ANTIMITOTIC EFFECTS OF SIMPLE SUBSTITUTED PHENOLS AND AROMATIC ORGANIC PHOSPHORUS COMPOUNDS

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has been accepted towards fulfillment
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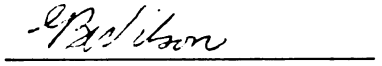

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ABSTRACT

ANTIMITOTIC EFFECTS OF SIMPLE SUBSTITUTED PHENOLS AND AROMATIC ORGANIC PHOSPHORUS COMPOUNDS

by Arthur Alan Nethery

An attempt was made to determine the cytologically recognizeable effects of a series of substituted phenols and of a group of aromatic organic phosphates derived from the simple phenols, and by comparison of the effects, to determine whether a relationship exists between the chemical structures and biological activity.

Treatment of the root meristems of the garden pea (Pisum sativum, var. Alaska) was accomplished by adding the chemicals to an aqueous nutrient medium used to culture the pea roots under standardized conditions. By microscopical examination of the root meristems, analyses were made of the cytological changes induced by the chemical treatments. The relative toxic levels of the chemicals were obtained from toxicity tests on Drosophila melanogaster.

Three types of antimitotic effect were produced by all members of these two series of compounds--pre-prophasic inhibition, late prophase blockage and spindle disruption--all of which occur at sub-toxic dose levels. The general pattern of pre-prophasic inhibition produced a depression in the mitotic index first occurring at from one and one-half to three hours after the initiation of treatment. The mitotic index remained depressed for two to eight hours, depending on the chemical used and the dose level.

Subsequent recovery was very rapid and resulted in a sharp peak of mitotic activity significantly above the control level, before the index returned to the normal level.

The prophase block caused an increase in the relative proportion of late prophase configurations after treatment. Spindle disruption was of the type produced by the alkaloid colchicine.

The nature, number and position of substituents affects the relative capabilities of the compounds to produce the antimitotic effects. Nitro groups were found to be most effective and methyl groups the least. Bromo and chloro substituents had an intermediate effect on the activity of the molecule. Three substituents caused a greater effect than two, and the ortho positions were the most influential in causing cytological changes.

The organic phosphorus compounds were found to be non-effective when the phosphorus atom was bonded to a sulfur atom, but were effective when the phosphorus was bonded to an oxygen atom.

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INTRODUCTION

This research program was initiated as an attempt to determine the differential cytological effects of a series of substituted phenols and of a series of aromatic organic phosphates, in order to establish whether a relationship exists between their chemical structure and biological activity. This type of approach has been fairly successful as applied to the structure-activity relationships of several groups of plant growth regulators, namely the phenoxy-alkyl acids (Linser, 1956 and Aberg, 1956) and the naphthyloxy-alkyl acids (Linser, 1956; Luckwill and Woodcock, 1956; van Overbeek, 1956), and in the area of the bactericidal and insecticidal activity of phenols (Kagy, 1941; Metcalf, 1955; Tattersfield, 1925). The value of the correlation of the structures of a series of related compounds to their relative biological activities may be several-fold. First, such a system can be of predictive value in the synthesis and manufacture of commercial pesticides, and in the routine analysis of these compounds, where reasonable expectations may be gained as to their primary and general usefulness, appropriate formulations, and possible deleterious side effects on plants and animals. Secondly, the elucidation of the chemical groups requisite for the biological action to occur provides an indication of the probable chemical and physical factors responsible for the effect, and may be helpful as a first step in the study of the mode of action of these compounds.

The study of antimitotic agents is important both from the standpoint of determining the action of specific causal factors and of inferring from such disruptions the "normal" activities of the mitotic cycle. Although a great deal of work has been done on the antimitotic effects of various physical and chemical agents (Biesele, 1958), it was felt that there was a need for a systematic comparative study of the effects of a series of closely related compounds. The experimental system for this comparison must necessarily be rigidly standardized insofar as possible, in order to recognize subtle differences in the effects of compounds of similar activities.

Since several phenols were known to have a recognizeable effect on mitosis at physiological concentrations and because of their commercial importance as disinfectants and pesticides, this group of compounds was selected as a model for developing a method for the comparative assay of structure-activity relationships. The phenol ring also has the capacity for ring substitution, by which means a large number of relatively stable compounds of only slightly different structures may be produced.

In order to describe antimitotic effects, we must first classify the various possible aberrancies arising from the disruption of the mitotic cycle. According to Wilson (1965), only two classes of events may be readily recognized as changes from the normal course of mitosis (other than gross chromosome damage). These are: 1. inhibition of the onset of

mitosis and 2. production of deviant figures in mitosis. In the second category, essentially two types of disruption occur, namely failure of the transition of late prophase to pro-metaphase and failure of the spindle to form or to function.

These three criteria of mitotic interruption are more or less separable as distinct effects on the division cycle, and are suggestive of the occurrence of several reactions or modes of action. An analysis of the comparative effects of a group of closely related phenolic compounds was made, based on these criteria. The ends were: 1. to develop an analytical method for the comparison of chemical structure with biological activity, 2. to describe a general type of antimitotic reaction for phenolic compounds and 3. to correlate the biological activity of the compounds with their chemical structures and physicochemical properties by pointing up specific differences between the effects of related compounds.

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LITERATURE REVIEW

A. Classification of Antimitotic Effects

The general field of antimitotic chemicals has been reviewed by Biesele (1958), who discussed several methods of classification of antimitotic action based on the susceptible stages of the mitotic cycle or on observable damage induced, and on the physiological changes or biochemical reactions affected. Bauch classified mitotic poisons into 1. spindle poisons, 2. cell division poisons and 3. chromosome poisons. Levan discussed chemical effects under 1. lethal and toxic reactions, 2. reversible physiological reactions and 3. mutagenic reactions. D'Amato grouped mitotic poisons into 1. inhibitors of cytokinesis, 2. spindle inhibitors and 3. preprophasic inhibitors (Biesele, 1958). Wilson (1960) classified chemical effects on the cell into four types--mutagenesis, fragmentation, carcinogenic activity and antimitotic activity--and briefly described the general nature of antimitotic action. Other systems include groupings for chromosome breakage, "perfect" or "partial" radiomimesis, prolongation of metaphase and faulty chromosome separation (Biesele, 1958).

The classification proposed here, for the sake of clarity, places chemical agents producing cellular effects under five headings - 1. irreversible lethal and toxic reactions, 2. mutagenesis, 3. fragmentation, 4. carcinogenic activity and 5. antimitotic activity. The last category is further subdivided into a) spindle disruption, b) prophase blockage,

c) pre-prophasic mitotic inhibition and d) failure of cytokinesis.

B. Spindle Disruption

The field of experimental cytology dealing with the chemical and physical factors which influence the mitotic cycle began in earnest with the work of Nebel and Ruttle (1938) and Levan (1938) on the effect of the alkaloid colchicine on mitosis. Nebel and Ruttle reported that colchicine was an important tool for producing polyploidy in plants, which led to an abundance of applied research, but not to the elucidation of the basic cellular effects or of the mode of action. Levan showed that colchicine suppressed spindle formation, and consequently resulted in the failure of chromatid separation. The term "c-mitosis" was applied to this distinctive action. Later the scope of the field was broadened by the reports of many other compounds giving "c-mitotic" effects (Levan and Sandwall, 1943; Levan and Ostergren, 1943; Ostergren, 1944; D'Amato, 1948; Levan and Tjio, 1948). These chemicals were classified as c-mitotic primarily because of the production of scattered configurations, and thus may not all fall under the description of c-mitosis as set forth below.

The independent work of Auerbach (1943) and Oehlkers (1943) proving that mutations can be induced both in plants and in animals by chemical agents gave an additional impetus to the study of chemicals exhibiting various cytological effects.

Although the many compounds classified as c-mitotic agents are, for the most part, completely unrelated both structurally and in chemically reactive groups, it must be supposed that, if the cytological effect truly is the same, the mode of action should be common to all. However, since a serious error could be made in attempting to infer a common mechanism from the observation of the end result, the aberrant configurations, the c-mitotic reaction will be defined here as adhering to the following criteria outlined by Hadder and Wilson (1958): 1. no inhibition of the onset of mitosis, 2. production of the following deviant configurations in order: a) "scatters" (c-metaphase) associated with partial effect, b) "clumps" (stalled pro-metaphase) associated with full reaction, 3. for a given dose under standard conditions there should be a steady increase in the degree of effect with time until either full effect or an equilibrium is reached, 4. polyploid or multinucleate cells or both should be recoverable.

C. Prophase Blockage

The prophase poison reaction was first described by D'Amato (1948 and 1949), and later by Hawthorne and Wilson (1952) and Hadder and Wilson (1958), the latter two papers describing the effects of the anti-fungal antibiotic Acti-dione in this regard. Since the activity of this chemical has been thoroughly described, it may be used as the type reaction for the prophase stalling effect. It should be added here that Acti-dione also affects the pre-mitotic

competence of the cell to a great extent.

D. Mitotic Inhibition

Although mitotic inhibition is sometimes thought of in terms of a physiological effect rather than a cytological one (Biesele, 1958), it should be considered in conjunction with prophase stalling and spindle disruption, particularly since it often occurs simultaneously with prophase blockage (Wilson, 1965).

E. Cytological Test Systems

Many variations of the Allium test, as described by Levan (1938) and D'Amato (1949), have been used widely in testing the effects of chemicals at the cytological level. While this is to some extent a standardized system, there are several drawbacks to this test, namely the variability in mitotic index over a period of time in the root tip, the lack of uniformity due to the differences in lengths and ages between individual roots, and the difficulty in maintaining uniformity among several stocks of onion bulbs.

The Pisum test, which has been described by Bowen and Wilson (1954) and re-examined in some detail by Wilson (1965) provides a greater uniformity of material, due to the possibility of selection of individual roots for testing and to the intrinsically more stable mitotic levels over a period of time. This particular system has also been highly standardized with respect to such variables as temperature, humidity, pH, and total ion concentration of the nutrient medium.

F. Biological Activity and Structure of Phenols

According to Sexton (1963), any biological activity which is recognized as being due to an exogenous chemical may be considered either "the end result of a series of interlinked chemical reactions or the observable manifestation of an interference with a delicately balanced system of interdependent chemical and physical processes." He also states that chemical compounds do not necessarily produce biological activity by inhibiting the function of specific essential metabolites, but also bring about such effects by mechanisms which may be determined by physicochemical factors. Therefore, the chemical structure may be correlated with the physiological effect, to the extent that the physicochemical properties relate to the structure.

Various substituted phenols have been studied with a view to their effect on mitosis or chromosome structure. Levan and Tjio (1948) reported a c-mitotic effect for the mononitrophenols, and also claimed radiomimetic effects. The antimitotic activity of various other phenols has also been reported (Loveless and Revell, 1949; Hindmarsh, 1951; Muhling et al., 1960; Clowes, 1951).

Very little is known about the mode of action of the phenols as mitotic inhibitors. A number of theories relating the physical or chemical molecular characteristics to the biological activity of phenols have been proposed.

According to Ferguson (1939), Richet stated that chemical toxicity is roughly inversely proportional to the sol-

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ubility of the compound. He also reported that Moore claimed that the toxicity of insect fumigants increased with the boiling point and consequently is in an inverse relationship with the vapor pressure. The Meyer-Overton lipoid theory of narcosis (Meyer and Hemmi, 1935) assumed that isonarcotic effects are produced by extremely diverse chemical structures when their molar concentrations in the cell lipoids are identical. Levan and Ostergren (1943), from a study of numerous chemicals inducing polyploidy in plants, found that the introduction of -OH, $-NH_2$, $-CO_2H$, and $-SO_3H$ radicals into the reactive molecules extinguished the c-mitotic effect. They also noted that $-NO_2$ and haloids sometimes had this extinguishing effect. Since most of these are typical hydrophilic groups which as a rule increase water solubility (and thereby decrease lipoid solubility), this correlation was taken to suggest that the partition of substances between water and lipoids plays an important part in the mechanism of c-mitotic action. Ferguson (1939) believed that a parallelism between physiological action and the oil/water distribution ratio is really a case of Richet's rule, and that the same may be said of the parallelism between physiological action and surface tension lowering. From the work of Fuhner, Ferguson (1939) draws the conclusion that it is apparent that there is no intrinsic connection between the two, and where a correlation exists, it is due to the fact that highly surface-active substances are also usually very slightly soluble.

THE

Comparative studies of structure-activity relationships for bactericidal activity of phenols have been carried out (Wolf and Westveer, 1952; Suter, 1941). Wolf and Westveer determined the phenol coefficients of 16 of the 19 possible chlorophenols and showed that they increase in germicidal activity in the mono-, di-, and trichloro- series, but decrease in the tetra- and penta- series. According to Tattersfield (1925), the toxic value of nitrocresols as contact insecticides was greatest when the nitro group was *para* to the hydroxyl group, and when the ring was alkylated in the *ortho* position as in 4, 6-dinitro-*o*-cresol (DNOC). With respect to the mononitrophenols, he found *para* >*meta* >*ortho*. He also found that the introduction of a third nitro group to 2, 4-dinitrophenol to form picric acid reduced toxicity to *Aphis rumicis*.

The physiological activity of phenols has been studied by Blackman *et al.*(1955 a and b) by the induction of chlorosis in *Lemna minor* and the reduction of radial growth of *Trichoderma viride*. They concluded that when the results are corrected for the degree of dissociation of the molecule, as an alkyl chain is lengthened or the number of chlorine atoms or alkyl groups substituted in the ring is increased, then the biological activity is progressively augmented. They also found that the position of the substituents was important. Blackman *et al.* (1955a) emphasized the importance of correcting comparative concentrations of different chemicals for the difference in dissociation of the mole-

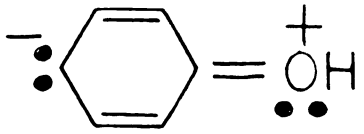
cules. They found (1955b) that all phenols tested showed a general linear relationship between pK value and the logarithm of activity, except those which are substituted in both ortho (2 and 6) positions, at least one of which substituents is a chlorine atom. All of these compounds failed to conform because the activity was considerably higher in relation to the pK values. Considering the hydroxyl group of prime importance in activating the biological response, they interpreted these results in terms of the enhanced ionization of the phenol by adjacent chlorine atoms and of the tendency to repel the compound from possible negatively charged bonding sites. According to Metcalf (1955), Dierick found that a 0.12% aqueous solution of 4, 6-dinitro-o-cresol (DNOC) applied to Ephestia eggs produced 100% mortality at pH 2 and none at pH 5. The importance of considering the amount of dissociation of the molecule is obvious, since DNOC is undissociated at pH 2 and completely dissociated at pH 7. It should also be noted that the amount of dissociation affects the oil/water phase distribution of the chemical--the greater the dissociation, the greater the affinity for the aqueous phase.

