INHIBITION OF CELL DIVISION WITH 8-METHOXYPSORALEN

by

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INTRODUCTION

Modern scientific investigations show an increasing tendency to cut across fields which once were insular. The merger of the words, ideas, and tools of physics and biology, of neurology and chemistry, etc., has produced exciting new concepts and approaches to old problems, and has revealed many new problems. To paraphrase Donne, no field of inquiry seems to be unto itself an island.

This thesis, from the Department of Anatomy, deals fundamentally with a biological problem, but the author has not hesitated, magpie-fashion, to accumulate a glittering array of ideas and techniques and terminology from the fields of cellular physiology, cytogenetics, mathematics, biochemistry, pathology, etc., which he hopes have not been too badly misused.

Since it has been only slightly over 100 years since Schleiden and Schwann postulated the cell theory, it is not surprising that the main line theories of growth and development and differentiation and organization have been developed mostly in this century. Following the widely encompassing researches of Jacques Loeb, around the turn of the century, there have been an ever increasing number of workers who have looked upon the understanding of the living cell to be fundamental to an understanding of life itself.

Hertwig (19,20,21), shortly after 1900, proposed the nuclear-cytoplasmic ratio theory of cell division which, in somewhat modified form, is still widely held today. In any case, whether it is valid or not, it has led to a great deal of research on the events of cell division, with particular regard to metabolic and physiologic changes in both

the nucleus and cytoplasm prior to, during, and after division.

Popoff[49], working with the microorganism, Frontonia, was perhaps the first worker to prolong artificially the intermitotic interval by removing bits of protoplasm at a certain time prior to an expected division. He regarded this as verification of Hertwig's theory.

These men postulated that "a uniform rate of cell growth is a property inherent in living protoplasm," and went on from there to investigate the ways in which this growth rate might be modified, and to what other biological events it might be correlated. This, one feels, is still a very useful approach.

Howard and Schultze[23], in 1911, published a monograph on the biology of tumor cells. In spite of the tremendous advances which have been made in this area since that time, much of the work still seems valid. They state the conditions under which growth and cell division take place in the adult metazoan, e.g., for renewals or replacement of cells or parts, repair of wounds, inflammatory new formations, production of sex cells, and tumor formation.

Fundamentally, it is still the comparison of events, biochemical, biophysical, etc., which are associated with these conditions, that we continue to explore today in the hope that our understanding will ultimately lead to increased control.

It was W.B.Canon[9] who, in 1929, coined the term "homeostasis," and thus gave great impetus to the notions, which now seem almost self-evident, concerning the self-regulatory nature of cell proliferation.

The most recent theory of homeostasis is that of Osgood[38],

who postulates an inhibitor substance or substances produced by cells which affect only closely related cells.

Of direct bearing on theories of cell inhibition have been the contributions made largely by the biochemists in producing a great number of substances which have a toxic or inhibitory effect on certain micro-organisms. This is clearly important in the possible control of pathogenic organisms subject to these substances, but perhaps more importantly than that, they have directed our attention to certain basic metabolic processes which are going on at a cellular level, and opened new avenues of attack--the use of metabolic analogues--on solving metabolic problems. Of particular interest are the processes involved in the synthesis of nucleoproteins, an understanding of which should contribute to biochemistry, virology, genetics and biology in general.

Furocoumarins

Recently there has been a revival of interest in the furocoumarins, and especially 8-methoxypsoralen, particularly among dermatologists in regard to its supposed action as a "suntan" pill. There has been a burst of clinical observations, and some experimental studies have been made.

Almost, if not all, of this work has centered on the property of furocoumarins like 8-methoxypsoralen to be photodynamically activated in the presence of long wave ultraviolet light

The present study was also originally planned to include an investigation of this property in regard to its action on cell populations. Instead, however, a large amount of data has been

accumulated on the dark reaction. It is possible, of course, that its dark reaction is but a part of a chain of biochemical reactions which are intrinsic to the photodynamic reaction also. It is of interest that Evenari (13), studying germination in plants, found a thermally linked skotomechanism to which coumarin was connected, and a photomechanism which was not sensitive to coumarin. No evidence is offered here concerning such a relationship.

It is intriguing that 8-methoxypsoralen, which Rodighiero (53) and Musajo (35) have shown to have an inhibitory effect on the growth of plant tissues, can be shown here to have the same effect on animal or animal-like cells. It does not necessarily follow that the mechanism for inhibition is the same in both cases, however.

DEFINITIONS

Throughout this paper certain terms are employed for which simple dictionary definitions do not always suffice. This is not to suggest that these terms should run counter to such definitions, but that it may be useful to state as precisely as possible what is meant by them here.

By "growth" is meant the developmental increase in the mass of a body, strictly. The confusion about this term possibly arises from its being applied to protista, or tissue cultures, on the one hand, and metazoa on the other. Considering the metazoan as a whole, it is obvious that developmental increase in mass is accomplished by increasing the number of cells, fluid, extracellular products, etc. When we speak of growth of human beings, for example, we cite increases in height, weight, etc., with the unstated proposition that this is due to cell multiplication.

By analogy, when speaking of a population of cells, the term "growth" is often used to mean an increase in the number of cells in that population, even though this is not precise.

Another point is that these two concepts are not independent. Cell divisions cannot take place very often without there being an accompanying process of growth. Hence, when we speak of the growth of a cell population, the underlying assumption is that there is a causal relation between the increase in the number of cells and the growth of each individual cell such that no increase in numbers can occur without accompanying growth. For very short periods of time this may not be strictly true--under some circumstances a series of cell

divisions may take place so rapidly that the daughter cells produced are actually smaller than their "parents." Eventually, however, growth must catch up with division, and the cells are established at a size presumably governed by their genetic make-up.

The term "cell" itself is not without some ambiguity. The classical use, or rather the classical scientific use, of the word may be said to have begun with the work of Schleiden[58] and Schwann[59], who meant by it any of the minute bits of protoplasm which make up organized tissue. As such, it should be considered as a part of a larger organization.

But the term is almost universally applied to bacteria, yeasts, fungi, protozoa, etc., which usually very clearly do not exist as a part of an organized tissue, but are themselves highly organized and complex organisms.

The term "unicellular organisms" has been used, but it is no more precise than the term "cell." The term "acellular organisms" has been proposed also, but not generally accepted. The term "protista" has enjoyed widespread use among people who work with them, but does not seem to be generally in vogue.

This is not sheer pedantry. A term such as "cell" insensibly carries with it some notions of how a cell should behave. If a cell is a bit of protoplasm which is but a part of an organized tissue, which is in turn part of an organ, which is part of a complex organism, then this bit of protoplasm, though subject to a bewildering variety of external and internal influences, follows a certain pattern of behavior. To what extent does it have a life of its own? What happens to this pattern when these various influences are removed

and different ones are substituted? A partial answer is that to date only a certain number of cells have been demonstrated to be culturable *in vitro.* And even more importantly, cells which will grow *in vitro* are subject to alteration in culture in such a way that they may exhibit different properties from those in an intact organism! They may become typically malignant cells (Leighton, 30; Moore, 34). In terms of organization they may no longer resemble cells so much as they do protista, though they still have a finite life span.

Here the term "cell" has been used in accordance with the popular use of the word to refer to individuals within a tissue culture, individual protists, and individual functional bits of protoplasm which are part of an organism.

There is, however, one more set of notions to develop in this regard. There are various modifying adjectives which serve as functional qualifiers for the term "cell." These are such terms as "immature," "mature," "differentiated," "undifferentiated," "malignant," "normal," etc., which may often include, in addition to anatomical description, certain ideas of function. It would be exceedingly difficult as well as immodest to attempt to give precise definitions to all these combinations of terms. In general, one attempts to give the same meaning to them as do the bulk of the workers in the fields which employ them.

