population when treated with either DNP, DCP, or

cyanide, requires explanations additional to those of cellular energetics. It is obvious that the situation as it exists at the fifth hour segment is different from the fourth and sixth hour segments, since treatment at hours other than the fifth do not result in an accelerated second cycle.

The facts concerning the fifth hour segment and the production of an accelerated second cycle are these:

1) neither 0.01 M fluoride nor 0.05 M fluoride produce a precocious second division, nor do they increase the duration of the second cycle,
2) DNP, DCP, and cyanide treatment all result in an accelerated second cycle, and
3) 0.01 M fluoride tends to prevent the production of a decrease in cycle time by either DNP or cyanide.

The effect of fluoride indicates that the production of a fast cycle does not occur when anaerobic glycolysis is inhibited. Moreover, this also shows that the maintenance of the normal cycle time is not affected by a decrease in anaerobic glycolysis and that the fifth hour segment is insensitive to a decrease in anaerobic glycolysis.

The effects of DNP, DCP, or cyanide, however, indicate that the fifth hour segment is highly responsive to an increase in rate of the Embden-Meyerhof pathway and this sensitivity is verified by the effect of fluoride on both DNP and cyanide treatments.

Perhaps the most important observation made in these studies is that a treatment performed during a given inter-

phase affects not only the mitosis following treatment but also the second mitosis after treatment (division II). This residual effect can be explained in only one way; that is, a cell while preparing for the next mitosis is also preparing for at least one mitosis beyond the next. Therefore, any treatment performed on dividing cells will have two effects; one on the immediate mitosis and another on the second division after treatment. Taking this into account, it is not surprising that Gray and Scholes (1951) observed a greater effect of radiation on cells that completed two revolutions of the mitotic cycle after treatment.

Swann's (1954) work with sea urchin eggs also implies that cleaving eggs are preparing for two consecutive mitoses simultaneously. Prescott (1960) while studying the relative rates of DNA, RNA, and protein synthesis during interphase of Tetrahymena also came to the conclusion that the RNA synthesized during a given interphase has little or nothing to do with the functions of that interphase but is produced to function during the subsequent interphase. Therefore, because the result of an increase in anaerobic glycolysis during the fifth hour segment is not observed until the second division following treatment, it is apparent that more consideration must be given to those systems that are more stable and less susceptible to the normal chemical fluctuations of the cell.

Swann's (1954) concept of an ATP storage mechanism fits these requirements. He hypothesizes that ATP is shunted off

somewhere in the cell and thus prevented from participating in normal cellular reactions. Swann's idea should not be taken lightly, for recently Hess and Chance (1961) presented evidence that the mitochondria of the ascites tumor cells are able to retain ATP. Hence, the possibility does exist that the mitochondria may function both as an ATP producing particle and an ATP storage unit. In terms of Swann's hypothesis, the results reported in this dissertation may be explained in the following manner. Both DNP and cyanide increased the amount of phosphate acceptor and inorganic phosphate, thus increasing the rate of anaerobic glycolysis. This increase would provide a surplus of substrate for Kreb's cycle activity which would produce a spark or spurt type of reaction. This sudden increase in Kreb's cycle activity would result in the production of an unusual amount of ATP. Assuming that any ATP over an established limit would be parcelled out to be stored for future cycle functions, the momentary increase in ATP would result in an increase in the mount of ATP contained in the storage mechanism. Since the ATP accumulation is ahead of schedule, a precocious second division should come about.

Another possibility that should be considered is the change in distribution of intracellular components as a result of treatment. Frédéric (1954) observed that DNP first stimulated and then inhibited mitochondrial movements. Kamiya (1960) concluded that cytoplasmic streaming almost without exception is increased with the increase in anae-

robic glycolysis. These two reports are important from the standpoint of intracellular architecture, for it is possible that one form of architecture would favor one kind of synthesis while another form may set up conditions favoring another. It is not difficult to imagine that an increased rate in cytoplasmic streaming and therefore increased rate in substrate distribution within a cell may be more compatable with a particular type of synthesis.

Another factor which must be considered with those already mentioned is the influence of an unusual concentration of the products of anaerobic glycolysis. These C-3 units could well be aminated by an aminase and eventually incorporated into specific proteins that normally are not synthesized until a later time.