2, 4-dinitrophenol and 2, 4-dichlorophenol are known to be uncouplers of oxidative phosphorylation, and several other phenols have been reported to have this activity at varying degrees of efficiency. Chance and Hollunger (1960) found uncoupling activity with 2, 4-dibromophenol; Gaur and Beevers (1959) list the mono-substituted nitro-, chloro-

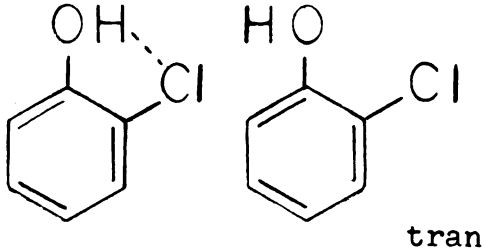
and bromo- phenols as exhibiting uncoupling of oxidative phosphorylation.

In his study of the herbicidal action of compounds with phenyl nuclei, Aberg (1956) shows a correlation between the lowering of the "intrinsic auxin activity" and the increase in the van der Waals radius of the para substituent. He found that the effect of para chlorination is to increase the dissociation constant, leading to the notion that para substitution could increase the affinity of a substance for receptor sites.

According to Pauling (1960), phenols form stronger hydrogen bonds than aliphatic alcohols because of the increase in electonegativity of the oxygen atom resulting from resonance with structures such as:



He reports that hydrogen bond formation is of importance in affecting the melting point, boiling point, dielectric constant, solubility of organic liquids in water and other solvents, and proton magnetic resonance. In phenol and substituted phenols the C-O bond has some double bond character, so that cis and trans forms are possible:



cis trans

Thus, intramolecular bonding results in the presence of more of the cis form, at least in the gaseous phase, since it is more stabilized. In the liquid and crystal phases, intermolecular bonding may tend to keep both forms in somewhat equal abundance.

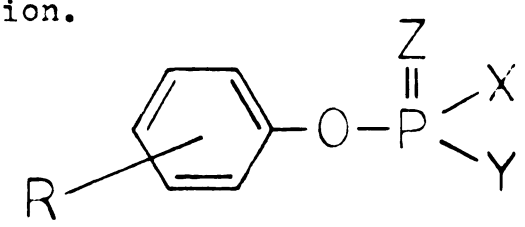
Sundt (1961) states that the influence of substituents on the physical properties of phenols is due partly to their nature and partly to the position of the substitution. The chief effects are: a) inductive effect, b) steric hindrance, c) hyperconjugation and d) mesomeric effect.

A comparison of structure-activity relationships of phenols made at the level of antimitotic activity was reported by Muhling et al. (1960). Their work indicated the importance of the ortho and para positions, in addition to describing to some extent the cytological effects of 2, 4-dinitrophenol and 2, 4-dichlorophenol. Clowes (1951) reported the inhibition of cell division in sea urchin eggs with nitro- and halo-substituted phenols. Inhibition occurred with the dinitrophenols and all dihalogenated phenols except those substituted in the ortho (2 and 6) positions. The meta and para mononitrophenols were less active, and the ortho-nitrophenol and all of the monohalophenols were inactive.

G. Biological Activity and Structure of Aromatic Organic Phosphorus Compounds

As far as the aromatic organic phosphates are concerned, the only report of their antimitotic activity was made by

Nethery et al. (1965) on the studies of the acaricide and anthelmintic ruelene. In considering the structure-activity relationships of these complex molecules one must take into consideration variations in groups X, Y, and Z, and both the position and the particular chemical group involved in the R substitution.



In discussing the insecticidally active phosphorus esters, Metcalf (1955) makes the generalization that methyl esters (at groups X and Y) are less toxic than the ethyl esters, and that activity falls again with increased chain length, although there are numerous exceptions. He also states that the stability of the dialkyl phosphorylated enzyme complex involved in acetylcholinesterase inhibition is approximately the same for various inhibitors containing the same dialkyl groups and varies in the order dimethyl phosphorylation< diethyl phosphorylation< diisopropyl phosphorylation. Group Z usually is either O, S or Se. The first two are the most common and are of commercial value as pesticides. The S substitutions are much less toxic, which is partially explainable by the fact that, at least in some cases, the thionophosphates must be converted enzymatically by the organism to the phosphate in order for the toxic action to occur (Metcalf, 1955). Such an enzyme system may also operate in some plants which are

selectively susceptible to herbicides such as Zytron (Crafts, 1961).

EXPERIMENTAL PROCEDURES

A. Cytological Experimental System

The experimental procedure used to determine the anti-mitotic effect of the phenolic compounds was a standardized system for the treatment of the root meristem of the garden pea, Pisum sativum, var. Alaska. The dried peas were for the most part supplied by the Ferry Morse Company and were guaranteed to be free of chemical treatment. The final experiments were conducted on peas of the same variety supplied by the Vaughan Seed Company; the results obtained were entirely equivalent to the earlier runs.

The dried peas were rinsed three times in de-ionized distilled water, then soaked for six hours in de-ionized distilled water at 22.5°C. ± 0.5°C. The soaked peas were rolled in absorbent paper toweling moistened with de-ionized distilled water. The rolls were wrapped with waxed paper, leaving about one inch uncovered at the lower end of the roll to facilitate water absorption, and placed in approximately one inch of water in 600 ml beakers. These rolls were left to germinate for 36-42 hours in an incubator at 22.5°C. ± 0.5°C., with the relative humidity increased by means of open pans of water on the lower shelf of the incubator. After germination, the paper toweling was unrolled, and the pea seedlings were selected for a root length of 1½-2 cm. These peas were suspended by means of wax-coated wire mesh with spacings of about 9 mm (3 to the inch) placed over 100 mm x 50 mm crystallizing dishes containing approx-

imately 390 ml of modified Hoagland nutrient solution made as follows:

12.5 ml of each of the five stock solutions was used per 937.5 ml of de-ionized distilled water to make each liter of nutrient solution.

Stock A - 3.80 g Calcium nitrate $Ca(NO_3)_2 . 4H_2O$ in 500 ml H_2O

Stock B - 5.16 g Ammonium nitrate NH_4NO_3 in 500 ml H_2O

Stock C - 7.20 g Magnesium sulfate $Mg SO_4 . 7H_2O$ in 500 ml H_2O

Stock D - 5.34 g Potassium monobasic phosphate KH_2PO_4 in 500 ml H_2O

Stock E - 0.28 g Potassium dibasic phosphate K_2HPO_4 in 500 ml H_2O

The pH of this solution was found to be 5.6-5.7.

Air filtered through a water trap and through a charcoal-glass wool filter was bubbled through the nutrient solution to provide oxygen and to supply a stirring action for homogeneous oxygen distribution.

The treatment dishes were maintained at 22.5°C. $\pm$ 0.5°C. by means of a controlled temperature water bath in an air-conditioned room with the relative humidity held at 40-50%. The peas were allowed to acclimatize to the culture conditions in the treatment dishes for four hours, at which time the wire grids carrying the peas were transferred to other crystallizing dishes containing the chemical being tested

in solution or suspended in the nutrient solution. The chemicals used for treatment were dissolved in the nutrient solution at the desired concentrations, if sufficiently soluble. Most of the phenols required heating up to 70°C., while being stirred on a magnetic stirrer-heater. At this temperature there should be no appreciable breakdown of the phenolic compounds studied. The abbreviations, symbols, and chemical structures of all compounds tested are given in Tables 1 through 5. Several of the less water-soluble compounds were suspended in the nutrient medium by the use of surface-active agents. The most commonly used was Tween 80, supplied by Nutritional Biochemicals Corporation, which was used at concentrations below 0.5 g/liter of nutrient medium. At this concentration, no cytologically detectable effect due to this compound occurred. The technique used was to add the weighed amount of chemical to the measured Tween 80, and then to pour on nutrient solution previously heated to 70°C. Stirring for up to one-half hour, while holding the temperature at 70°C. sufficed to produce apparently homogeneous suspensions with no sedimentation in most cases.

Samples were taken at various regular time intervals after the treatment for cytological analysis. A zero hour control (before treatment) of each treatment dish was taken. and a continuous non-treated control dish was run for the duration of each experiment. Samples were taken by removing at random five pea seedlings for each composite sample

at a given time, breaking off approximately the first centimeter of the distal end of the root and immediately killing and fixing the specimens in a shell vial containing approximately five ml of fixative consisting of 6 parts methanol, 3 parts chloroform, and 2 parts propionic acid.

The vials containing the fixed pea root tips were evacuated for 10-15 minutes. The material was prepared for being made into slides by fixation for at least twelve hours under refrigeration or 20 minutes in a 60°C. oven. The fixative was decanted and 60°C. 1.0 N HCl poured on. The vials were left in the 60°C. oven for 18-20 minutes, to allow mild hydrolysis of the deoxyribonucleic acid, liberating aldehyde groups which react with leucobasic fuchsin (Schiff reagent) to give a purplish color. The acid was poured off and Schiff reagent was added. The meristematic tissue developed a deep purple color in 15-30 minutes.

Slide preparation was carried out by removing the darkly stained meristematic region (approximately 1 mm), and macerating it with a flat-end glass rod in a drop of 0.5% fast green stain in 45% acetic acid. A cover glass was placed over the material with 1/8 inch extending beyond the edge of the slide. The slide was heated gently over an alcohol flame and squashed firmly between several layers of paper toweling. The preparation was then dehydrated for at least six hours in tert.-butyl alcohol, diluted to 90% with absolute alcohol.

After dehydration, the cover slip was removed, and the

slide was made permanent by adding a drop of diaphane mounting medium.

Four of the five slides prepared for each sampling time were usually scored by microscopical examination, and the fifth saved as a spare in case of breakage or inferior slide quality.

In order to retrieve meaningful information from observation of these slides, it was first necessary to set up guidelines for types of analyses required, as based on preliminary examination of the material. The type of data obtained depends on the analyses used, which in turn are dependent on the range of observable effects that could possibly occur.

From the preliminary work, it was seen that three types of effect could be distinguished: 1. mitotic inhibition, 2. prophase stalling and 3. c-mitotic action. Analyses were therefore arranged to distinguish between the presence or absence of each of these effects, singly or in combination.

Mitotic inhibition results from any action which prevents the cell from entering mitosis. There are many points (in time) where inhibition may occur, and presumably as many different reaction mechanisms involved, depending on the specific causal factor. In many cases mitotic inhibition is linked with the inhibition of the prophase-to-prometaphase transition, but this is not always so. Presumably this correlation holds only for inhibition at some

specific site or for some specific induced chemical change and not for others. Inhibition of mitosis is recognizeable by the lower proportion or complete lack of dividing figures at some time subsequent to the initiation of treatment. An analysis known as the mitotic index consistently proved to indicate the presence or absence of mitotic inhibition. The mitotic index is defined as the number of dividing cells per one thousand total cells counted. The analysis is performed by the counting of cells in random microscope fields, covering as much of the total slide area as possible, until 1000 cells are counted.

Chemicals which induce prophase stalling have been found always to have an associated effect on mitotic inhibition, although, as noted above, the reverse is not necessarily true. This suggests the probability that only one mode of action exists for inhibition of late prophase, and that this same reaction is one of several means by which cells may be prevented from entering mitosis. Prophase stalling has the result of preventing the normal transformation of a nucleus from the late prophase to the pro-metaphase stage. There is an associated failure of the nuclear boundary to break down and failure of the chromosomes to become oriented toward the center of the cell, as in a normal pro-metaphase. In fact, all subsequent orientational movements (including alignment on the metaphase plate, separation of the chromatids and polar movement and grouping of the separated chromatids) are typically absent, although

the morphological changes of the chromosomes continue at about the same rate as the normal to the interphase condition. Such affected nuclei therefore have the appearance of late prophase configurations with considerable over-contraction of the chromosomes relative to a normal late prophase, and eventually the chromosomes show a telomorphic appearance. A computation of the number of dividing cells in each mitotic stage was found to be useful in determining prophase stalling. A comparison of the ratio of early prophase stages to late prophases (E/L ratio) at a given time showed a relative accumulation of late prophases where significant stalling occurred. Admittedly, this reduction in the ratio was due both to the piling up of cells which were prevented from leaving late prophase and to the lower proportion of cells in early prophase due to the inhibition of mitosis. However, a significant drop in the ratio could be taken as proof of a late prophase block. This analysis was carried out by counting 200 dividing cells, and classifying each according to its mitotic stage. Again, the fields were selected at random over the major area of the slide.

C-mitotic action is defined in terms of the effect of colchicine on cell division. Colchicine is a spindle poison, causing partial or complete disruption of the spindle mechanism, and resulting in the formation of c-mitotic aberrations, namely "scattered" and "clumped" chromosome configurations. Due to the failure of both karyokinesis and

cytokinesis, a certain number of polyploid cells may be found in the second division cycle following the treatment. The c-mitotic effect may be analyzed in several ways. In some cases, a count of the abnormal post-prophase stages was made. This group of abnormal configurations was made up largely of "scatters" and "clumps". Although qualitatively showing the presence of a c-mitotic effect, these data gave no indication of the quantitative efficiency of such an effect relative to colchicine or to the effect of other phenols. For this reason, some scoring was also done by counting the number of "scatters" and "clumps" and comparing these data by means of the colchicine index:

$$\text{Colchicine index} = \frac{\text{no. of "scatters"} + 2(\text{no. of "clumps"})}{\text{total post-prophase cells}}$$

Chromosome damage was assayed primarily by scoring anaphase configurations for chromosome fragmentation and for the formation of chromosome bridges.

B. System for Determining Toxicity

The toxic dose levels of the various chemicals were obtained on Drosophila melanogaster. Chemicals in acetone solution were added to nutrient agar medium, which was poured into disposable plastic petri dishes. Four dishes each containing 25 ml of nutrient were poured for every dose level tested. Thirty flies, Oregon R strain, were placed in each dish, providing four replications of thirty flies each for every dose. After 24 hours at 22.5°C. ±

0.5°C., the dead flies were counted. By plotting the percent kill from several doses on a probability scale versus log dose, the LD_{50} concentrations could be interpolated for each chemical. The various chemicals used in this test system also are listed and the chemical structures are given in Tables 1 through 5.