For the purposes of this paper, the term "differentiated cell" means a morphologically and chemically mature cell of a metazoan, as the term "undifferentiated cell" means an immature cell of a metazoan. Only undifferentiated cells are considered as being capable of division. Protista might be classified as undifferentiated cells, but not in the

sense of maturity; it is better not to classify them as either differentiated or undifferentiated cells (which is one of the arguments for not classifying them as cells at all).

There is, however, repeated reference made to "malignant" cells in this paper. This is in accordance with the root meaning of the Latin word, malignare, to act maliciously. These cells are characterized by morphological alterations--increased nuclear size, hyperchromatism, etc.--by means of which they are identified and classified, and are certainly cells which act "maliciously" toward the organism in which they are growing, although no notion of intent is implied here.

"Normal" cells generally should be taken to mean cells which are functionally and anatomically characteristic of the organism. Generally it is possible to show that they are useful to the organism, although this involves a value judgment which it is often preferable to avoid. Used in reference to protista, "normal cells" means organisms which are growing, dividing, metabolizing, etc., in ways which are characteristic of that species. This use of the term is admittedly not precise, but it is convenient.

"Cell division" is the process, paradoxically enough, which produces "cell multiplication." It is almost always preceded by the process of mitosis among the protista and cells in tissue culture, although in the latter it is not uncommon for mitosis to occur without a subsequent cell division. Thus, cell division is actually a broader term than mitosis, since it usually includes mitosis. Amitotic divisions probably do not occur in holozoic organisms, but in any case need not concern us here.

A "population" means a group of cells or protista spatially bounded, i.e., in a growth chamber of some sort. "Population density" means the number of cells per unit volume. "Population doubling" means that a population has become two times as dense as a given measurement. "Population doubling time" is the interval of time during which a population doubles. When dealing with a single cell, which divides to become two, "population doubling" means the same as "generation." In this case "generation time" is the same as "population doubling time."

In the case of many cells, however, it may be that some divide at different rates and also that some cell death occurs. In this event, in order to double the density it would be necessary for some cells to divide more than once, or for cells to divide and their daughter cells also divide within this time period. In this case, it is only possible to refer to "mean generation time", as if one is dealing with time per generation in a population in which there is, in that interval, no cell death and in which each cell has the same division rate. Mean generation time, usually "T/G," is determined by dividing a time interval by the number of population doublings within that interval.

An example may be clarifying: if a population doubles twice in 24 hours, mean T/G is 24/2, or 12 hours. It is quite possible, of course, that the first doubling occurred in the first 8 hours, and the second in the last 16. T/G could only be constant during true exponential growth.

"Mitotic time" is the time course of the events of karyokinesis. "Intermitotic time" is the interval from the formation of a daughter

cell to the commencement of mitosis in that cell. A cell incapable of further divisions has no true intermitotic or mitotic time. In this case, it is proper to speak of the "life span" of the cell: that interval from its formation from a pre-existing cell to its death. For certain purposes it may be convenient to think of the intermitotic time plus the mitotic time as equivalent to "life span".

A "growth curve" is a plot of density of organisms, or cells, against time (Fig. 1). Most kinetic studies (Van Niel, 60) are based on growth curves. The interval (a-b) in Fig. 1 is termed the "lag" phase. It occurs whenever older cultures are transferred to fresh medium, but can be abolished by rapid transfers (Phelps, 46). It is followed (e) by a period of exponential growth. The period (m) represents the maximum density. If it is prolonged it is often called the "maximum stationary phase". Finer divisions of the curve may be made, but these are sufficient for present purposes.

PROBLEM AND APPROACH

With due recognition of the fact that a problem can rarely be entirely formalized, set out in a completely clear-cut, preconceived plan, and then pursued in a logical fashion following that plan, one may still state the general objectives of a project, subject to certain reservations.

In point of fact, since a thesis is composed after experimentation has been carried out, it would be all too easy to write, *ex post facto*, a set of propositions under this section, the truth of which is presumed to be demonstrated in sections following. In practice, the operation is very much different from this. In the beginning it is possible to entertain a wide spectrum of ideas and notions which range in clarity from relatively simple propositions which admit of a "true" or "false" answer, to complex and conditional hypotheses which may or may not have any answer. As investigation proceeds, a few questions may be answered, but meanwhile many more problems and questions arise. The formal statement of a problem almost certainly varies according to the particular time it is stated, and the criterion of success which is perhaps the most useful to apply is the extent to which the investigation has created new problems.

The specific objective of this investigation was to test the action of a chemical compound, 8-methoxypsoralen, one of the furo-coumarins, on living animal organisms at the cellular level.

The idea of the "unity of biochemistry" — that, to use Hutner's phrase, "life shows uniformities in molecular morphology" — has given rise to the hope that information gained from isolated cells of multi-

cellular animals, from protists, and from metaphytes may often be relevant to metazoa.

As cited above, it had already been shown that 8-methoxypsoralen, as well as related compounds, has an inhibitory effect on the growth of some higher plants. The question naturally arises as to what, if any extent does this compound show an inhibitory effect on the growth of animals? If it does have such an effect it should be of great interest primarily because it would then operate to the same end in species which are poles apart, biologically. And the sorts of substances which can be shown to act both in plant and animal cells, such as glucose, oxygen, etc., are considered to be intimately related to fundamental life processes.

Parenthetically, it should be added that this class of compounds is a normal constituent of, that is, is produced by, a great variety of plants, particularly adult plants (Pathak, 45) and therefore these compounds are ingested by a great variety of animals, perhaps in considerable quantity.

As has also been mentioned, furocoumarins in general, and 8-methoxypsoralen in particular, have recently been studied by biochemists, dermatologists, bacteriologists, etc., in special regard to their property of producing photosensitization in a large number of organisms, macro and micro. It is clear, however, that if this compound (8-methoxypsoralen) is capable of so altering fundamental life processes in such a way as to change or inhibit the growth rate of an organism <u>independent of</u> its photosensitization effect, this role should be better understood.

To assay to study the action of a compound on animals raises many

technical problems. It is true that metazoans exhibit certain types of organization which may yield qualities different from, or greater than, the sum of the properties of all their parts. This attribute of organization, therefore, may produce an alteration in the response of such organisms from that of the isolated response of any simpler system. But as stated before, the hope is that the study of simpler systems may be useful and applicable to more complex systems, an analytical device which is suggested in general terms by Polya (48).

Common laboratory animals demand space, quantities of food, and time, in dimensions which almost preclude their use in assaying a population response to an alteration in metabolism, useful as individual studies have been in the past. In the present case it would require working out an effective means of administration, noting physiological and growth alterations, and sacrificing the animal and studying sections taken from various tissues of the body.

These complications have led, not only in the present study, but in general, to a search for biological systems which, on a micro scale, retain and reflect the salient features of multicellular organisms in respect to metabolism and growth. They have led to intensive research in broad and over-lapping fields: the use of bacteria in micro-bio-assay; the studies on cell and tissue cultures of multicellular organisms; the use of protozoa.

The last two of these methods have been used in this study. The isolation and propagation in tissue culture of human cells permits the carrying out on living human protoplasm of experiments which would not otherwise be possible. Even though profound changes, often of an unknown nature, may take place both before these cells are cultured and

after, nevertheless it is highly probable that they retain certain characteristics of human cells in general, and of the particular tissue or cell series from which they were taken.

The fact that such alterations occur, however, may, at least for the present, preclude the possibility of studying wholly normal human cells by this technique. Many cells have not yet been grown, in the sense of long-term growth, in tissue cultures, particularly cells of adult tissues. Further, there is evidence (Moore, et.al., 34; Leighton, 30) that even when successful lines of some cells are established from normal tissues, neoplastic changes may subsequently develop in vitro.