Further, if a G_1 period occurs in the interphase of a pea root meristem cell at approximately the same time as in other cells, it is possible that the fifth hour segment represents the beginning of DNA synthesis and that an increase in anaerobic glycolysis at this time benefits DNA synthesis in some way. The work of Allfrey and Mirsky (1957) suggests a possible connection between DNA synthesis and respiration. They found a respiratory chain in the nuclei of calf thymus cells that is sensitive to cyanide and DNP but reacts differently to other uncouplers. It is conceivable that this particular respiratory chain is specifically linked with the enzymatic synthesis of nucleoside triphosphates to be used by the nucleus or nucleolus in forming RNA and DNA. Though

these speculations are interesting, experimental proof that the favoring of anaerobic glycolysis has anything to do with DNA synthesis remains to be adduced.

Early interphase or G_1 cells are not only affected by an increase in rate of the Embden-Mayerhof pathway. Pelc and Howard (1955) found that root cells of Vicia faba in the G_1 portion of the mitotic cycle were also inhibited from synthesizing DNA by low doses of radiation. The work of Lajtha et al (1958) substantiates the findings of Pelc and Howard. He and his co-workers found that the G_1 cells of bone marrow are hypersensitive to low doses of radiation and that they too were inhibited from synthesizing DNA. Therefore it seems as if the fifth hour segment and the G_1 radiation sensitive segment may be one and the same. This possibility however, remains to be tested.

CONCLUSION

The purposes of these investigations were, first, to develop a technique that was applicable to the study of differences between cells in the various segments of the mitotic cycle, and second, to study the effects of physiologically active chemicals on cells in these segments.

The technique developed was one utilizing colchicine as a polyploidizing agent, and producing a small population of tetraploid cells that divided more or less synchronously for at least three divisions. Using this technique and short time treatments with DNP and DCP, a difference between early and late interphase cells was demonstrated.

Another difference was demonstrated by the effect of DNP, DCP, cyanide and fluoride on the fifth hour segment of interphase. This segment is peculiar in that treatment with DNP, DCP, or cyanide at this time delays the first division of the population but accelerates the second mitotic cycle of the population.

The difference between early and late interphase cells and the delay in appearance of the tetraploids in division can be explained satisfactorily in terms of ATP requirements. There is uncertainty however, as to the explanation of the responsiveness of the fifth hour segment to a momentary increase in anaerobic glycolysis.

SUMMARY

1. A synchronously dividing population of tetraploid cells was produced in the root meristem of pea seedlings by a 30 minute treatment with the appropriate concentration of colchicine.
2. The proper amount of colchicine was determined by treating seedlings for 30 minutes with various concentrations and then measuring the rate of disappearance of cells showing an effect and the appearance and disappearance of the resulting tetraploids in mitosis.
3. A quantitative relationship between the number of cells affected and the number of tetraploid cells was shown to exist at higher colchicine concentrations.
4. The existence of a threshold in the production of a colchicine effect was demonstrated by treating seedlings for varying lengths of time with a given colchicine concentration and then determining the size of the tetraploid population produced.
5. An experiment involving three revolutions of the mitotic cycle indicated that useful synchrony remained during this time in spite of an increase in the distribution of the population due to the different speeds of cells revolving in the cycle.
6. Treatment of the population with DNP or DCP in early interphase caused a delay in appearance in the subsequent mitosis and an improvement in the synchronization.
7. Treatment of the population with DNP or DCP when it

was in late interphase resulted in a decrease in synchrony and a slight delay in its entrance in division.

8. When the population was treated with DNP, DCP, or cyanide at the fifth hour after polyploidizing, the second division following treatment made a precocious appearance in division as compared to the controls, thus indicating a decrease in the mitotic cycle time.

9. Neither cyanide nor fluoride in low concentrations affected the degree of synchrony of the population.

10. The acceleration of the second cycle following treatment was prevented to a greater or lesser degree by combining fluoride with either DNP or cyanide.

11. The decrease in cycle time of a treated population appears to be the result of creating cellular conditions favorable to an increase in the rate of anaerobic glycolysis at the fifth hour segment of interphase.

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