OBSERVATIONS

A. C-mitotic Activity

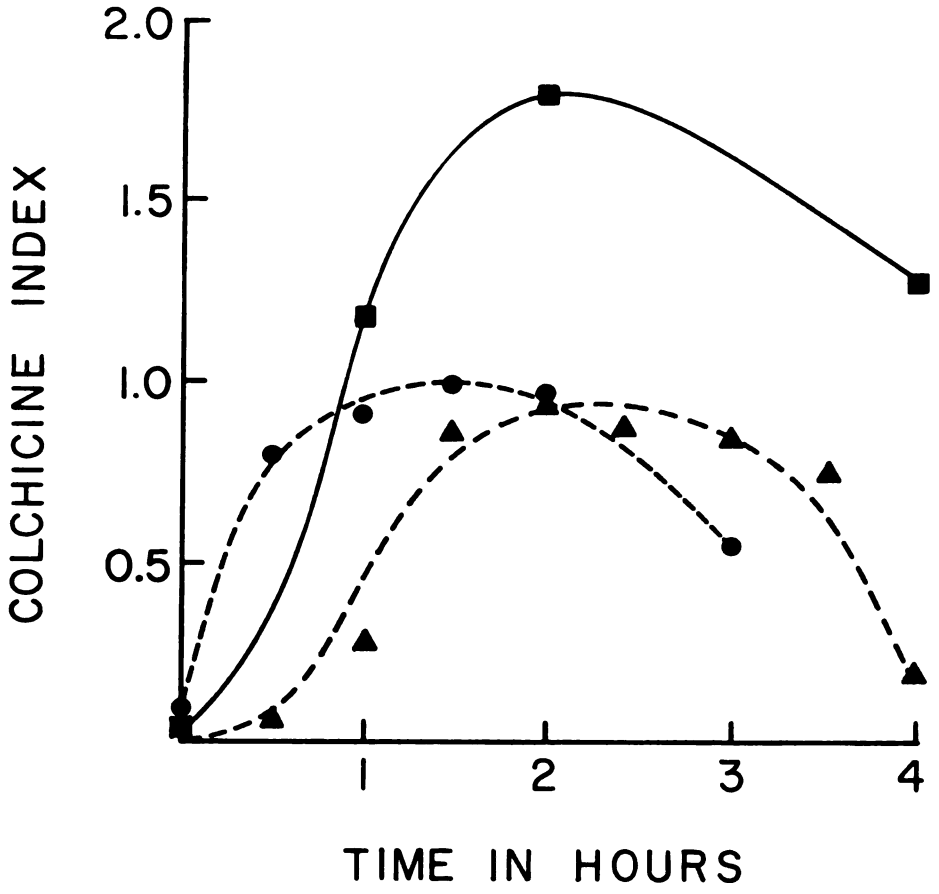
Some c-mitotic activity, as evidenced by the presence of "scatters" and "clumps" was found with virtually all of the simple substituted phenolic compounds tested. The effect is compared by means of the colchicine index. The colchicine indices of all compounds tested during the first four hours of treatment are found in Table 6.

From studies on the effects of colchicine (Hadder and Wilson, 1958), it was found that for maximum effectiveness, the initial peak of c-mitotic activity should occur within two hours after treatment. One of the clearest demonstrations of the c-mitotic activity of a phenol is shown by the effects of 2,4,5-TCP. Used at a dose level of 40 mg/liter of treatment solution, the 2,4,5-TCP-treated cells reached a colchicine index peak of 0.97 at 2 hours, as compared to 1.75, the effect produced by 200 mg/liter of colchicine (Figure 1). It was noted that dose levels of 2,4,5-TCP up to 80 mg/liter resulted in no further increase in c-mitotic effect, as measured by the colchicine index (Table 6). Doses greater than 80 mg/liter proved toxic.

2,4,6-TBP at 200 mg/liter also reached a peak slightly greater than 1.00 at 2 hours; 2,3,6-TCP at 100 mg/liter peaked at 0.87 at 1½ hours; 2,4,6-TCP at 40 mg/liter reached 0.50 at 1½ hours; and 200 mg/liter of 2,3,5-TMP at 1 hour reached a colchicine index of 0.69.

The di-substituted phenols had a somewhat less well-

Figure 1. Colchicine indices of colchicine, Ruelene and 2,4,5-TCP plotted versus time.


2.0
1.5
1.0
0.5
COLCHICINE INDEX
1
2
3
4
TIME IN HOURS
Colchicine
Ruelene
2,4,5-TCP

defined effect on the spindle. One of the most effective was 3,4-DCP. 60 mg/liter caused a peak of 0.46 at 1 hour; 90 mg/liter resulted in an index of 0.61 at 1 hour. 2,3-DCP at 100 mg/liter produced an index of 0.97 at 1 hour; 50 mg/liter of 2,6-DCP reached 0.43 at 2 hours; 3,5-DCP at 50 mg/liter peaked at 0.81 in 1 hour; 2,4-DBP caused an index of 0.41 at 2 hours with a dose of 50 mg/liter; and 2,4-DMP at 200 mg/liter resulted in a peak of 0.47 at 2 hours.

The highest sublethal dose (200 mg/liter) of the organic phosphate, Ruelene (II), is compared to the effect of 200 mg/liter of colchicine in Figure 1. Again, the maximum effect reached is considerably below that induced by colchicine, since the bulk of the deviant figures may be classified as "scatters" (partial effect) rather than "clumps" (complete effect). The maximum index obtainable with Ruelene is about 1.00, as compared to a theoretical maximum of 2.00, if all deviant figures were "clumps". The other actively antimitotic organic phosphate compounds (IV and VI) did not show a particularly striking tendency toward spindle disruption as measured by the colchicine index, but did produce great enough numbers of "scattered" and "clumped" configurations to indicate that at least some such action does occur (Table 6).

One of the diagnostic features of c-mitosis is the appearance of polyploid cells in several division cycles subsequent to treatment. Slides were scored at these times for polyploid cells, but only in a few cases were any sig-

nificant amounts found. Figure 2 shows the percent polyploidy recovered after treatment with 155 mg/liter of Ruelene (II). Low levels of polyploidy were also found with 2,4-DNP, 2,4,6-TBP and 2,4,5-TCP.

B. Prophase Stalling

The extent of prophase stalling was gauged primarily by the drop in the E/L ratio. This change was noted in almost every treatment to a greater or lesser extent. As can be seen from Table 7, this drop may be recognized within the first hour of treatment, and usually reaches its lowest point at from one to two hours after the initiation of the treatment.

Figure 3 shows the change with time in the E/L ratio obtained by treatment with 60 mg/liter of 2,4,5-TCP as compared with the control. All of the substituted phenols showed a very definite drop in the ratio, all falling below 1.00 at some time within the one to two hour period, and at some effective concentration. It was also noted (Table 7) that increasing the dose level did not materially increase the prophase inhibition. Probably the lack of a direct proportional relationship of the effect to dose is due in part to the fact that only a fairly complete inhibition is recognizeable, because of the tremendous variability of E/L ratios in untreated material.

The di-substituted phenols appeared to be at least as effective as the tri-substituted phenols in producing a drop in the E/L ratio; all chemicals of this type tested

Figure 2. The percent of polyploid cells recovered after treatment with 155 mg/liter of Ruelene (II) versus time.

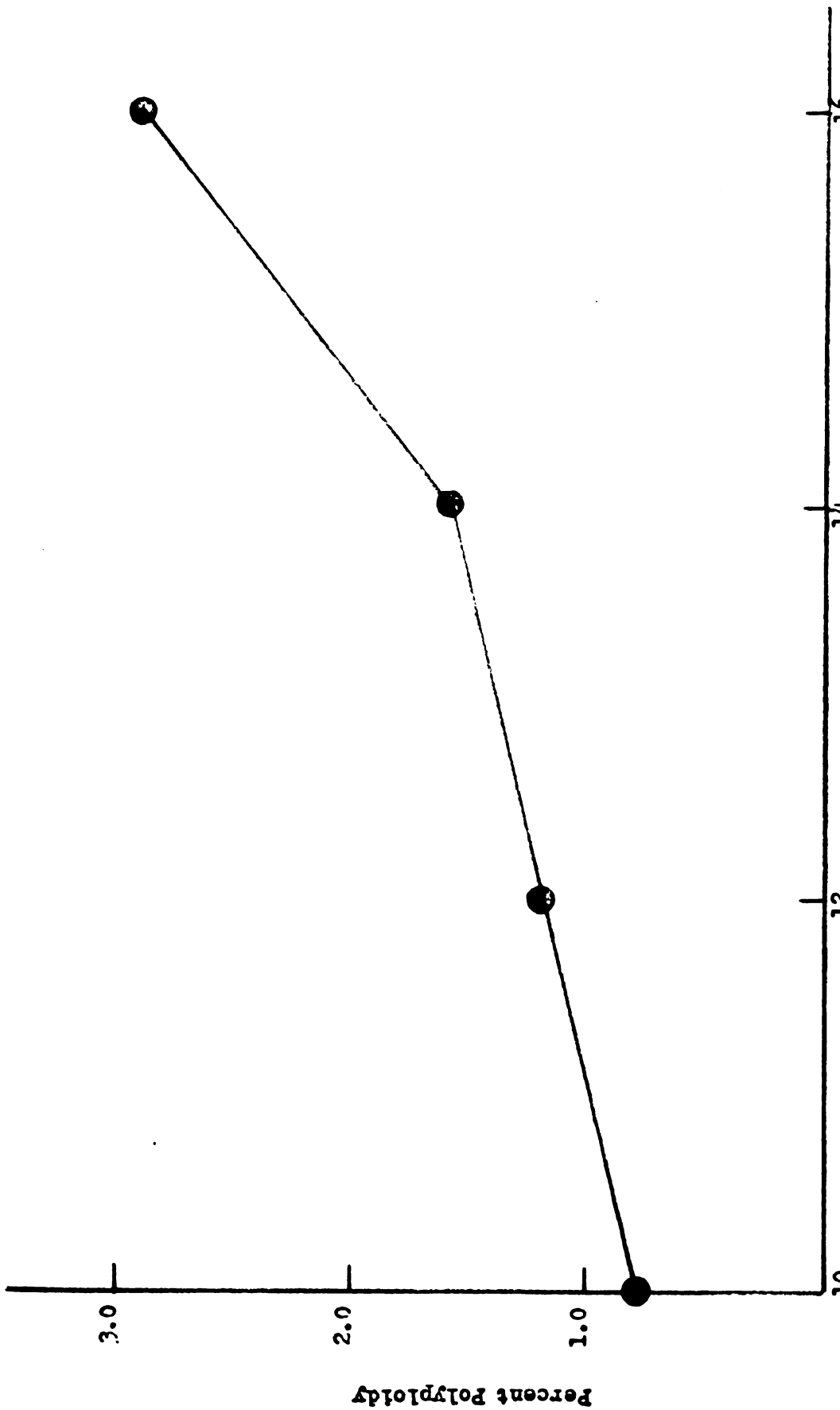


Figure 2.

Figure 3. Ratio of early to late prophases resulting from treatment with 60 mg/liter of 2,4,5-TCP, compared with the control.

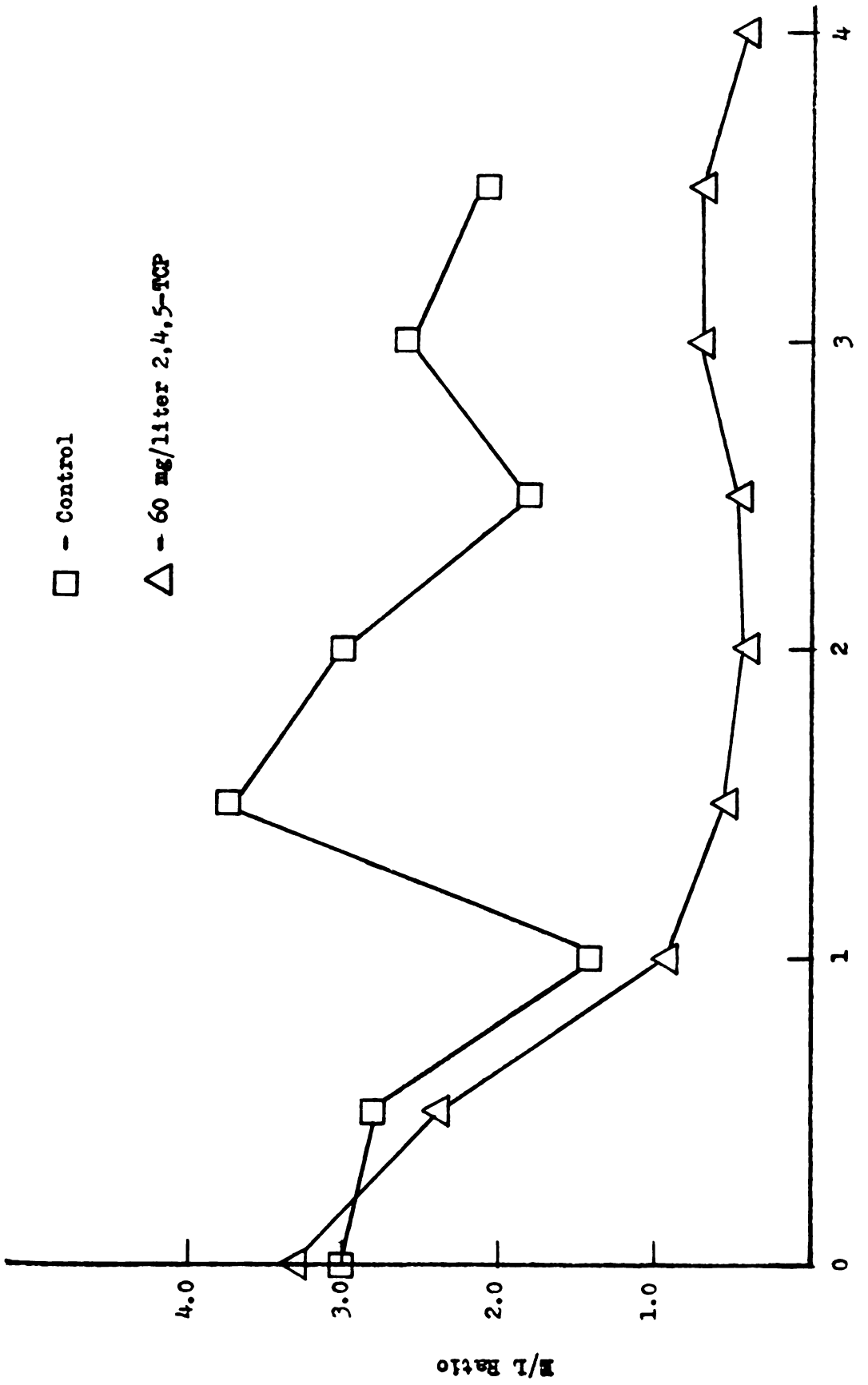


Figure 3.

showed a very definite effect of this nature.

Of the antimitotically effective organic phosphorus compounds, **para-oxon**(VI) showed the best evidence of prophase stalling, reaching a low value of 0.53 two hours after treatment with a dose of approximately 100 mg/liter. The other chemicals of this type tested showed a drop, but the values did not fall significantly below 1.00, and are therefore only indicative of the presence of such an effect.

As a result of the release of large segments of root meristem cells from the depression of the transition rate from late prophase to pro-metaphase, the subsequent recovery from the treatment of these segments may lead to an extreme reversal in the ratio, with peak figures of 4.00 or 5.00 in some cases (Table 7).

C. Mitotic Inhibition

In order to compare the changes in the mitotic cycle arising from inhibition at various points of the cycle, which resulted in longer average cycle times, a control mitotic index was scored for each chemical tested. The control mitotic indices averaged over the duration of the sampling period are tabulated in Table 8. The overall mean mitotic index was 60, with the individual averages ranging from 45 to 75, a deviation of ± 15 index points, or $\pm 25\%$. The mitotic indices from each of the treatments have been computed as the percent of the control mitotic index for that experiment, and are found in Table 9. Any deviation from the control value of greater magnitude than $\pm 25\%$ will

be considered significant.