Since it is known that such changes alter the size and shape of the nucleus, increase numbers and types of chromosomes, etc., it is entirely likely that the metabolism and subsequent growth and cell division of such cells are altered also. If the in vitro culture of human cells produces an environment conducive to neoplastic changes in these cells, it would seem that the attempt to study the action of 8-methoxypsoralen on a cell undergoing normal metabolic processes in vitro would be foredoomed to failure.

In the face of such mutually exclusive actions, it is necessary to seek an entirely different and alternative cell system.

The establishment of certain ciliate protozoa in axenic culture has been particularly stimulating to research, providing for metabolic, morphologic, genetic, etc., investigations unhampered by the presence of bacterial or algal contaminants. And for one ciliate, Tetrahymena sp., it is now possible to obtain growth and reproduction on chemically defined media (Seaman, 58; Kidder & Dewey, 28).

Seaman (59) notes the similarity between the requirements for

Tetrahymena and of mammalian tissue: e.g., the amino acid requirements of the ciliate are almost identical with those of the growing rat and man, and *Tetrahymena* appears to metabolize carbohydrates in the conventional manner.

In some respects the metabolic behaviour of *Tetrahymena* is very much like that of a metazoan such as the chick or rat. The use of *Tetrahymena* merely reduces the scale of analysis.

The problem of investigating the action of 8-methoxypsoralen falls conveniently into two parts: the first, dealing with tissue cultures of cells cultured from the blood of a leukemic patient, which are represented as malignant human cells, and the second, dealing with axenic cultures of the ciliate protozoan, *Tetrahymena*, which are represented as normal animal cells.

Because of this, the presentation of material is made in two distinct parts, following the course of the investigative procedure. Under each part there is a presentation of the materials and a description of the methods used, followed by a presentation of each individual experiment in the section, with a discussion of particular problems and results obtained in that, and in closely related, experiments.

Following these two parts there is given a general discussion which relates the findings of all the experiments to the problem as a whole, and in which is included projected investigations which might profitably be carried out, together with some comments on techniques which might be useful to such an investigation.

PART I

Malignant Cells

A. Description of Oregon J 96.

The cell strain Oregon J 96 was started April 7, 1954, from the blood of a 29-year-old patient with subacute leukemic monocytic leukemia. It was initially grown in a medium composed of Earle's balanced salt solution[1] 60%, and human pleural fluid, 40%. In 20 ml. of medium with an initial count of 1500 cells per cubic millimeter in an 120 ml. flat-sided pharmaceutical bottle, lying on the flat side, this gave a depth of 8 mm., and a "gradient factor" (Osgood, 43) of 1.2 (that is, 0.8 x 1.5). Later this strain went into rapid growth, many subcultures were made, and it has been widely distributed. (Osgood, 42).

This cell strain is usually referred to as simply "J 96," and this shortened name will be generally used in this paper.

The use of the term "malignant" in referring to this strain stems from the description of its history and characteristics. It was isolated from the myelomonocytic, or Naegli, type. Osgood's (39) description in 1957 is still valid today. He comments on the extreme pleomorphism of these cultures which has existed since J 96 went into rapid growth. Many of the cells closely resemble fibroblasts, many resemble tissue histiocytes or reticulum cells, and in fact, these are predominant. In addition, there are cells resembling Langhan's, foreign-body giant cells, and Dorothy Reed cells. According to Osgood (39), "Nearly all the types of giant cells so well described and pictured by Rebuck (52), which might be derived from histiocytic or monocytic tissue, are seen in these cultures." We

[1]Difco, item 0351

have seen giant cells with a giant nucleus (mononuclear giant cells) as well as multinuclear giant cells with one or more nuclei in division.

Further evidence of the malignant character of these cells may be adduced from the work of Burchenal (8) in making animal transplants.

B. The slide cap culture method.

The slide cap culture method uses Kahn antigen mixing vials whose lips have been ground flat with emery powder on a plate glass surface (Osgood and Brooke, 42). These shell vials are effectively sterilized by aluminum foil wrapping and heating in a dry oven. After sterilization, the lips of each vial are dipped into beeswax which has been brought to smoking temperature and then slightly cooled. The vials are then held vertically upside down until the wax has congealed and then reverted to an upright position (preferably under a sterile hood), and allowed to cool. A convenient amount of fluid is pipetted into the vial and a sterile glass microscope slide is placed over the vial with sterile forceps.

When a complete set has been made in this way, and placed on a tray, the vials may be moved to an incubator at 37.5°C. for $\frac{1}{2}$ to 3 hours to permit gas equilibration before sealing. Or, as was later discovered, if the air temperature of the hood is kept at this temperature or higher, the period of equilibration may be eliminated.

The vials are then removed, still held upright, a Bunsen flame passed lightly over the slide and, if properly done, the melting beeswax will flow to the slide, making an airtight, moisture-tight seal. The chambers are once again inverted so that they rest on the micro-

scope slide, and placed in the incubator.

The cells in the culture medium tend to fall to the surface of the slide, attach, multiply, and form colonies.

C. Preparation of the culture media.

Culture medium is generally composed of a balanced salt solution to which certain enriching substances are added. In this series of experiments the balanced salt solution is composed of NaCl, 7.46 gm.; KCl, 0.37 gm.; $CaCl_2$, 0.224 gm.; $MgCl_2$, 0.20 gm.; Na_2HPO_4, 0.12 gm.; KH_2PO_4, 0.03 gm.; $MgSO_4 \cdot H_2O$, 0.07 gm.; CH_3COONa, 0.50 gm.; dextrose, 4.00 gm.; and water to make 1000 ml.

A convenient method is to make up one liter of these salts in 10x concentrations with the exception of the $CaCl_2$ which should be made up separately as autoclaving this salt with the others results in the formation of a fine precipitate. This 10x concentration may safely be kept non-sterile in stoppered bottles under refrigeration and diluted and autoclaved as needed. The $CaCl_2$ is made up in 10x concentration and autoclaved in vaccine-stoppered bottles (which are evacuated before heating). This reagent is added by sterile syringe after the calcium-free balanced salt has cooled, just before use.

A variety of nutrients and vitamin mixtures have been tried. For a protein source we have used human pleural fluid which was sterilized by force-filtration through Corning TC sintered glass filters, reconstituted lyophilized human serum, and Difco TC Horse Serum. Perhaps the best results were obtained, however, with the use of 10% human serum, whole, from Courtland Laboratories, Los Angeles, and Difco TC Vitamins Eagle, item 0792.

To obtain media containing appropriate amounts of 8-methoxypsoralen (which will subsequently be referred to as "8-MOP"), this compound was weighed out and diluted in calcium-free balanced salt solution to give 50 mg./ liter. This was autoclaved, cooled and the proper nutrients were then added to bring it up to complete medium. Serial dilutions were made directly in the shell vials, using a comparable medium as a diluant, but not containing 8-MOP.

An alternate procedure, used later, was to make serial dilutions of 8-MOP in calcium-free balanced salt solution in several bottles which were vaccine-capped, autoclaved, and cooled. The nutrients were then added to each of these bottles.

Each bottle containing 8-MOP was wrapped with aluminum foil to protect it from the light.

The highest concentration of 8-MOP before adding cells was 2.315 x 10^{-4} Molar, and after adding cells this became approximately 1.7 x 10^{-4} Molar. Serial dilutions were made to give a ratio of 1:10, 1:100, 1:1000, etc., and in some cases an additional series was made with ratios of 1:20, 1:200, 1:2000, etc.

In other terms, the final highest concentration was always 36 µg./ ml., followed by 3.6 µg./ ml., etc., in the first series, and 18 µg./ ml., 1.8 µg./ml., etc., in the other series, when the latter was made.

It might be of some interest to observe at this point that the LD/50 of 8-MOP in mice has been established to be in the range of 685-700 mg./ kg. (Pathak, 45). For several reasons these dosages cannot, however, be directly compared to the dosages given J 96.