All treatments which showed any cytologically apparent effect inhibited the onset of mitosis to some extent. The drop in mitotic index did not occur immediately; in some cases, the higher concentrations particularly seemed to produce even a slight stimulation at one-half to one hour after treatment. The 2,4,5-TCP provides a good example of stimulation at higher dose levels. From Table 9, it may be seen that, at one-half hour following treatment, levels of 114% and 120% of the control value were caused by dose levels of 70 mg/liter and 80 mg/liter, respectively. With 2,4,6-TCP, indices of 118% and 132% of the control level occurred at one-half and one hour subsequent to treatment with 25 mg/liter, and 128% of the control at one-half and one hour after treatment with 40 mg/liter. This apparent stimulatory effect was also noted with the di-substituted phenols (125% at one-half hour with 50 mg/liter 2,4-DBP) and with the organic phosphorus compounds (118% at one-half hour with 100 mg/liter of para-oxon (VI)).

After the initial stimulation or maintenance at control level, the drop became apparent at from one and one-half to three hours, depending at least in part on the dose level. The lowest point was reached at three and one-half to four hours. An eventual rise, usually quite steep, to supra-control levels was found in most cases during the recovery period.

It may be seen from Table 9 that all of the simple

substituted phenols at four hours following treatment had a mitotic index below 75% of the control value at almost all detectably effective dose levels. The higher dose levels of the organic phosphates also resulted in indices below 75% of the control at four hours, except for para-oxon (VI) which reached a low of 80%. The amount of the drop was in most cases not very dependent on the dose level, possibly since the drop must be of fair magnitude to be recognizeable. However, the rate of recovery of the tissue may be in part dependent on the dose. Figures 4, 5 and 6 show respectively the recovery legs of the effect curves of 2,4-DNP, 3,4-DCP and 2,4,5-TCP. Once recovery began, the build-up of dividing cells appeared to ensue rapidly. Therefore, a straight line between the last sub-control point and the first supra-control point should be a good approximation of the true shape of the curve. Although it may be seen that, in general, higher dose levels tended to retard recovery, and that a relationship approaching linearity was found with an arithmetic dose progression, no rigorous proof of linearity can be offered, due to the approximated shape of the recovery curves.

The mitotic index also serves as a means of comparing the minimal effective molar dose levels of different compounds in order to classify them according to their relative effectiveness. The minimal effective and maximal subtoxic dose levels for all compounds tested which were found to be antimitotically effective are listed in Table 10.

Figure 4. Mitotic indices as percent of control of the recovery legs of the effect curves from treatment with 5 mg/liter and 7 mg/liter of 2,4-DNP.

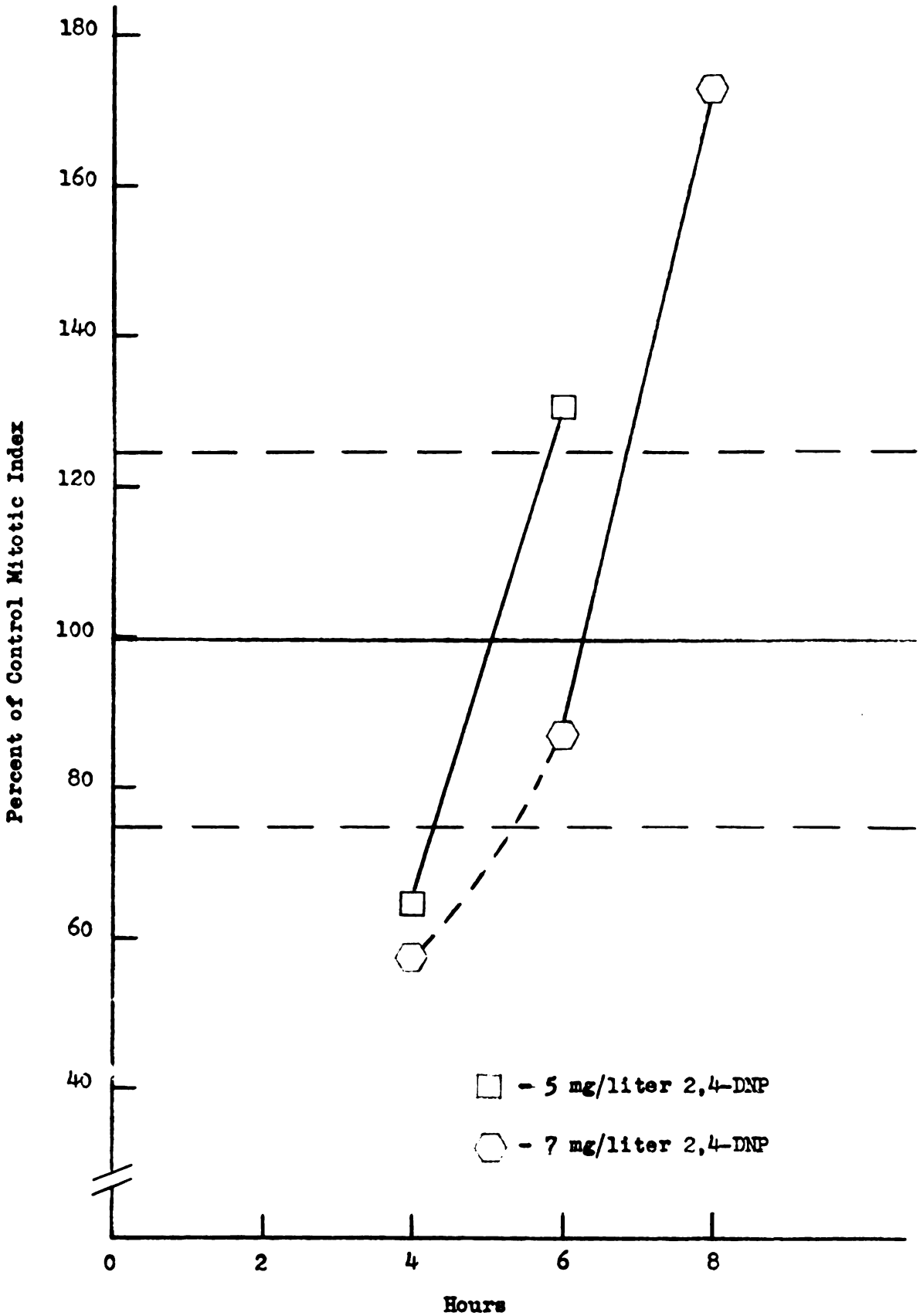

180
160
140
120
100
80
60
40
Percent of Control Mitotic Index
0
2
4
6
8
Hours
□ - 5 mg/liter 2,4-DNP
○ - 7 mg/liter 2,4-DNP

Figure 4.

Figure 5. Mitotic indices as percent of control of the recovery legs of the effect curves from treatment with 50 mg/liter, 60 mg/liter, 70 mg/liter, 80 mg/liter, 90 mg/liter and 100 mg/liter of 3,4-DCP.

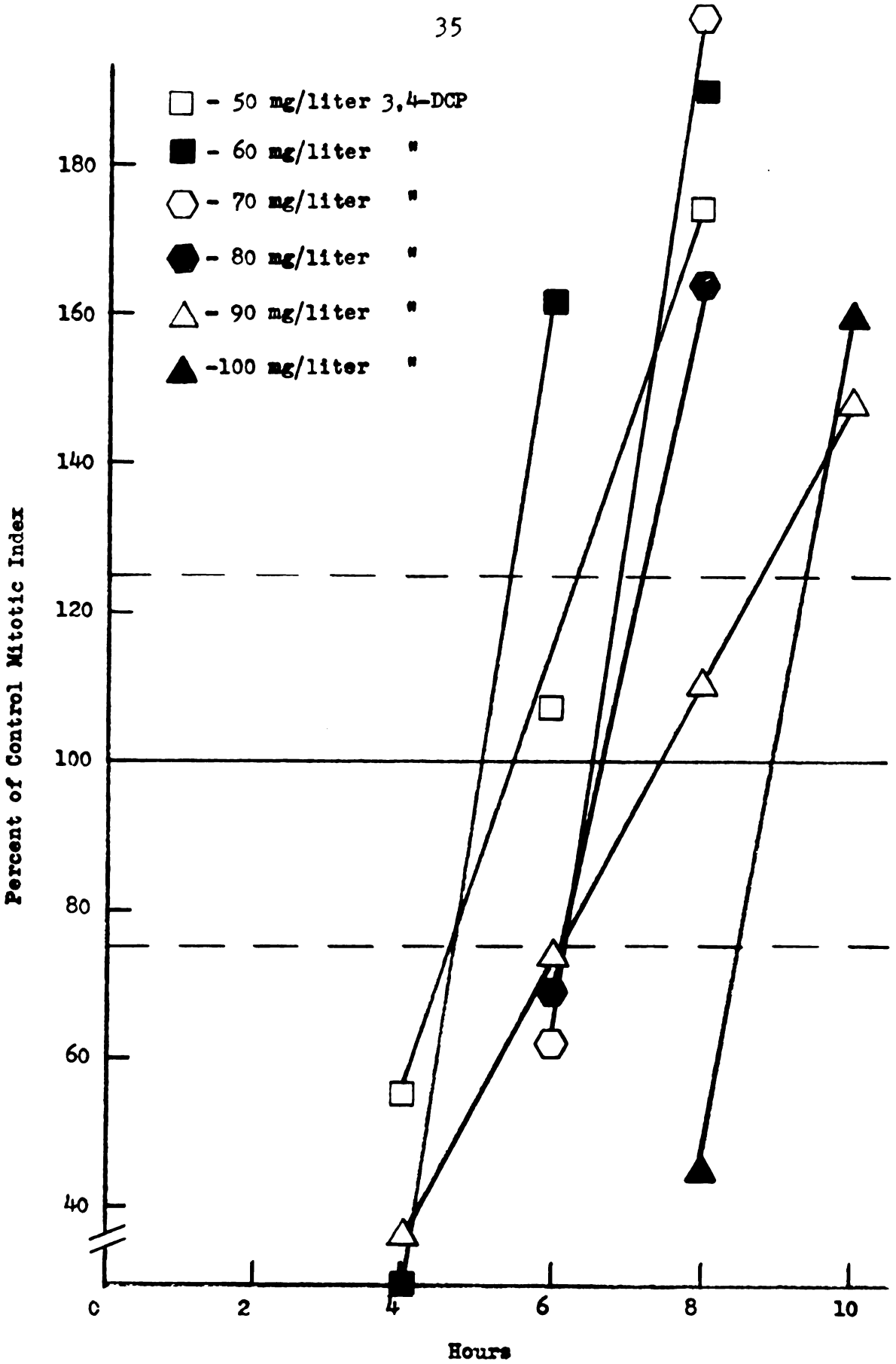

□ - 50 mg/liter 3,4-DCP
■ - 60 mg/liter "
⬡ - 70 mg/liter "
⬢ - 80 mg/liter "
△ - 90 mg/liter "
▲ -100 mg/liter "
Percent of Control Mitotic Index
180
160
140
120
100
80
60
40
0
2
4
6
8
10
Hours

Figure 5.

Figure 6. Mitotic indices as percent of control of the recovery legs of the effect curves from treatment with 40 mg/liter, 50 mg/liter, 60 mg/liter, 70 mg/liter and 80 mg/liter of 2,4,5-TCP.

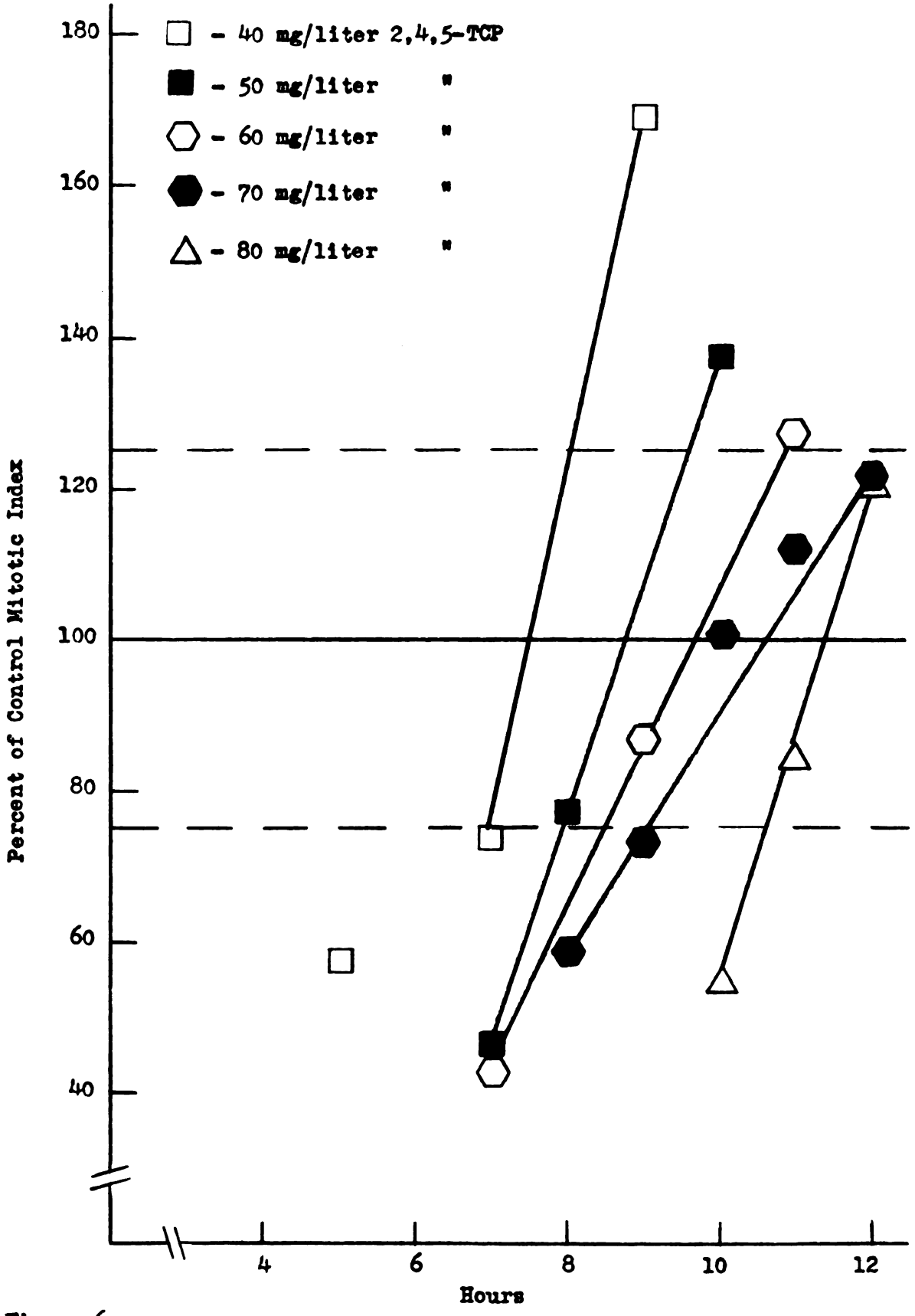

□ - 40 mg/liter 2,4,5-TCP
■ - 50 mg/liter "
⬡ - 60 mg/liter "
⬢ - 70 mg/liter "
△ - 80 mg/liter "
180
160
140
120
100
80
60
40
Percent of Control Mitotic Index
4
6
8
10
12
Hours

Figure 6.

Concentrations are given both in mg/liter and in molarity. The various compounds may be classified in several ways in order to compare their relative effectiveness. First, they have been listed in order of effectiveness, from the most effective to the least, by placing them in order from the lowest minimal effective dose to the highest. The relative toxic values may also be compared by listing the compounds according to their maximal sub-toxic dose levels. Both of these orders of effectiveness have been recorded in Table 11.

It can be seen readily that the nitro substituents are the most influential in causing the phenols to disturb the mitotic cycle, and that the methyl substituents are the least effective. Both the bromo and chloro groups fall within these two extremes, and are more difficult to separate as to order of effectiveness. Several interpretations of these data will be offered later in order to distinguish between the biological activity of these two chemical groupings.

D. Toxicity Data

The comparative LD_{50} values in Table 12 were derived by plotting the actual experimental doses used on a log scale versus the probit percent kill of Drosophila melanogaster. An intercept was dropped from the 50% line to determine the LD_{50} value. A characteristic plot is shown in Figure 7, from which the LD_{50} value of 30 mg/liter was determined for 2,4-DMP.

Figure 7. Effective doses of 2,4-DMP plotted as probit percent kill of Drosophila melanogaster versus log dose.

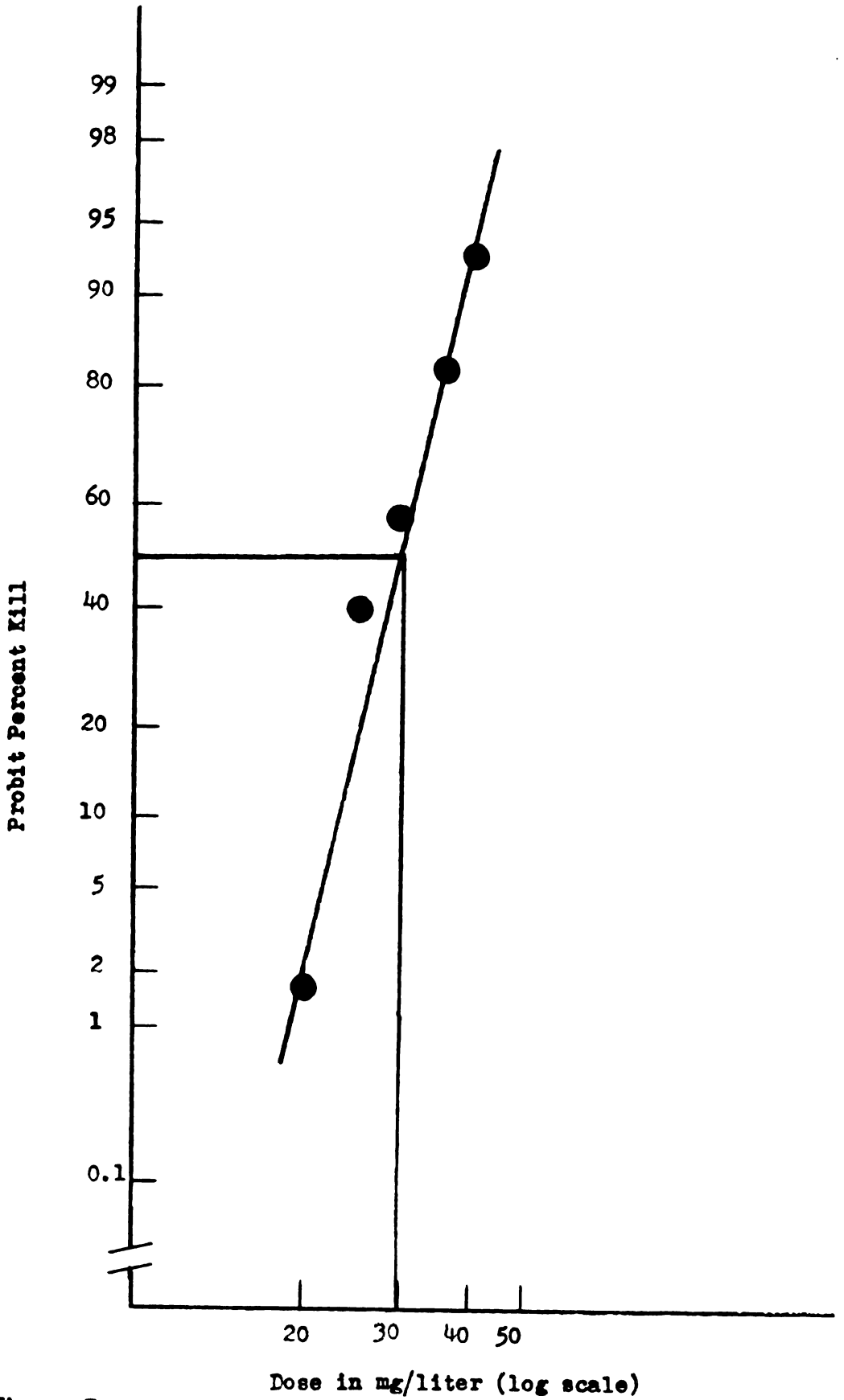

99
98
95
90
80
60
40
20
10
5
2
1
0.1
Probit Percent Kill
20
30
40
50
Dose in mg/liter (log scale)

Figure 7.

In general, the same order of effectiveness was found for the compounds in regard to toxicity to Drosophila melanogaster as with the pea system. The 2,4-DNP was by far the most toxic, resulting in 50% mortality with a concentration of only 2.0 mg/liter or 1.1×10^{-5}M. As was the case with mitotic inhibition of the pea root meristem cells, the methylphenols required the highest concentrations to produce 50% mortality. The bromo- and chlorophenols had effects ranging between the two extremes. Generally, the tri-substituted phenols were more toxic than the di-substituted ones, the only exception being 2,4,6-TCP, which was less toxic than 2,4-DCP, 2,3-DCP, 3,4-DCP and 3,5-DCP, but more toxic than 2,6-DCP. In regard to the minimal effective dose and the maximal sub-toxic dose in the pea system, 2,4,6-TCP was both more effective in producing mitotic inhibition and toxicity than any of the di-substituted phenols.

DISCUSSION

Although the three antimitotic effects just described appear to be distinctly separable, at least in many cases, they may occur also in combination. This, of course, does not necessarily preclude the possibility of a distinct mode of action being required to initiate each effect. Therefore, it should be pointed out that no contradiction exists when mitotic inhibition occurs simultaneously with a c-mitotic reaction. According to our definition, a true c-mitotic reaction does not cause inhibition of mitosis, and indeed this does not occur with colchicine treatment. Alternatively, the mitotic inhibition found with treatment by many phenols may be interpreted as a distinct effect acting simultaneously with the c-mitotic reaction. In fact, the inhibition may or may not be observable, depending on the chemical used and the dose level.

We have shown that the general effect of substituted phenols as a group on mitosis is three-fold. Firstly, an inhibition of mitosis occurs, secondly the transition of late prophase to pro-metaphase is inhibited and thirdly the spindle mechanism is disrupted. Each of these should be considered a separate reaction or the recognizeable effect of a separate reaction for several reasons. First, these mechanisms may not all be associated with the use of certain chemicals. For instance, colchicine is probably the most effective chemical for producing spindle damage below toxic levels, and for allowing the recovery of poly-

ploid cells during subsequent division cycles. However, no recognizeable mitotic inhibition or prophase stalling occur. A second reason for considering the effects as separate is the specific point during the mitotic cycle at which each effect must be produced. That is to say that cells in the presence of the chemical will continue their normal mitotic changes up to the specific susceptible point.

Figure 8 shows diagrammatically the susceptible points of the mitotic cycle in the pea root meristem. Van't Hof et al. (1960) reported an average mitotic cycle duration of twelve hours for the pea root meristem by colchicine tagging. This figure has since been confirmed in the same laboratory by a somewhat different method by Bekken (1965). More recently, Van't Hof (1965) reported a fourteen hour cycle with colchicine tagging, and, from tritiated thymidine treatments has calculated mitosis and "G_2" to be 3.5 hours, the "S" (DNA synthetic) period to be 5.8 hours and "G_1" to be 4.7 hours, by difference. However, he calculated cycle time from the beginning of colchicine treatment, rather than from the colchicine index peak which usually occurs about two hours later. The polyploid cells which one sees during the second mitotic period correspond to those which are affected by the colchicine and are apparent two hours after treatment. This timing, then, should correlate with the other reports of a twelve hour cycle, and the "G_1" period, which is computed by difference, would then be about 2.7

Figure 8. Diagrammatic interpretation of the points of mitotic disruption, compared to the Pisum mitotic cycle.

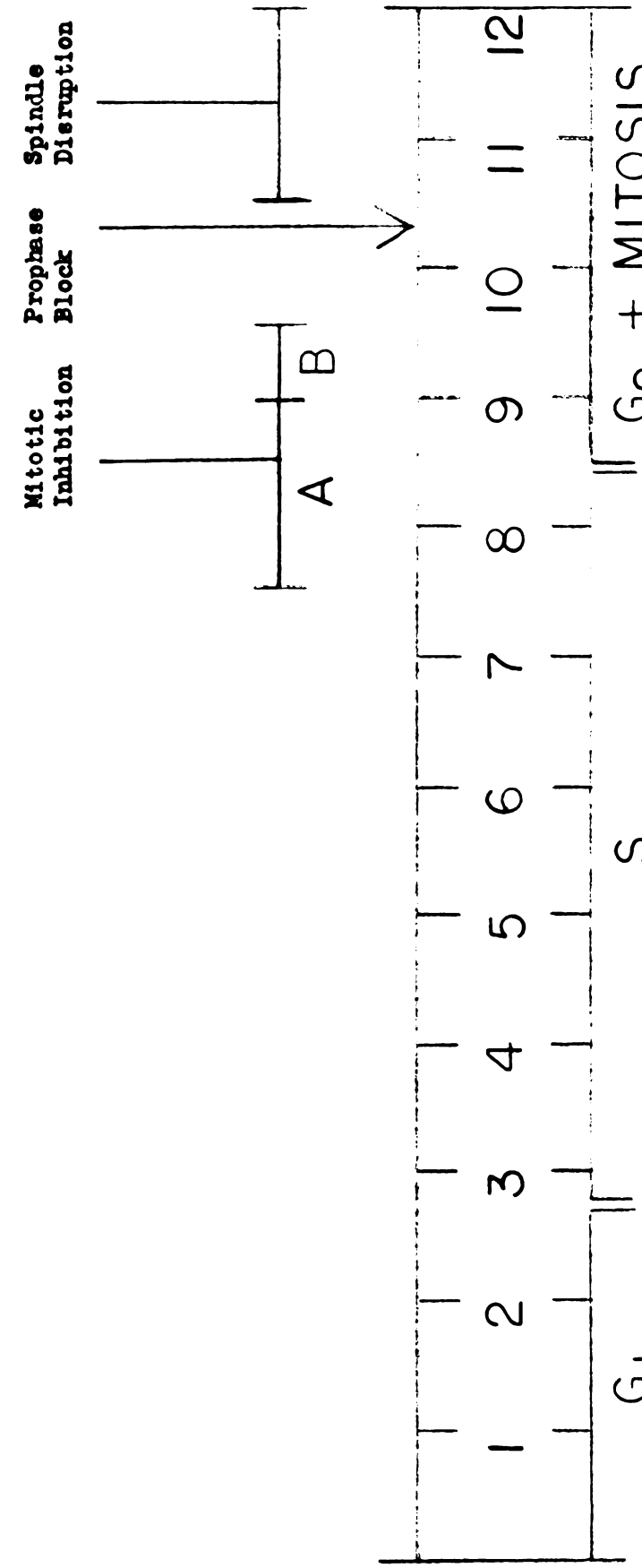

Mitotic Inhibition
Prophase Block
Spindle Disruption
A
B
1
2
3
4
5
6
7
8
9
10
11
12
G1
S
G2 + MITOSIS

Figure 8.

hours in length.

In order to correlate the chemical effects with various portions of the mitotic cycle, mitosis will be considered first. Van't Hof (1965) allotted 3.5 hours to "G_2" and mitosis, 1.8 of this to mitosis itself. However, mitosis may require as long as 2.0 to 2.5 hours. The length of mitosis, as measured by microscopical examination of the nuclear configurations, may vary considerably, depending on the investigator's subjective judgement as to the beginning of early prophase stages and the end of the telophase stage. Since the post-prophase stages occupy less than half of the total mitotic time, spindle disruption occurs within the last hour or slightly earlier. That c-mitosis is induced during the actual presence of the spindle is shown by the almost immediate production of c-mitotic configurations by chemicals with very rapid ingress and egress, such as the carbamates (Wilson, 1965). Prophase stalling occurs just slightly prior to this point, since the accumulation of late prophases can be seen within one-half hour subsequent to treatment. The point of mitotic inhibition is somewhat more difficult to localize, and it probably occurs over an extended portion of the cycle. Since no immediate response is noted, there is certainly no inhibition of cells very near the onset of mitosis. For this reason, a period known as "B" has been indicated, which probably corresponds to some segment of the "G_2" period. Once into the "B" period, cells are not prevented

from continuing mitosis, unless they encounter a later blockage point. However, cells in the "A" period are inhibited. Since no reduction in the number of dividing cells is seen until after two hours after treatment, the center of period "A" must occur at least two hours prior to the center of the mitotic period. Therefore, the "A" period has been extended back into the "S" period. Although the inhibition may be due in part to reactions occurring in "G_2", the susceptibility probably also is present in the late "S" phase.

Each of these three types of activity is manifested in a definite pattern over a period of time. Figure 9 shows the mitotic index pattern produced by treatment with 40 mg/liter of 2,4,5-TCP. The curves of the other phenols closely follow this type of pattern. As noted previously, certain chemical treatments seem to produce a slight stimulation during the first one or two hours after treatment. The low point is usually near four hours, as in this case. Although the depression is seen to be from two to eight hours in duration, depending on the chemical and dose used, the inhibition is not necessarily still in force at the time at which the depression is recognized by the lack of dividing cells. In a moving cyclic system such as the mitotic cycle, an inhibition which occurs several hours prior to the presence of recognizeable configurations is not noticed until several hours after it occurs. Likewise, the release from inhibition is not seen until several hours later.

Figure 9. Mitotic index in percent of control from treatment with 40 mg/liter of 2,4,5-TCP versus time.