D. Counting procedures.

A bulk culture bottle of rapidly growing J 96 was selected and

given a medium change two or three days before the experiment. At the time of the experiment the bottle was shaken slightly and the supernatant fluid with floating or loose cells was removed. Ten ml. of culture medium was used to wash the surface of the cells, this also being discarded. Another 10 ml. of medium was added, and to this 1 ml. of 1.5% EDTA was added along with a few drops of 5% Na_2CO_3 to adjust the pH to the alkaline side (Phenol Red being used as an indicator in all culture media). The bottle was placed back on its side such that this fluid covered the cells and it was allowed to sit for 30 minutes, or until the cells came free with gentle agitation.

Depending on the amount of growth to be seen when previously examined under low power, another 30 ml. or more of medium was then added to the bottle to suspend the cells and dilute the EDTA. Counts were taken on this suspension and appropriate dilutions were then made to give the desired number of cells.

This "desired" number changed during the course of the experiments. Initially, we tended to think in terms of the parameters of the first successful isolation and cultivation of this cell strain, and hence were dealing in suspensions of 1000 cells per cu. mm., or more. As experimentation progressed, as will become clearer, it was seen that J 96 had altered enough in continuous culture to permit much wider latitudes of culture conditions, making it possible in this work to come down to suspensions of 10 cells per cu. mm. I am now informed (Brooke, 5) that Puck, using his special techniques (Puck, 50; Marcus, 32) has succeeded in producing a clone from a single cell.

For accurate establishment of the maximum number of cells which could be present in the initial inoculum, nuclei counts were made.

For this purpose, 1 ml. of well-suspended cells was taken up in a volumetric pipette and diluted with 1 ml. of 1% citric acid solution and 1 ml. of citric acid-cresyl violet solution. This was shaken vigorously, and the purple stained nuclei were counted in an hemacytometer. This number gave the greatest number of cells which could possibly grow since some of the nuclei must be derived from non-viable cells, and perhaps in some cases several nuclei came from a single cell which may not be destined to give rise to other cells.

The slide caps were sacrificed and the cells enumerated after a certain period of time to be described below. The supernatant fluid was discarded without a count, and this requires an explanation. An examination of this fluid revealed that there were very few viable cells in this supernatant. When this material was centrifuged and stained smears made, the cells were seen to be almost invariably in some stage of disintegration. They probably represented the non-viable and injured cells present in the original inoculum, plus some others trapped on the walls during inversion of the shell vial. Over the range of this experiment, the actively growing cells, for the most part, attach to the glass slide and multiply there.

Thus the glass slides were saved, very lightly rinsed with balanced salt to remove any cell debris (especially valuable in reading older slides), fixed with absolute methanol, and stained with Wright's stain, Giemsa, or the stain of Chen-hui Liu[1].

Early counts were often made with the 20x objective, whereas later counts were made under oil immersion, which permits some detailed examination of cellular morphology during the counting procedure.

[1] Dr. Chen-hui Liu is presently at the National Taiwan University Hospital, Taipei, Taiwan.

A Miller disc was fitted into the eyepiece, the field of which was calibrated for each objective with a stage micrometer. The fields were selected for counting by moving the slide in a straight line equivalent to 10 times the side of a Miller disc before counting the next field. Twenty-five to 100 such fields were counted on each of two or three replicate slides for each concentration of 8-MOP and controls, at each sampling time. A total of 200 to 1000 cells were counted for each point. Slides were filed for review, recounts, and reexamination for any changes in morphology which might have occurred during the course of the study.

RESULTS AND DISCUSSION

First of all it should be stated that, in the hands of this investigator, the techniques described above are not entirely satisfactory. There are far too many variables, the sensitivity of which is not known, for there to be the kind of repeatability which we desire in experimentation.

There are differences from batch to batch of pooled human serum, perhaps slight differences in the balanced salt solutions depending on weighing errors and even in the time for autoclaving. There are slight differences in the temperature of the room, in the hood, and perhaps even in the incubator, any of which may alter the amount of dissolved gases, cause convection currents, etc., which could possibly shift the microenvironment of the growing cells.

We do not yet know in which areas and in what ways these cells are most sensitive. Therefore it is that two different experiments may give two different absolute values. The saving feature, however,

seems to be that with any one experiment, excluding such obvious faults as contamination, leaks around the seal, etc., the results are internally comparable. That is, within any one experiment, by setting up triplicate or quadruplicate controls, these will be found over a period of time to be approximately the same, and the experimental group with various concentrations of 8-MOP will be found to differ from the controls by an amount that may be highly significant.

There follows a discussion of the individual experiments, how they may have been carried out in ways which differ from the others, and the results obtained by each of them.

Experiment 1

Cells from a bulk culture of J 96 were removed from the glass in 10 ml. of culture medium to which 1 ml. of 1.5% EDTA had been added. The concentration of EDTA necessary to remove cells from glass is about 150 mg. per cent. Approximately 30 ml. of medium was added and counts were made. The suspension was computed to have 145 cells/ mm^3.

Rows of 6 shell vials were set up and to the first was added 2.5 ml. of medium containing 50 µg./ ml. 8-MOP (which is 2.315 x 10^{-4} Molar). Serial dilutions (1:10) were made by carrying 0.25 ml. of the 8-MOP medium to 2.5 ml. of normal medium in the next vial, mixing, transferring 0.25 ml. of this mixture to the next vial, etc. Controls were made using 2.5 ml. of normal medium.

To each of the vials 1 ml. of cell suspension was added, the slides placed on top, the chambers then allowed to equilibrate in the incubator for 3 hours, brought out, sealed, inverted and replaced in the

incubator, undisturbed until sampled.

It should be noted that a suspension of 145 cells/ mm^3 means that there are 145,000 cells put into the vials. When inverted, the cells should drop onto an area of about 175 mm^2, giving an initial count of 8,300 cells / mm^2. Actually, this is a crude approximation, and too high.

The problem of getting accurate counts is a little complicated, but should be appreciated. All tissue cultures are kept in sterile, closed containers, usually stoppered with vaccine caps. Transfers are made by going through a pool of 70% alcohol with a sterile needle and syringe. Thus, volumes are only as accurate as can be measured with a syringe.

Furthermore, the ideal counting suspension turns out to give about 64 cells per large square (o.1 mm^3) of an hemacytometer. But this is much too large (640,000 cells/ml.) for an ideal inoculum. Therefore, it would be greatly diluted. But the number of cells ideal for an inoculum would be so small that it would take dozens of counting chambers to count a proper number of cells. The compromise is to take an ideal counting suspension and then try to dilute it as accurately as possible with the syringe. In this kind of experiment the matter is not of crucial importance since we are interested in differences, rather than absolute values.

From an examination of Table 1 it will be seen that at 120 hours the control slides (which revealed typically healthy cultures) showed an average of 239 cells / mm^2. We have already said that computations from the inoculum predicted 8,300 cells/mm^2 initially. One might conclude that these cells are not growing at all. This is clearly

not the case, and perhaps the paradox can be resolved by turning to a previous experiment.

Osgood and Brooke (42), in an experiment using J 96 in slide caps to measure normal rates of growth, found 5 day cultures had increased nearly 8 times the inoculum. More precisely, slide caps sampled on day 5 in this series had increased 7.6 x that of the slide caps sampled on day 1 (24 hours). This really says little about the initial inoculum.

At this time it was customary in Osgood's laboratory to set the slide caps with an initial "gradient factor" (43) of 2--calculated by multiplying the number of cells in thousands per cubic millimeter times the depth in centimeters. Since the standard depth of 3.5 turns out to be 2 cm., this means that there were 1000 cells/mm^3 in the inoculum, or 3,500,000 cells in each slide cap. Falling on an area of 175 mm^2, this would mean 20,000 cells/mm^2 on the slide. The calibration of the microscope used to make these counts was such that 109 fields of the Miller disc under oil immersion equaled 1 mm^2. Thus, there would have been initially 183 cells per field, under oil. A referral to Fig 2C, a plate about the size of such a field taken under oil immersion, shows one binuclear and six mononuclear cells at this magnification. One can imagine the crowded living conditions were 183 cells crowded into such a field!