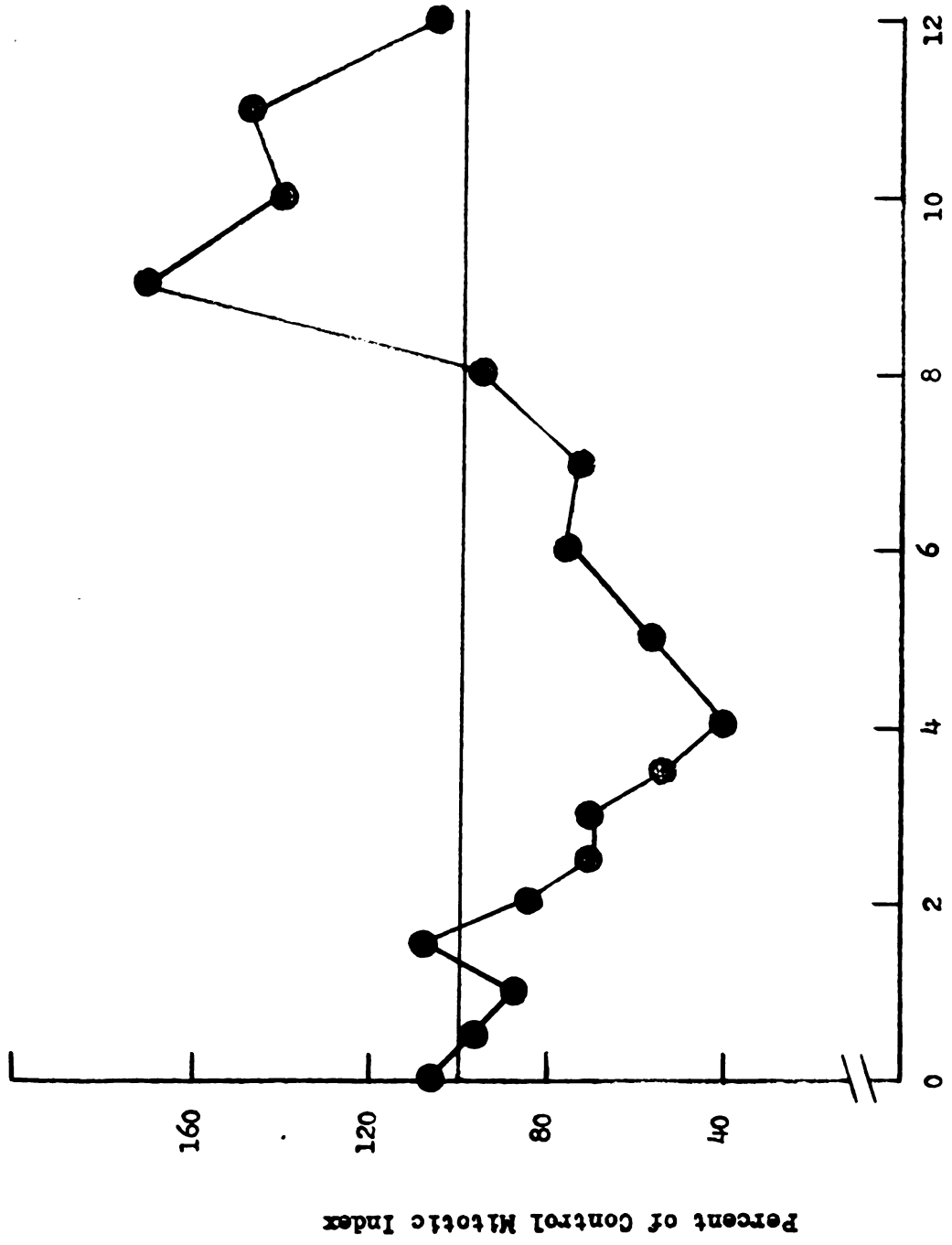

Percent of Control Mitotic Index
160
120
80
40
0
2
4
6
8
10
12
Hours

Figure 9.

The recovery portion of the curve reaches a point considerably above the control level. The time required for the index to cross the 100% line on the recovery leg is dependent on the dose level. This may be due to the need of a "threshold" concentration in order for the reaction to ensue.

The rates of movement of the phenols into and out of the cell are not known, but it is evident from the immediacy of some of the antimitotic effects that their entry is fairly rapid. This is not surprising, since all of these compounds are very lipid-soluble, and should certainly pass through the cell membranes with ease. However, the exit of the chemicals at the end of treatment (and the consequent reduction in their concentration at the active site) does not occur with as much ease. The phenols have good binding qualities which permit inter-molecular hydrogen-bonding and hydrogen-bonding between the phenols and other sites of appropriate attraction. Since most of the phenols also dissociate with fair ease at the proper pH values, ionic bonds also could occur. Lipophilic attractions may also serve to maintain high levels of these compounds in the tissues even after removal from the treatment solution. Thus, the cell may require a longer period of time to reduce the higher concentrations of phenols to levels below the effective "threshold" level.

The plot of the E/L ratio versus time resulting from treatment with a 60 mg/liter dose of 2,4,5-TCP in Figure 3

is typical of the effects of prophase stalling induced by the phenols. A very immediate drop is noted, indicating an immediate blockage of the transformation of late prophase nuclei to the pro-metaphase stage. This results in an increase in late prophases as compared to early prophases. By the time of the lowest point, usually 2-2½ hours after treatment, the mitotic inhibition may also begin to affect the ratio, in that most early prophases have moved on to late prophase, and no more cells are permitted to enter early prophase. However, the ratio begins to increase from this point, indicating that cells are being allowed to move on to later stages from late prophase. Since the mitotic inhibition is still in effect, very few cells are permitted to enter the prophase range, but of these few cells, the ratio of early to late prophases returns to more normal levels, usually by four hours. Although supra-normal levels may occur with recovery, the great variation in normal control root meristems (from about 1.50 to almost 3.50) makes it difficult to distinguish a significant increase.

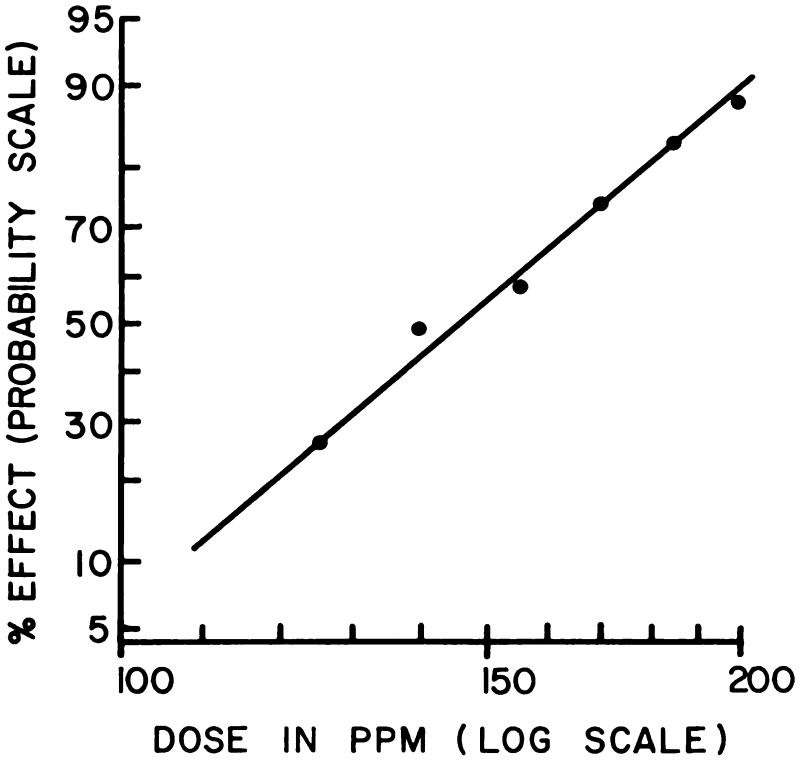
The pattern of c-mitotic action of the phenols, where it occurs at fairly high levels, is similar to the specific curves shown in Figure 1. Again, the rising portion of the curve is much more rapid than the drop-off, which tends to form a shoulder. The colchicine index may be used as a guide for the prediction of the amount of polyploid cells in subsequent division cycles. Since the maximum indices obtainable with the phenols does not exceed 1.00, one would

expect very few polyploid cells to be recovered. This was found to be true, and only small, but significant, numbers of polyploid cells were found with several treatments. The c-mitotic effect was used to determine the dose-dependence of this reaction as induced by the organic phosphorus compound, Ruelene (II). The effect was scored as the percent of abnormal post-prophase cells, consisting entirely of "scattered" and "clumped" configurations, without weighting the "clumps" as two and the "scatters" as one, as is done with the colchicine index. The curves were plotted for six doses of Ruelene (II) ranging arithmetically from 125 mg/liter to 200 mg/liter. The area under the curve of each of these concentrations was calculated in arbitrary units for the period of maximum effect, 1/2 to 2-1/2 hours after treatment. These arbitrary units were computed as the percent of total area possible between these limits and then plotted as the percent effect on a probability scale versus the log dose (Figure 10). A straight line was obtained, confirming the sigmoid character of the dose-effect relationship.

The fact that mitotic inhibition is fairly sensitive to dose makes possible the comparison of the relative biological activity of the various phenols, from which certain general principles regarding the nature and position of the substituent groups may be drawn.

It should be pointed out that inhibition of mitosis and toxicity may be the end results of two very different

Figure 10. Probit percent effect plotted versus log dose of Ruelene treatments of 125, 140, 155, 170, 185 and 200 mg/liter.


95
90
70
50
30
10
5
% EFFECT (PROBABILITY SCALE)
100
150
200
DOSE IN PPM (LOG SCALE)

reactions or mechanisms, so that no correlation need necessarily exist between the minimal effective doses and the maximal sub-toxic doses. Rather, the order of the compounds classified by the toxic levels should differ from the anti-mitotically effective order depending on the breadth of the dose range of each compound below the dose necessary to produce the toxic reaction.

Many factors are undoubtedly involved in the biological activity and effective dose levels of compounds at the cellular level. Any one compound may undergo various separate reactions within a cell, and may be involved in several metabolic pathways. The simultaneous action of a compound on numerous enzyme systems is a common phenomenon. Effects may even be caused without chemical reaction, by the alteration of the physical environment at enzymatic or structural sites. From the diversity of structure in the many compounds found to exhibit the c-mitotic reaction and other mitotic aberrations, there arises the possibility that at least part of the biological reactivity of these molecules may be due to physical characteristics such as energy potentials, particularly regarding electronegativity, rather than, or, in addition to, generalized biochemical reactions. Factors influencing the biological effects of compounds such as the phenols include such considerations as the rate of entry and exit of the chemical, which are dependent on such physical properties as the lipid solubility of the compound, its charge, and on its propensity to hydrogen-

bond or otherwise form attachments of less than covalent bond strength; the specificity of the chemical for the active site; and the ability of the compound to concentrate at the site of action.

Different physical or chemical factors may be influential for each reaction which a compound undergoes. Conversely, the same factors may influence the action of one compound differently from that of another. Thus, it is not surprising that different relative activities are found for the same compounds when the criteria of biological activity are mitotic inhibition, pea root toxicity or toxicity to Drosophila melanogaster, although a certain general order is common to them all.

Comparisons have been made on the assumption that an equilibrium exists between the lipid and water phases, and that the chemical potentials of the compounds are the same in each phase, so that the chemical potentials may be obtained from measurements in the circumambient phase. It should be cautioned that, although any measure of comparative toxicity must involve comparison between the amounts required of different chemicals to produce the same effect, the measured toxic concentration of a given compound is not necessarily an indication of the concentration at the site of action.

Without consideration of the position or number of substituent groups, it can be stated that the nitro group is the most effective in inducing mitotic inhibition, and

that the methyl group is the least effective. The bromo and chloro groups are midway between these activities, and are rather close in comparable effectiveness. However, 2,4-DBP is both more effective antimitotically and more toxic (in both test systems) than are any of the dichlorophenols. For this reason, it is likely that the bromo substitution results in more biological activity than the chloro substitution. The failure of 2,4,6-TBP to be more active than the trichlorophenols (although close to them in value) may be due in part to stereochemical interference at the active sites due to the larger size of the bromine atom (van der Waals radius of 1.95 Å) as compared to the chlorine atom (van der Waals radius of 1.80 Å). In 2,4,6-TBP, two bromine atoms flank the hydroxyl group of the phenol.

In general, also, the tri-substituted phenols are more active than the di-substituted ones. This might be expected if one considered the substituents to influence the reaction of the compound as a whole; thus, if one group created a given amount of activity, two groups could produce a greater activity, and so forth. There is the exception of 2,4,6-TBP being less effective than 2,4-DBP in the pea test system. Again, this may be accounted for by steric hindrance.

The position of the substituent groups is undoubtedly of some importance also. From the order of mitotic inhibition, it is seen that amongst the chlorinated phenols,

the four most effective ones are all substituted in both ortho (2 and 6) positions. The next lower one in activity, 2,4,5-TCP, has only one ortho position filled, but would be expected to manifest considerable activity because of the triple substitution. 2,4-DCP is the next lower chlorophenol and has one ortho position substituted. The 3,5-DCP and 3,4-DCP are indistinguishable from 2,4-DCP in activity, although they lack the ortho substitution entirely. In the same manner, 2,4,6-TMP with two filled ortho positions inhibits mitosis at considerably lower doses than 2,3,5-TMP, which has only one ortho substituent. It would appear also that the combination of only one ortho group plus possible steric hindrance renders the 2,3,5-TMP less active than 2,4-DMP, which in turn is less active than 2,4,6-TMP.

2,4-DNP is well known as an uncoupling agent. 2,4-DCP also has some uncoupling capacity, but considerably less than that of 2,4-DNP. The order of magnitude, and the reports of uncoupling by 2,4-DBP and several mono-substituted phenols have led to the possibility that the antimitotic activity of phenols is related to their uncoupling capacities. However, there is no proof that this is the primary action which results in the cytologically apparent disruptions.

The question of how the various substituent groups mediate their effects on the activity of the molecule is a problem falling into the realm of physical chemistry. Several possibilities of their physical effect on the mol-

ecule, however, stand out because of the good correlation with the biological data.

The more electron-withdrawing substituents are found to result in higher activities, when incorporated into the phenolic compound. Fieser and Fieser (1957) list as electron-attracting NO_2, Cl and Br, with dipole moments of 3.97, 1.56 and 1.53 respectively. CH_3 is classified as electron-releasing, with a dipole moment of 0.41. The electron withdrawing substituents tend to deactivate the ring to electrophilic attack, while the electron-releasing substituents, by displacing electrons into the ring and increasing electron density at the available carbon centers, activate them for electrophilic attack. Thus, several factors may be involved. If we consider the theory of Blackman et al. (1955b) of negatively bonded active cellular sites, it can be seen that the rings deactivated by electron-attracting substituents to attack by positive ions are still free to bind to negative sites. Further, the phenols activated by electron-releasing substituents to electrophilic attack may become much more susceptible to hydrolysis and metabolic degradation or detoxification.

The capacity of the phenols to accept or give up electrons could also be involved in a possible mechanism of interference with hydrogen bonding (for instance, in the tertiary structure of proteins) essential for the normal transition of a nucleus from one stage to another.