From such a consideration two things are clear: evidently only a fraction of the original inoculum survives to grow and divide, and further, that the best estimate of the true inoculum of viable cells is to be gained by extrapolating points back to the origin. The data given do not permit this to be done with accuracy, but it is

possible to draw a smooth curve with not more than 1/30th of that inoculum, namely, 100,000 cells, as the number which actually attached to the slide and began to grow and divide.

To return to the present experiment 1, suppose one applies this crude rule, and divides the inoculum by 30. Then there would be 4,833 cells destined to attach and grow, or 27 cells/mm^2. In these terms it is possible that the 120 hour controls, with a mean of 239 cells/mm^2, show quite good growth, comparable to that of the normal growth rate calculated by Osgood and Brooke (42).

In any event, there is a striking difference in the controls and those vials having 36 ug./ml. of 8-MOP. In the latter there was not only no growth, but at the end of five days there were no viable cells.

The differences in some of the other concentrations as compared to the controls is much less, and at this time it was felt that they were not greater than chance differences.

The graphs of data from experiments 1,3,4, and 5 are shown in Fig. 4. Tabulations of the data are shown in Table 1. In all of these runs, the sampling was done on day 5, at 120 hours; in experiment 5 samples were also taken at other times. On Fig. 4 the plot is Log Number of cells/mm^2 on the ordinate and concentration of 8-MOP on the abscissa. The origin of the abscissa represents zero concentration of 8-MOP, or the controls. The inoculum is the same for all slide caps for a given experiment.

It will be noted that in spite of differences in the size of the inoculum, as well as other differences in procedure to be noted below, the curves are remarkably similar, especially those for

experiments 1 and 4.

Experiment 2

All slide caps in this experiment were contaminated after 24 hours, the contaminant probably being introduced in the medium or cell suspension. Even after a great deal of experience, setting up an experiment takes from 6 to 10 hours and may cost from $20 to $40. This is probably the most annoying way to produce large cultures of bacteria.

Experiment 3

Cells from a bulk culture of J 96 which had had a medium change three days earlier were removed and suspended as in experiment 1. Direct counts were made after diluting to a volume of about 40 ml. This was computed to have a count of about 350 cells/mm^3 and was accordingly further diluted, almost 50 per cent. Direct counts then gave 155 cells/mm^3.

Slide caps were prepared and dilutions of the medium containing 8-MOP were made as previously. One ml. of the cell suspension was added to each vial and the vials were equilibrated, sealed and incubated as before.

At this time, however, a portion of the cell suspension that had just been used was taken for nuclei counts. To a one ml. sample of cell suspension one ml. of 1% citric acid solution was added in a micro test tube, the top covered with Parafilm and the contents vigorously shaken. Then 1 ml. of citric acid-cresyl violet was added and the contents shaken again.

On 8 fields in the hemacytometer 64 nuclei were counted, or

8.5 per field, or $85/mm^3$. The dilution was 1:3, so the final count on nuclei was $255/mm^3$.

This is quite different from the direct cell count on the cell suspension. In general, nuclei counts are more accurate for the physical reason that a suspension of nuclei flows under the cover slip of the hemacytometer better, and gives a better distribution. On the other hand, these are abnormal, heteroploid cells, often multinucleate.

As a matter of side interest, if these counts were entirely reliable, they would afford a means of computing the average number of nuclei per cell. Above, this would clearly be 1.6 nuclei per cell. Obviously this distribution could be such that for each cell with only one nucleus there is another with two, or that for each three cells with one nucleus there is another with three, or for each five cells with one there is another with four, etc.

Since there is evidence (given above) that direct counts actually give a figure greater than the probable true viable inoculum, and since nuclei counts give even a higher figure, it is really probable that heteroploidy exists to a marked degree. Studies in the morphology of these and closely related cell populations, such as Oregon J 111 (Osgood, 39), have been and are being carried on. (Goldstein, 16, Hsu, 24).

Table 2 shows the results of this experiment. It was quite disappointing as compared to the first experiment. The differences are such as could be ascribed to chance. Several points are actually higher than the controls, one of which, at 3.6×10^{-3} µg./ml. 8-MOP, was as much higher than the control as the highest concentration,

3.6×10^1 µg./ml., was lower.

In the light of these findings, the following experiment was changed in the way the cells were suspended, in the size of the inoculum, and in the counting method.

Experiment 4

The preparation of the cell suspension was rather more elaborate, beginning on 7-12-57 with the washing and EDTA extracting of a culture of J 96. Five ml. of this suspension was added to a new culture bottle containing 15 ml. of medium. This was then incubated until 7-15-57 and used as a source of cells for this experiment.

The object in the above manipulations lies in observations of the subcultures. Cells treated in this fashion attach to the fresh bottle, spread out and appear to be in active growth and division for the next several days. After a while, however, cells begin to pile up several cell layers deep, and probably as a result of this, many become necrotic and begin to disintegrate. Making a subculture three days prior to use is probably superior merely to giving a medium change at that time; a medium change is stimulating, but the subculturing technique reduces the population to a monolayer, as well as providing new medium.

The bottle thus selected was removed from the incubator, the medium poured off, and 30 ml. of Ca-free balanced salt solution was added and the cells were gently rinsed with this. Then this was poured off (taking any loose cells or cellular debris) and another 30 ml. of the Ca-free balanced salt was added.

Two ml. of 1.5% EDTA solution was added (100 mg.%) to the 30 ml. volume, the solution was made alkaline to phenol red (1%) with

a few drops of 5% Na_2CO_3, and the cells came off in a few minutes.

This material was thoroughly shaken and a sample removed for counts. The direct count gave 175 cells/mm^3.

It was desired, however, that this experiment should begin with a very low count, reasoning that in previous experiments the count had been too high initially, so that the results were probably confused with the secondary effects of crowding, difficulty of accurate counting, and possible deterioration of some of the cultures.

In this run, it was decided to use 10 cells/mm^3, arrived at as follows: growth over the test period will probably not exceed 10 times the original number. Approximately 20 cells per Miller disc, under oil immersion, are as many as can be counted accurately. Therefore, it would be convenient to start with 2 cells per Miller disc. This would be roughly 200 cells/mm^2 on the slide. There are just over 175 mm^2 available for the total population of cells (as a "landing field"), therefore we should have about 35,000 cells in each shell vial. This must be added in a volume of 1 ml., hence the inoculum must have 35,000 cells/ml., or 35 cells/mm^3. When this is added to the 2.5 ml. of medium already in the shell vial, the volume will be 3.5 ml. and the final count will be 10 cells/mm^3.

Therefore 8 ml. of the cell suspension with a count of 175 cells per mm^3 was diluted to 40 ml. with fresh medium. This gives 1,400,000 cells/40 ml., or 10 cells/mm^3.

The results of this experiment are shown in Table 3 and Fig. 4. The results closely parallel those of experiment 1. The concentration of 36 μg./ml. of 8-MOP has apparently completely blocked the growth, or division, of these cells.

The question of toxicity of the 8-MOP in this concentration may be raised, but it cannot be effectively treated at this time. If it is toxic, one can only conclude that it is toxic to a very low degree. Toxicity studies in living organisms have centered around the interest in the photodynamic effects of this compound (Fowlks,15; Patek, 45) etc., and hence are not comparable to this type of experimental design.

Nevertheless, the mere cessation of division would not account for the reduction in cell numbers at the end of five days. But Osgood (40,43,44) has adduced evidence that the life span of cells of the monocytic series may be around 108 hours. Even if this is subject to errors of the magnitude of 50%, one might expect that, at the end of 120 hours in the absence of division, these cells, failing to divide, have reached the end of their life span and died.

Experiment 5

This was the final and most elaborate of this series of tests, and the design of the experiment was modified from the previous ones.

The 8-MOP was weighed out and diluted in balanced salt solution to the various dilutions needed, each of these being distributed to separate bottles. These were vaccine capped, evacuated, autoclaved, cooled, and to each the nutrients of Eagle's medium added.

Cells from rapidly growing J 96 were given EDTA, suspended, counted and diluted to give 35,000 cells per ml., just as in experiment 4.

Each of the six dilutions used (3.6×10^{1}, $\times 10^{0}$, and $\times 10^{-1}$ and 1.8×10^{1}, $\times 10^{0}$, and $\times 10^{-1}$ µg./ml.) plus the controls were in separate bottles. The dilutions were volumetric, and the cell sus-

pensions were such that 10 ml. could be added with a 10 ml. syringe to each bottle. Under these circumstances, the volume of cell suspension is probably not greatly different from bottle to bottle. Further, the replicates for each dilution are quite comparable without the usual pipetting errors made when the dilutions were made in the slide caps and the cells added later.

In this experiment each dilution and the controls were set up in slide caps to be sampled on each of four days, each in duplicate. The sampling times were at 72, 120, 144, and 168 hours (3,5,6 and 7 days). During the course of the run, however, a few slide caps leaked and a few showed gross contamination, resulting in a reduction of numbers such that on the 7th day there were not enough duplicates left for several of the concentrations. In these cases, only one slide cap was counted.

The results are given in Tables 4 and 4A and Fig. 4. The inclusion of the results along with other experiments in Fig. 4 has been done by using only the data at 120 hours.

Table 1. Mean Population Densities of Oregon J 96 Cells Grown 120 Hours in the Presence of Various Concentrations of 8-Methoxypsoralen.

Experiment 1

Concentration of 8-MOP* (µg./ml.)	No. of fields counted	Micro-scope Objective	No. of cells counted	Area counted (mm^2)	Cells/ mm^2	Mean
Controls (no 8-MOP)						
1 (contaminated)	-	-	-	-	-	-
2	25	20x	1007			
3	25	20x	1147			
4	25	20x	1297			
5	25	20x	1188			
				19.36	239	239
3.6×10^{-4}						
1	25	20x	953	4.84	197	
2	25	20x	850	4.84	176	
						187
3.6×10^{-3}						
1 (contaminated)	-	-	-	-	-	
2	25	20x	1135	4.84	234	
						234
3.6×10^{-2}						
1	25	20x	813	4.84	168	
2	25	20x	878	4.84	181	
						175
3.6×10^{-1}						
1	25	20x	857	4.84	181	
2	25	20x	1214	4.84	251	
						214
3.6×10^{0}						
1	25	20x	545	4.84	113	
2	25	20x	808	4.84	167	
						140
3.6×10^{1}						
1	25	20x	none	4.84	0	
2	25	20x	none	4.84	0	
						0

* 8-MOP is 8-methoxypsoralen

Table 2. Mean Population Densities of Oregon J 96 Cells Grown 120 Hours in the Presence of Various Concentrations of 8-Methoxypsoralen.

Experiment 3

Concentration of 8-MOP (μg./ml.)	No. of fields counted	Micro-scope objective	No. of cells counted	Area counted (mm^2)	Cells/ mm^2	Mean
Controls (no 8-MOP)						
1	50	oil	649	0.48	1415	
2	50	oil	748	0.48	1631	1526
1.8×10^{-4}	50	oil	970	0.48	2115	2115
3.6×10^{-4}	50	oil	826	0.48	1800	1800
1.8×10^{-3}						
1	25	20x	834	4.84	1551	
2	25	20x	600	4.84	1116	1333
3.6×10^{-3}						
1	25	20x	944	4.84	1756	
2	25	20x	1008	4.84	1875	1817
1.8×10^{-2}						
1	25	20x	858	4.84	1596	
2	25	20x	676	4.84	1258	1427
1.8×10^{-1}						
1	25	20x	815	4.84	1516	
2	25	20x	479	4.84	891	1203
3.6×10^{-1}						
1	25	20x	921	4.84	1713	
2	25	20x	824	4.84	1533	1623
1.8×10^{0}						
1	25	20x	821	4.84	1527	
2	25	20x	613	4.84	1140	1333
3.6×10^{0}						
1	25	20x	992	4.84	1845	
2	25	20x	765	4.84	1423	1634
1.8×10^{1}						
1	25	20x	688	4.84	1280	
2	25	20x	527	4.84	980	1130
3.6×10^{1}						
1	25	20x	626	4.84	1164	
2	25	20x	633	4.84	1177	1170

Table 3. Mean Population Densities of Oregon J 96 Cells Grown 120 Hours in the Presence of Various Concentrations of 8-Methoxypsoralen.

Experiment 4

Concentration of 8-MOP (µg./ ml.)	No. of fields counted	Micro-scope objective	No. of cells counted	Area counted (mm^2)	Cells/ mm^2	Mean
Controls (no 8-MOP)						
1	100	oil	749	0.92	816	
2	100	oil	666	0.92	726	
3	100	oil	702	0.92	765	
						769
3.6×10^{-4}						
1	100	oil	817	0.92	891	
2	100	oil	659	0.92	718	
						804
3.6×10^{-3}						
1	100	oil	558	0.92	608	
2	100	oil	618	0.92	674	
3	100	oil	627	0.92	683	
						655
3.6×10^{-2}						
1	100	oil	623	0.92	679	
2	100	oil	844	0.92	920	
3	100	oil	689	0.92	751	
						783
3.6×10^{-1}						
1	100	oil	809	0.92	882	
2	100	oil	291	0.92	317	
3	100	oil	52	0.92	57	
						418
3.6×10^{0}						
1 (contaminated)	-	-	-	-	-	
2	100	oil	32	0.92	35	
3	100	oil	25	0.92	27	
						31
3.6×10^{1}						
1	100	oil	0	0.92	0	
2	100	oil	0	0.92	0	
3	100	oil	0	0.92	0	
						0

Table 4. Mean Population Densities of Oregon J 96 Cells Grown for Various Times in Various Concentrations of 8-Methoxypsoralen.

Experiment 5

Concentration of 8-MOP (µg./ ml.)	No. of fields counted	Micro-scope objective	No. of cells counted	Area counted (mm^2)	Cells/ mm^2	Mean
TIME: 72 hours.						
Controls (no 8-MOP)						
1	100	oil	257	0.92	280	
2	100	oil	284	0.92	310	
						295
1.8×10^{-1}						
1	100	oil	211	0.92	230	
2	100	oil	270	0.92	294	
						262
3.6×10^{-1}						
1	100	oil	218	0.92	238	
2	100	oil	270	0.92	294	
						266
1.8×10^{0}						
1	100	oil	338	0.92	368	
2	100	oil	308	0.92	336	
						352
3.6×10^{0}						
1	100	oil	225	0.92	245	
2	100	oil	222	0.92	242	
						244
1.8×10^{1}						
1	100	oil	165	0.92	180	
2	100	oil	169	0.92	184	
						182
3.6×10^{1}						
1	100	oil	87	0.92	95	
2	100	oil	83	0.92	90	
						93
TIME: 120 hours.						
Controls						
1	100	oil	547	0.92	596	
2	100	oil	580	0.92	633	
						615
1.8×10^{-1}						
1	100	oil	465	0.92	507	
2	100	oil	446	0.92	486	
						497

Table 4 (cont.) Mean Population Densities of Oregon J 96 Cells Grown for Various Times in Various Concentrations of 8-Methoxypsoralen.