The theoretical consideration of the problems involved

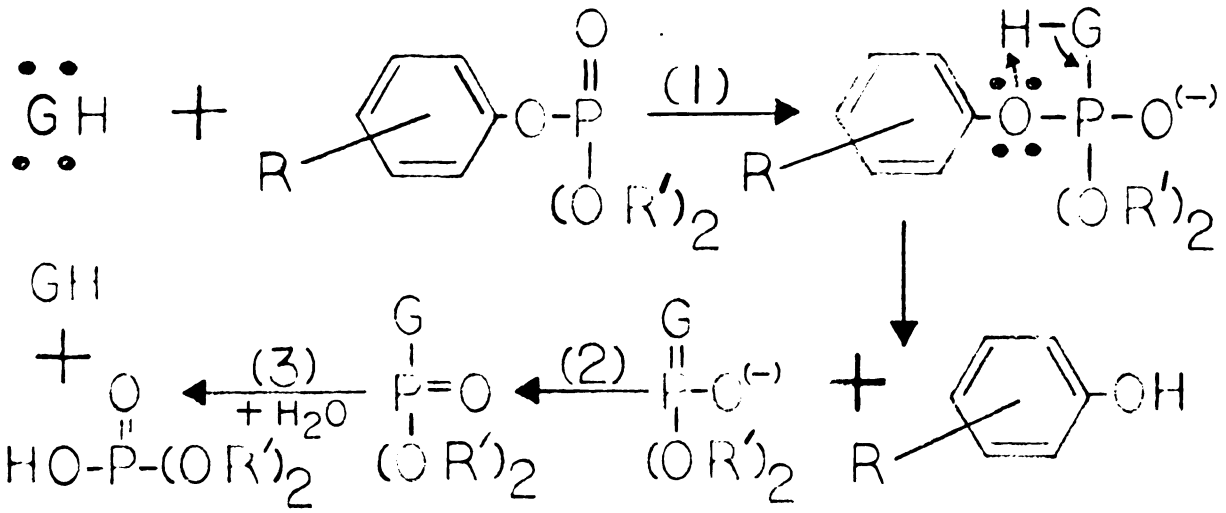
in comparing the relative activities of a series of compounds such as the phenols are complicated further by the practical aspects of providing mechanical contact of the test chemicals with the experimental materials. For the most part, these chemicals are soluble in water up to the concentrations used in these experiments, and present no serious hindrance to uptake. However, 2,4,5-TCP, 2,4,6-TCP and the organic phosphates IV and VI, were not soluble in aqueous solutions at the concentrations desired for testing. These chemicals were suspended in the treatment solutions by means of the surface-active agent Tween-80, which was found to have no cytologically detectable affect when used alone. It is not known what interactions occurred between the chemicals and the surfactant, nor how strong were the forces of attraction (and thus the ease of dis-association), nor the effect of this complex on the uptake by and localization within the cell. However, when used in conjunction with other chemicals, which also could be dissolved in water alone, the results could not be distinguished.

No attempt was made to correlate the relative effectiveness of the organic phosphorus compounds, because of the influence not only of the nature, number and position of substituents on the ring, but also the influence of the chemical groups attached to the phosphorus atom.

The most important finding in regard to the organic phosphates was the total inactivity of all compounds with a sulfur atom bonded to the phosphorus, regardless of the

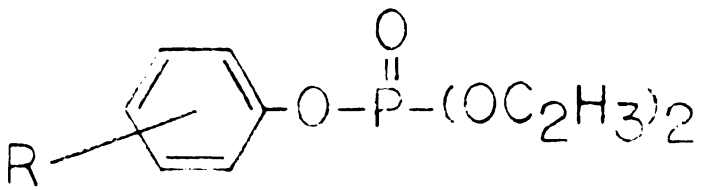
ring substituents or the esteratic groups attached to the phosphorus atom. Insofar as toxicity to insects is concerned, the P = S form is also inactive, and must be enzymatically converted to P = O, which is considered the active form (Metcalf, 1955). If the P = S form is also inactive in causing lethality in plants, then enzyme systems similar to those in the insects must be present in certain plants, since Zytron (X) is used as a selective herbicide for crabgrass (Crafts, 1961). Pisum sativum apparently is not capable of such an enzymatic conversion at a rate sufficient to supply effective levels of the active form.

The P = O forms of these organic phosphates are very active phosphorylating agents, and it is by this means that they mediate their toxic action on insects. The following is an interpretation of the phosphorylation mechanism suggested by Metcalf (1955):



G represents an electron transferring group and H an acidic portion of some susceptible enzyme. By the phosphorylation of the enzyme, it is deactivated. At the same time, the

corresponding simple substituted phenol is released. Applying this system to the data obtained from treatment of the pea root meristem, it could be suggested that the antimitotic action of the organic phosphorus compounds is due to the liberation of the phenol. Further, step 3 is rate determining, and if sufficiently slow, will allow the build-up of inactivated enzyme, which could result in other side effects or toxicity. Comparative toxicity data given by Metcalf (1955) of diethyl aryl phosphates on topical application to Musca domestica agree well with our results. With compounds of the type



the LD_{50} values in γ/g for substitutions in the para position were: nitro--0.5, chloro--150, tert.-butyl-->500 and methyl-->500. With Apis mellifera the LD_{50} values were: nitro--0.6, chloro-->100, tert.-butyl-->100 and methyl--200. Thus, the order of effectiveness of the substituent groups in the phenolic organic phosphates appears to follow the same trend as in the simple phenols, i.e. $NO_2 > Cl > CH_3$.

From this reasoning, then, it follows that any effects of the groups on the phosphorus atom are not directly involved in the reaction causing mitotic aberrations, but influence the solubility, the stability to hydrolysis or other physical properties which prevent uptake, movement

or phosphorylating capacity of these compounds.

The analysis of chemically induced mitotic deviations in the standardized pea root meristem test system was found to be as useful in determining the relative biological activity of a series of related compounds as are the more usual insect toxicity tests, growth regulator systems and bactericidal systems. Biological activity of one type, such as antimitotic activity, is not, however, strictly comparable to activity of other types, such as toxicity or metabolic disturbances of other types. Although parallels in activities and relative effectiveness may be drawn from data obtained on separate test systems, the divergent metabolic schemes and protective devices of different organisms prevent them from reacting in the same way to the same stimulus. Because of this, it is to be hoped that data from other workers in agreement with ours will tend to strengthen our conclusions, while that in opposition can be attributed to inherent biochemical and physical differences.

SUMMARY

All compounds tested of the series of substituted phenols and the series of organic phosphorus compounds with phenyl nuclei were found to have three distinct effects on the mitotic cycle. These are: pre-prophasic inhibition, prophase blockage and spindle disruption.

The substituent groups on the phenol ring have an influence on the antimitotic activity of the compounds. Those compounds with nitro substitutions were most effective and those with methyl substituents were least effective. The bromo- and chlorophenols were intermediate in effectiveness.

Compounds with three substituent groups generally showed an antimitotic action at lower doses than those with only two groups. The ortho positions on the ring appeared to be especially important for activity.

Presence of the P = S group in the organic phosphorus compounds eliminated their antimitotic effect. Those with P = O were all effective.

The structure-activity relationships may be explained in terms of the physical and chemical characteristics of the molecules insofar as these are determined by the structures.

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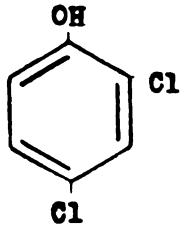
APPENDIX

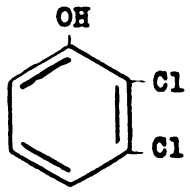
Table 1. Abbreviations for Substituted Phenols

2,4-DCP	2,4-dichlorophenol
2,3-DCP	2,3-dichlorophenol
2,6-DCP	2,6-dichlorophenol
3,4-DCP	3,4-dichlorophenol
3,5-DCP	3,5-dichlorophenol
2,4-DNP	2,4-dinitrophenol
2,4-DBP	2,4-dibromophenol
2,4-DMP	2,4-dimethylphenol
2,4,6-TMP	2,4,6-trimethylphenol
2,3,5-TMP	2,3,5-trimethylphenol
2,4,6-TBP	2,4,6-tribromophenol
2,4,6-TCP	2,4,6-trichlorophenol
2,3,6-TCP	2,3,6-trichlorophenol
2,4,5-TCP	2,4,5-trichlorophenol

Table 2. Structures of Simple Substituted Phenols Tested.



2,4-DCP



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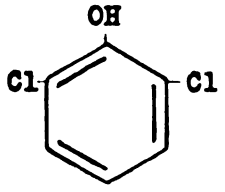

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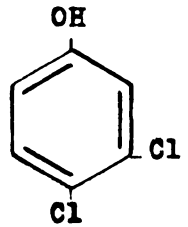

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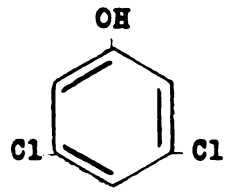
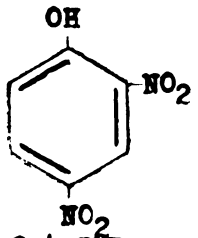
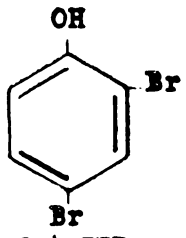


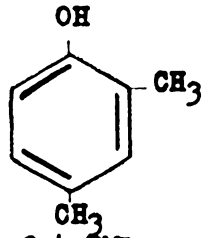
3,5-DCP



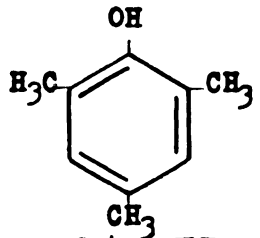
2,4-DNP



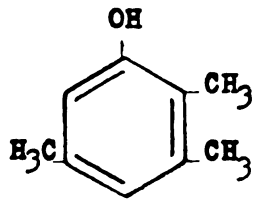
2,4-DBP



2,4-DMP



2,4,6-TMP



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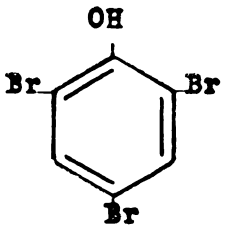
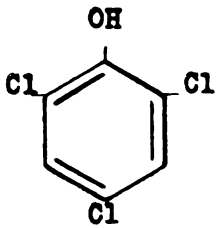
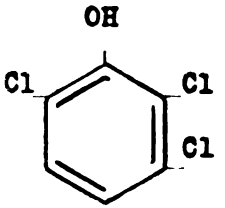


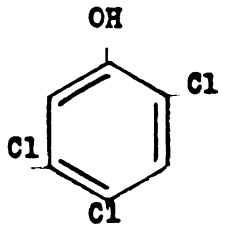
2,4,6-TBP



2,4,6-TCP



2,3,6-TCP



2,4,5-TCP

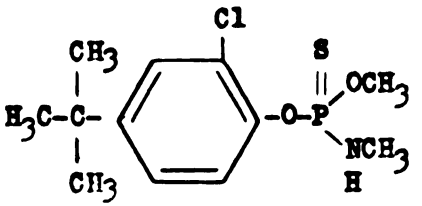
Table 3. Chemical Nomenclature of Organic Phosphorus Compounds.

I	O-4-*tert.*-butyl-2-chlorophenyl, O-methyl methylphosphoramidothionate
II	O-4-*tert.*-butyl-2-chlorophenyl, O-methyl methylphosphoramidate
III	O-4-*tert.*-butyl-2-chlorophenyl, O,O-dimethylphosphorothionate
IV	O-4-*tert.*-butyl-2-chlorophenyl, O,O-dimethylphosphate
V	O-*p*-nitrophenyl, O,O-diethylphosphorothionate
VI	O-*p*-nitrophenyl, O,O-diethylphosphate
VII	O,O-dimethyl O-(2,4,5-trichlorophenyl) phosphorothionate
VIII	O-ethyl O-(2,4,5-trichlorophenyl) ethylphosphonothionate
IX	O,O-dimethyl O-(2-chloro-4-nitrophenyl) phosphorothionate
X	O-(2,4-dichlorophenyl) O-methyl isopropylphosphoramidothionate
XI	O,O-dimethyl O-(4-methylthio-*m*-tolyl) phosphorothionate

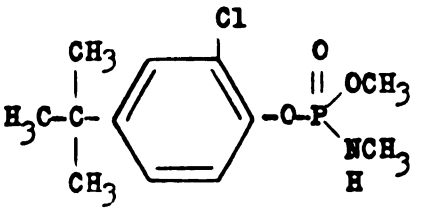
Table 4. Structures of Organic Phosphorus Compounds Tested.

Non-effective

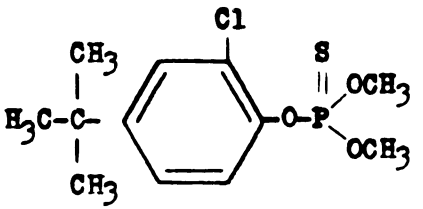
I



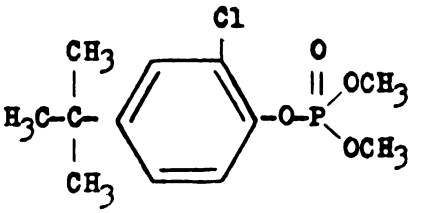
II



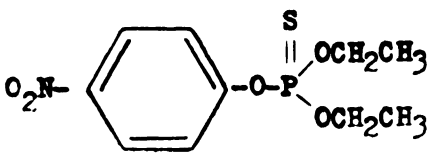
III



IV



V



VI

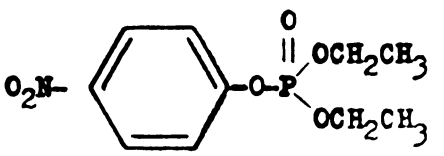
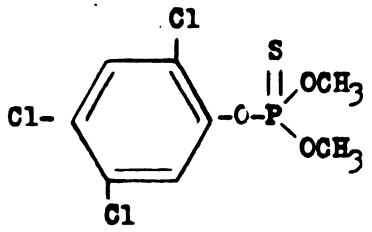
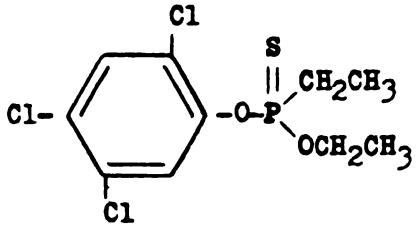


Table 5. Structures of Other Non-effective Organic Phosphorus Compounds Tested.

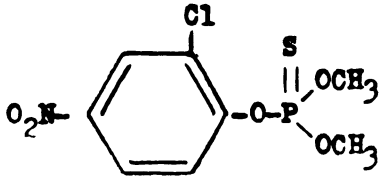
VII



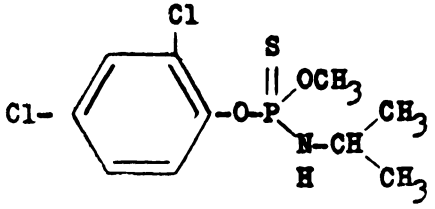
VIII



IX



X



XI

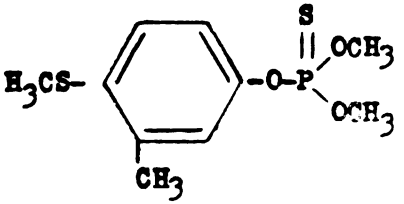


Table 6. Colchicine Indices of Substituted Phenols and Organic Phosphorus Compounds.