Concentration of 8-MOP (µg./ ml.)	No. of fields counted	Microscope Objective	No. of cells counted	Area counted (mm^2)	Cells/ mm^2	Mean
3.6×10^{-1}						
1	100	oil	473	0.92	515	
2	100	oil	439	0.92	479	
						497
1.8×10^{0}						
1	100	oil	589	0.92	642	
2	100	oil	492	0.92	536	
						589
3.6×10^{0}						
1	100	oil	483	0.92	526	
2	100	oil	467	0.92	509	
						518
1.8×10^{1}						
1	100	oil	202	0.92	220	
2	100	oil	205	0.92	224	
						222
3.6×10^{1}						
1	100	oil	74	0.92	81	
2	100	oil	52	0.92	57	
						69
TIME: 144 hours						
Controls (no 8-MOP)						
1	100	oil	456	0.92	497	
2	100	oil	464	0.92	507	
						502
1.8×10^{-1}						
1	100	oil	438	0.92	477	
2	100	oil	449	0.92	489	
						483
3.6×10^{-1}						
1	100	oil	383	0.92	417	
2	100	oil	395	0.92	431	
						424
1.8×10^{0}						
1	100	oil	391	0.92	426	
2	100	oil	398	0.92	434	
						430
3.6×10^{0}						
1	100	oil	323	0.92	352	
2	100	oil	267	0.92	291	
						322

Table 4 (cont.) Mean Population Densities of Oregon J 96 Cells Grown for Various Times in Various Concentrations of 8-Methoxypsoralen.

Concentration of 8-MOP (µg./ml.)	No. of fields counted	Micro-scope objective	No. of cells counted	Area counted (mm^2)	Cells/ mm^2	Mean
1.8×10^1						
1	100	oil	60	0.92	65	
2	100	oil	73	0.92	80	
						73
3.6×10^1						
1	100	oil	28	0.92	31	
2	100	oil	27	0.92	29	
						30

TIME: 168 hours.

Concentration of 8-MOP (µg./ml.)	No. of fields counted	Micro-scope objective	No. of cells counted	Area counted (mm^2)	Cells/ mm^2	Mean
Controls (no 8-MOP)						
1	100	oil	490	0.92	534	
						534
1.8×10^{-1}						
1	100	oil	344	0.92	375	
						375
3.6×10^{-1}						
1	100	oil	402	0.92	438	
						438
1.8×10^0						
1	100	oil	447	0.92	487	
						487
3.6×10^0						
1	100	oil	409	0.92	446	
2	100	oil	329	0.92	359	
						403
1.8×10^1						
1	100	oil	128	0.92	140	
						140
3.6×10^1						
1	100	oil	30	0.92	33	
						33

Table 4A. Resume of Table 4. Chart to Show Means Only of Cell Populations against Sampling Times

Concentration of 8-methoxypsoralen (µg. / ml.)	Times sampled, hours 72	120	144	168
Controls	295	615	502	534
1.8×10^{-1}	262	497	483	375
3.6×10^{-1}	266	497	424	438
1.8×10^{0}	337	589	430	487
3.6×10^{0}	244	518	322	403
1.8×10^{1}	182	222	73	140
3.6×10^{1}	93	69	30	33

PART II

Protozoan Cells

A. Description of Protozoan Cells.

Tetrahymena sp., a small ciliate protozoan, was first isolated in pure culture by Lwoff (31) at the Pasteur Institute in 1929. The organism is ubiquitous in nature and has subsequently been isolated by different workers all over the world. Dr. Austin Phelps, at the University of Texas, has started cultures of *Tetrahymena* from samples of water taken from icy mountain streams as well as hot springs whose temperature was as high as 40° C.

From the outset it became clear that this organism afforded a special means of studying a variety of problems. Its organization and metabolism proved to be of a complexity on the order of that of a mammalian cell, but it provided the technical approach that had, until its isolation, been more generally reserved for bacteriological problems.

The nutritional requirements for *Tetrahymena* were worked out primarily in the laboratories of Kidder and Dewey (28, 29) over a period of about 10 years, with the result that it is possible to design for this organism a purely synthetic and completely defined diet.

This opens the possibility of making biochemical studies with an accuracy and definition not possible with any other organism of this complexity.

On the other hand, it is possible to grow *Tetrahymena* on an extremely simple medium from the standpoint of maintenance. They

grow well on 0.5 or 1% proteose-peptone (Difco, item 0121) or 1% yeast extract (Difco, item 0127). The latter was used in the following experiments.

The degree of predictability and repeatability of experiments with <u>Tetrahymena</u> depends on a variety of factors. The growth characteristics vary with species, nutrients, temperature, light, pH, etc. (Phelps, 46, 47).

Changes in one of these factors may clearly affect the population in such a way as to change other factors. For example, Browning (6) showed that the time per generation was at a minimum when cultures were stirred with 20% O_2. Of course in nature, in the shallow pools where they often thrive, their environment is in equilibrium with atmospheric O_2 which is just about 20%.

By sampling various depths of a still pond, one will find the bulk of the population at the surface—which, of course represents the highest O_2 gradient. And in the laboratory, in test tubes, one will find that in a few hours the bulk of the population is in the top few centimeters.

This provides a rational explanation for the fact that cultures bubbled with sterile air present shorter division times, and reach a higher maximum phase, than those bubbled, e.g., with 50% O_2.

From another standpoint, however, the bubbling of flasks introduces a number of problems. One must guard more carefully against contamination, care must be taken against evaporation, bubbling systems are liable to clogging, etc.

In general, for each step taken to yield maximum growth rates, maximum populations, etc., there is a price to be paid in complexity

of apparatus, medium expense, time, etc. The decision has to be made as to whether the particular experiment must be made in terms of maximum growth, or whether it is equally satisfactory to measure in terms of standard growth.

In the following experiments using five strains represented by two species the decision was to rely on a large volume of studies under a standard set of conditions, i.e., to standardize growth conditions.

In the last 15 years a tremendous wealth of literature has appeared on and about *Tetrahymena* in much of which, as here, the interest has been primarily focused on some problem of biochemistry, genetics, growth, antibiotics, virus culture, etc., rather than on the organism itself. It would not be pertinent to review this literature here; a glance into a recent issue of *The Journal of Protozoology*, for example, will lead into a vast number of references in each particular field of interest.

For the present study cultures were obtained from Dr. Kozloff of Lewis and Clark College, Portland, Oregon. They consisted of *T. pyriformis E.*, *T. pyriformis WH 14*, *T. pyriformis WH 6*, *T. vorax* V_1, and *T. vorax PP*.

B. The culture method.

The method was as simple as possible. Cultures were made in 250 ml. Erlenmeyer flasks, cotton stoppered. Test tubes were used for the experimental work, for the most part, although in one experiment, to be described, small flasks were used. Medium was autoclaved in the flasks or tubes and subcultures were made with sterile cotton

plugged serological pipettes. The test tubes were placed in racks and incubated at 27° C.

Tubes were generally set up in duplicate for each concentration of 8-MOP tested and for each time point.

Stock cultures were maintained at room temperature.

C. Preparation of the culture media.

The medium employed was also as simple as could be found. It consisted of 1% yeast extract made up in ordinary tap water.

In order to prepare medium containing 8-MOP, however, the decision was made not to autoclave 8-MOP with the yeast extract. Therefore, a flask containing 50 µg./ml. 8-MOP solution was mixed in equal volumes with 2% yeast extract, yielding 25 µg./ml. 8-MOP in 1% yeast extract. Alternatively, a flask containing 10% yeast extract and another flask containing 56 µg./ml. of 8-MOP in water were prepared, each being autoclaved separately and mixed when cool in the proper proportion to give a final concentration of 50 µg./ml 8-MOP and 1% yeast extract.

Serial dilutions were made of this medium with ordinary medium several days before an experiment, the tubes being held at room temperature, in the dark, during this time. By this means, it was possible to catch certain contaminants which might have grown up during this period, it being difficult in making a large number of such dilutions to avoid some air contaminants.

Precautions were taken to protect solutions of 8-MOP from the light during the preparation of medium and after the tests had been prepared.