Compound	Dose	Hours after initiation of treatment								
		0	½	1	1½	2	2½	3	3½	4
2,4-DCP	70 mg/liter	0.02	0.05	0.11	0.19	0.13	0.08	0.03	0.00	0.11
2,3-DCP	100 mg/liter	0.04	0.76	0.97	——	0.42	——	——	——	0.61
2,6-DCP	50 mg/liter	0.15	0.21	0.35	0.39	0.43	——	——	——	0.43
3,4-DCP	60 mg/liter	0.00	0.39	0.46	0.23	0.44	——	——	——	0.23
	90 mg/liter	0.07	0.50	0.61	0.61	0.23	——	——	——	0.23
3,5-DCP	50 mg/liter	0.20	0.55	0.81	——	0.63	——	——	——	0.65
2,4-DNP	5 mg/liter	0.00	0.05	0.03	0.00	0.03	0.00	0.03	0.00	——
	7 mg/liter	0.03	0.06	0.29	0.17	0.31	0.08	0.04	0.05	0.00
2,4-DBP	50 mg/liter	0.13	——	0.63	——	0.41	——	0.32	——	0.33
2,4-DMP	200 mg/liter	0.10	——	0.57	——	0.47	——	0.34	——	——
2,4,6-TMP	200 mg/liter	0.04	——	0.50	——	0.54	——	——	——	0.13
2,3,5-TMP	200 mg/liter	0.00	0.36	0.69	0.25	0.38	——	——	——	0.13
2,4,6-TBP	200 mg/liter	0.08	——	1.00	——	1.03	——	——	——	1.18
2,4,6-TCP	40 mg/liter	——	0.08	0.28	0.50	0.22	0.18	0.57	0.15	0.13
2,3,6-TCP	100 mg/liter	——	0.36	0.74	0.87	0.83	——	——	——	1.00

Table 6. (Continued)

Compound	Dose	Hours after initiation of treatment								
		0	½	1	1½	2	2½	3	3½	4
2,4,5-TCP	40 mg/liter	0.00	0.08	0.25	0.86	0.97	0.91	0.82	0.70	0.18
	50 mg/liter	0.02	0.03	0.29	0.77	0.85	0.77	0.79	0.97	0.84
	60 mg/liter	0.04	0.12	0.71	0.89	1.00	0.97	0.89	0.61	0.91
	70 mg/liter	0.09	0.04	0.50	0.88	0.94	0.88	0.89	1.00	0.97
	80 mg/liter	0.03	0.03	0.24	0.67	0.92	0.88	1.00	1.03	1.04
II	200 mg/liter	0.08	0.85	0.92	1.01	0.98	——	0.57	——	——
IV	75 mg/liter	0.03	0.14	0.13	0.22	0.14	——	0.05	——	0.05
VI	100 mg/liter*									
	a)	0.11	0.18	0.15	——	0.22	——	——	——	0.09
	b)	0.07	0.20	0.22	——	0.33	——	——	——	——
	c)	0.09	0.12	0.17	0.15	0.15	——	——	——	0.18
	Average	0.09	0.17	0.18	0.15	0.23	——	——	——	0.14
Colchicine	200 mg/liter	0.05	——	1.20	——	1.75	——	——	——	1.25

*Three individual runs made at the apparent concentration of 100 mg/liter.

Table 7. E/L Ratios of Substituted Phenols and Organic Phosphorus Compounds.

Compound	Dose	Hours after initiation of treatment								
		0	½	1	1½	2	2½	3	3½	4
2,4-DCP	70 mg/liter	3.30	2.15	0.74	0.25	0.80	0.86	0.78	1.07	1.33
2,3-DCP	100 mg/liter	2.31	1.19	0.57	----	0.29	----	----	----	2.43
2,6-DCP	50 mg/liter	1.50	1.58	0.97	0.73	0.48	----	----	----	1.57
3,4-DCP	60 mg/liter	----	1.95	0.61	0.56	0.37	----	----	----	5.75
	90 mg/liter	4.60	0.37	1.04	0.71	0.68	----	----	----	5.14
3,5-DCP	50 mg/liter	3.38	2.33	1.56	----	0.44	----	----	----	1.55
2,4-DNP	5 mg/liter	1.35	0.81	0.60	0.42	1.06	2.25	1.23	1.68	----
	7 mg/liter	1.95	1.23	0.64	0.28	0.31	0.91	1.46	1.77	1.61
2,4-DMP	50 mg/liter	1.33	----	0.77	----	0.97	----	0.37	----	0.44
2,4-DMP	200 mg/liter	2.24	----	0.86	----	1.13	----	0.97	----	----
2,4,6-TMP	200 mg/liter	1.67	----	0.91	----	0.29	----	----	----	1.31
2,3,5-TMP	200 mg/liter	1.28	1.24	0.55	1.17	2.37	----	----	----	2.80
2,4,6-TBP	200 mg/liter	2.71	----	0.86	----	1.24	----	----	----	2.80
2,4,6-TCP	40 mg/liter	----	3.57	1.80	0.22	0.35	0.86	0.25	0.84	2.75
2,3,6-TCP	100 mg/liter	----	2.10	0.68	0.86	0.48	----	----	----	0.49

Table 7. (Continued)

Compound	Dose	Hours after initiation of treatment								
		0	½	1	1½	2	2½	3	3½	4
2,4,5-TCP	40 mg/liter	3.23	2.25	0.97	0.62	0.64	0.56	0.86	0.84	4.53
	50 mg/liter	2.69	2.13	0.77	0.76	0.58	0.43	0.67	0.73	0.60
	60 mg/liter	3.33	2.39	0.85	0.51	0.40	0.45	0.73	0.70	0.38
	70 mg/liter	3.00	2.36	1.17	0.64	0.35	0.59	0.60	0.74	0.52
	80 mg/liter	2.15	1.60	1.62	0.60	0.40	0.39	0.58	0.46	0.46
II	200 mg/liter	2.14	1.76	1.98	----	----	----	1.17	----	----
IV	100 mg/liter	3.04	2.69	2.11	1.30	1.26	----	0.78	----	1.50
VI	100 mg/liter*									
	a)	1.84	2.26	1.71	----	0.50	----	----	----	----
	b)	1.90	1.80	1.37	1.07	0.55	----	----	----	0.69
	c)	2.24	1.29	1.63	----	----	----	----	----	1.92
	Average	1.99	1.78	1.57	1.07	0.53	----	----	----	1.31

*Three individual runs made at the apparent concentration of 100 mg/liter.

Table 8. Average Control Mitotic Indices.

2,4-DCP	67
2,3-DCP	62
2,6-DCP	64
3,4-DCP	58
3,5-DCP	54
2,4-DNP	56
2,4-DEP	48
2,4-DMP	63
2,4,6-TMP	45
2,3,5-TMP	56
2,4,6-TEP	62
2,4,6-TCP	64
2,3,6-TCP	60
2,4,5-TCP	67
II	65
IV	75
VI	55
Overall average:	60
Deviation from the mean:	± 15

Table 9. Mitotic Indices of all Compounds Tested as Percent of Control Value.

Compound	Dose	Hours															
		0	½	1	1½	2	2½	3	3½	4	5	6	7	8	9	10	11	12
2,4-DCP	50 mg/l	84	77	79	79	83	---	---	---	35	---	68	---	131				
	70 mg/l	101	88	74	61	62	---	---	---	41	---	68	---	137				
2,3-DCP	100 mg/l	115	113	105	---	95	---	---	---	32								
	120 mg/l	118	100	103	90	98	---	---	---	35								
	140 mg/l	98	102	89	79	81	---	---	---	27								
	160 mg/l	108	115	102	98	71	---	---	---	24								
	180 mg/l	100	111	100	81	90	---	---	---	26								
	200 mg/l	94	111	88	98	76	---	---	---	47								
2,6-DCP	25 mg/l	100	---	106	---	94	---	---	---	56								
	50 mg/l	83	89	100	41	39	---	---	---	38	---	58	---	106				
	100 mg/l	100	---	59	---	25	---	---	---	22								
	200 mg/l	100	---	66	---	69	---	---	---	27								
3,4-DCP	50 mg/l	112	97	97	81	83	---	---	---	55	---	107	---	174				
	60 mg/l	103	93	105	81	52	---	---	---	29	---	162	---	190				
	70 mg/l	97	66	79	74	55	---	---	---	40	---	62	---	200				
	80 mg/l	88	83	91	90	47	---	---	---	38	---	69	---	164	---	159		
	90 mg/l	83	91	105	76	62	---	---	---	36	---	74	---	110	---	148		
	100 mg/l	93	91	105	62	52	---	---	---	33	---	45	---	55	---	160		
3,5-DCP	50 mg/l	104	---	78	---	63	---	---	---	67								
	100 mg/l	94	---	113	---	78	---	---	---	20	---	---	---	20				
2,4-DNP	5 mg/l	100	105	116	108	82	---	---	---	64	---	130	---	169				
	7 mg/l	107	103	105	80	80	---	---	---	57	---	87	---	173				
2,4-DNP	25 mg/l	100	---	---	---	---	---	56	---	60								
	50 mg/l	92	125	77	77	67	---	42	---	52	---	77	---	79	---	131	---	175

Table 9. (Continued)

Compound	Dose	Hours																
		0	½	1	1½	2	2½	3	3½	4	5	6	7	8	9	10	11	12
2,4-DMP	100 mg/l	95	---	103	---	---	---	43	---	40	---	---	---	---	---	75		
	200 mg/l	92	---	103	---	49	---	38	---	29	---	---	---	---	---	100		
	300 mg/l	100	---	57	---	40	---	33	---	40	---	---	---	---	---	132		
2,4,6-TMP	100 mg/l	89	---	91	---	96	---	93	---	40	---	---	---	---	---	133		
2,3,5-TMP	50 mg/l	---	89	71	---	95												
	100 mg/l	---	88	98	---	75												
	150 mg/l	---	102	96	---	95												
	200 mg/l	138	104	89	80	57	---	---	---	46	---	93	---	109	---	152		
2,4,6-TBP	50 mg/l	85	---	113	---	71	---	---	---	53	---	97						
	100 mg/l	89	---	76	---	74	---	---	---	40	---	77						
	200 mg/l	119	---	66	---	97	---	---	---	53	---	55	---	84	---	88		
2,4,6-TCP	10 mg/l	103	115	112	93	73	---	---	---	75	---	90	---	153	---	118		
	25 mg/l	113	118	132	93	114	---	---	---	62	---	60	---	164	---	159		
	40 mg/l	98	128	128	81	73	---	---	---	31	---	26	---	76	---	162		
2,3,6-TCP	25 mg/l	98	98	85	88	47	---	---	---	33	---	43	---	50	---	158		
	50 mg/l	112	95	90	67	63	---	---	---	33	---	25	---	52	---	207		
	75 mg/l	103	95	100	88	48	---	---	---	37	---	43	---	23	---	122	---	190
	100 mg/l	103	98	82	52	53	---	---	---	39	---	45	---	53	---	58	---	125
2,4,5-TCP	40 mg/l	107	97	86	108	83	70	70	54	40	57	75	73	95	169	139	146	103
	50 mg/l	94	107	80	86	89	97	78	70	53	60	76	46	77	64	137	110	145
	60 mg/l	93	102	100	89	88	69	57	49	40	49	31	43	85	87	97	127	135
	70 mg/l	94	114	104	94	77	64	55	33	52	48	37	37	59	73	101	112	122
	80 mg/l	101	120	100	97	62	112	85	70	43	64	45	---	62	---	55	84	122

Table 9. (Continued)

Compound	Dose	Hours															
		0	½	1	1½	2	2½	3	3½	4	5	6	7	8	9	10	11	12
II	125 mg/l	105	115	117	109	95	78	100	94	105	---	---	---	132				
	140 mg/l	108	88	95	126	134	94	108	109	100	---	---	---	162				
	155 mg/l	102	89	108	106	100	98	75	105	86	---	---	---	143				
	200 mg/l	85	100	102	62	86	74	62	48	68	---	---	---	165	---	---	---	111
IV	75 mg/l	121	85	80	---	109	---	76	---	104	---	96	---	156	---	120	---	103
	100 mg/l	89	87	115	64	75	---	45	---	49	---	25	---	31	---	---	---	63
VI	100 mg/l*	82	118	75	---	82	---	---	---	80	---	98						

*Apparent concentration; mitotic index values are averages of three runs.

Table 10. Minimal Effective Doses and Maximal Sub-toxic Doses of all Chemicals Tested Causing Mitotic Inhibition.

Compound	Minimal		Maximal	
	mg/liter	X 10^{-4}M.	mg/liter	X 10^{-4}M.
2,4-DCP	< 50	< 3.07	70	4.29
2,3-DCP	100	6.13	200	12.27
2,6-DCP	< 25	< 1.53	200	12.27
3,4-DCP	< 50	< 3.07	100	6.13
3,5-DCP	< 50	< 3.07	100	6.13
2,4-DNP	5	0.27	7	0.38
2,4-DBP	< 25	< 0.99	100	3.97
2,4-DMP	100	8.19	> 300	> 24.56
2,4,6-TMP	100	7.34	> 300	> 22.03
2,3,5-TMP	200	14.69	> 300	> 22.03
2,4,6-TBP	< 50	< 1.51	200	6.05
2,4,6-TCP	10	0.51	> 40	> 2.03
2,3,6-TCP	< 25	< 1.27	> 100	> 5.06
2,4,5-TCP	< 40	< 2.03	80	4.05
II	125	4.29	200	6.86
IV	75	2.56	125	4.27
VI	< 100	< 3.63	> 100	> 3.63

Table 11. Orders of Effectiveness for Mitotic Inhibition and Toxicity Based on Minimal Effective Doses and Maximal Sub-toxic Doses.

Mitotic inhibition		Toxicity	
Compound	Dose (X 10^{-4}M.)	Compound	Dose (X 10^{-4}M.)
2,4-DNP	0.27	2,4-DNP	0.38
2,4,6-TCP	0.51	2,4,6-TCP	> 2.03
2,4-DBP	< 0.99	VI	> 3.63
2,3,6-TCP	< 1.27	2,4-DBP	3.97
2,4,6-TBP	< 1.51	2,4,5-TCP	4.05
2,6-DCP	< 1.53	IV	4.27
2,4,5-TCP	< 2.03	2,4-DCP	4.29
IV	2.56	2,3,6-TCP	> 5.06
2,4-DCP	< 3.07	2,4,6-TBP	6.05
3,5-DCP	< 3.07	3,5-DCP	6.13
3,4-DCP	< 3.07	3,4-DCP	6.13
VI	< 3.63	II	6.86
II	4.29	2,6-DCP	12.27
2,3-DCP	6.13	2,3-DCP	12.27
2,4,6-TMP	7.34	2,4,6-TMP	> 22.03
2,4-DMP	8.19	2,3,5-TMP	> 22.03
2,3,5-TMP	14.69	2,4-DMP	> 24.56

Table 12. LD_{50} Values for Chemicals Tested by the *Drosophila* System in Order of Effectiveness.

Compound	LD_{50} (mg/liter medium)	X 10^{-4}M.
2,4-DNP	2.0	0.11
2,3,6-TCP	13.0	0.66
2,4,6-TBP	22.5	0.68
2,4,5-TCP	14.0	0.71
2,4-DBP	22.0	0.87
2,4-DCP	15.8	0.97
2,3-DCP	19.5	1.20
3,5-DCP	20.0	1.23
3,4-DCP	23.0	1.41
2,4,6-TCP	28.5	1.44
2,6-DCP	31.0	1.90
2,4,6-TMP	28.0	2.06
2,3,5-TMP	30.5	2.24
2,4-DMP	30.0	2.46