D. Sampling techniques.

The sampling of a population of these protozoa growing under the conditions described must be rigidly controlled. As mentioned above, in the resting tube the bulk of the population tends to swim to the top layers of medium, probably seeking a higher oxygen tension. It is necessary to swirl such a tube several times in order to get a uniform distribution of cells.

It would be possible, and simpler, to remove the cotton plug and invert the contents of the tube, thus getting a well-mixed sample. It is often desirable, however, to keep the tube sterile for observations for some time after the sample is taken. Often, a contaminant which may have been slowly growing will not be apparent when the sample is taken, but shows up in the culture later. In this event, the counts on samples from this tube are considered invalid, since the presence of a bacterium or mold may have altered the experiment, even though it was there in a quantity too small to be detected at the time of sampling.

These protista, being ciliated, are highly motile. Subjectively, they seem fairly to zip across the microscopic field. Clearly they cannot be counted under these conditions, so they must be killed. In this study, a few drops of 10% formalin in which a small amount of methylene blue had been dissolved was used. The presence of the dye is certainly optional, but it seemed to increase the contrast and make the organisms easier to count.

Unfortunately, after they are killed, in 15 or 20 minutes they tend to stick together and form clumps. These clumps are impossible to break up without also destroying some individual organisms. For

this reason, counts were made at a uniform time after killing.

The question of resampling, or repetitive sampling, arises. It would seem for some purposes better to grow a culture in a flask which could be sampled over a course of time. The flaw in this lies in the sampling method. When the cultures are stirred prior to sampling, this must violently change the kind of "structuring" of the growth flask. Cells which are dead fall to the bottom of the flask, while living cells swim to the top. When this is stirred up, the material undergoing autolysis in the bottom is brought to the top where it may compete with the living organisms for oxygen. In any case, it is demonstrable that the count will drop dramatically when a culture is resampled a few hours after it has been sampled first, whereas a duplicate culture that has been growing under the same conditions, but had not been sampled, will show continued growth.

Counts were made in an ordinary hemacytometer. The number of cells counted depended on the information desired from the count. In most cases interest was directed to a difference in densities of the population at a particular time. In order for the difference in two densities to be significant at a 0.05 level of confidence, the following formulae, kindly supplied by Dr. Carl Hopkins, of this institution, were used:

$$\frac{\frac{N_1}{V_1} - \frac{N_2}{V_2}}{\sqrt{\frac{N_1}{V_1} + \frac{N_2}{V_2}}} > 2$$

, where N= number of cells and V= volume) or, when counting the same volume, this

reduces to,

$$\frac{C_1 - C_2}{C_1 + C_2} \quad 2 \quad (C_1 \quad C_2)$$

, where c = count.

E. Specific Problems

Initially pilot studies were run comparing the growth of <u>T. pyriformis</u> <u>E</u> and <u>WH 14</u> and <u>T. vorax</u> <u>PP</u> and <u>V1</u> with and without 8-MOP. Within two days visual examination of the tubes revealed turbidities in the control group up to two or three times that of the group containing 8-MOP.

Although careful analysis done later does not support this view, at this time in the investigation it was believed that the J 96 cells had shown some ability to "detoxify" or in some fashion neutralize the effects of 8-MOP. With this belief in mind it was natural to try to find out if these strains of <u>Tetrahymena</u> had the ability to remove the 8-MOP from the medium.

One of the sub-problems then was to test the possibility that over a period of time actively growing cells would metabolize or neutralize the 8-MOP present.

To this end paper chromatograms were made on a series of tests: one group of tubes contained protozoa and 1% yeast extract; one group contained protozoa, 1% yeast extract and 8-MOP; one group contained no protozoa, but 1% yeast extract and 8-MOP.

After incubation each of these was sampled over a period of several days and paper chromatograms were prepared from each sample.

The results were essentially negative.

In all cases the spot on the paper made by 8-MOP (which is fluorescent in U-V light) showed up equally well in both samples from protozoa-containing tubes and those tubes without protozoa.

Under the conditions of the experiment, we could detect no difference in the size or intensity of the spot to offer any support to the proposition that this material was being removed from the medium and altered in any way such as to change its characteristics on the chromatogram.

One of those mysteries which beleaguer and bewilder every investigator occurred at this point. A test was prepared with T. vorax PP testing the afore mentioned ideas. It was negative as usual with respect to showing any diminution of 8-MOP, but a curious thing appeared: a spot with Rf value of 0.22 appeared on the chromatogram of a sample from protozoa in normal media, whereas no such spot showed on the sample from 8-MOP media. Repeated sampling showed the same results. It seemed that cells growing normally were producing some material which was being blocked in 8-MOP.

Samples were taken from a second and third set on the 3rd and 5th day with essentially the same results. But then on the 16th day the fourth and final set was sampled. These showed no such spot.

Since each sample was taken from a separate set of replicate tubes contamination of the previous samples was suspected. Therefore the stock cultures of the protozoa were put through a special apparatus, described below under Experiment 2, designed to remove any bacteria which might be present.

Using these "washed" protozoa the experiment was run again. At this time no spots were seen at this Rf, seeming to point to a contam-

inant in the first experiment.

The mystery is, however, what material did appear at that spot? It was negative to phosphomolybdic acid, ninhydrin, and diazatized sulfonilic acid. It was fluorescent in U-V light. With the small quantity of material it was possible to run only one sample on the spectrophotometer: it appeared to have a slight peak at 260 mu. Since subsequent runs never produced that spot, it was impossible to run further tests. On reflection, however, it would seem that if there were bacteria present in these "false" tests, and if these were responsible for this spot, it probably should have given a positive ninhydrin test.

RESULTS AND DISCUSSION

Following the organization pattern of Part I, the various experiments conducted using species of Tetrahymena will be presented below, one by one. For some purposes data will be grouped from several experiments and presented graphically. In addition, data and discussion of individual experiments will be given.

The first four experiments, using three strains or species, were for the purpose of demonstrating and measuring the simple effect of growing cells with and without 8-MOP. To this end high concentrations of 8-MOP were employed.

The next four experiments, using all strains and species, were to attempt to determine the effect on growth of various concentrations of 8-MOP. For this purpose the following concentrations were always used, stated in µg./ ml.: 50, 25, 15, 5, 2.5, 1, and 0.5. Control flasks contained simply 1% yeast extract medium.

The final experiment was conducted to determine if after being cultured in 8-MOP for various lengths of time cell populations tended to exhibit any change in growth patterns upon being transferred back to normal medium.

Experiment 1

Tubes were prepared with 9 ml. of medium in two groups. The first group contained 9 ml. of 1% yeast extract medium. The second group contained 4 ml. of 2% yeast extract medium to which 5 ml. of a solution of 50 µg./ ml. 8-MOP was added after autoclaving each of these solutions separately.

Cultures of T. vorax PP grown in 1% yeast extract at room temperature were used. Direct counts on the stock solution gave 16,350 cells/ ml. This stock was diluted with fresh medium to give suspensions of about 1600 cells/ ml. One ml. of this suspension was added to each tube, hence the initial inoculum in each test tube was 160 cells/ ml.

All tubes were placed in the incubator at a temperature of 26-27° C.

Samples were taken for direct counts at 24, 72, 120, and 360 hours. Results are shown in Table 5.

As described under Section E., above, when samples were taken paper chromatograms were prepared from an aliquot of this material, the study of which did not, however, contribute to our understanding of the mode of action of the 8-MOP.

The differences in growth characteristics between the test group and the controls are significant. The control achieved a higher density and a greater maximum than the group with 25 µg./ ml. 8-MOP. A study of

of the first 24 hours reveals that the number of times the population doubled, N, which is given by the equation,

$$N = \frac{\log f - \log a}{\log 2}$$

(where log f = last reading, log a = first reading), is 4.8 for the test group and 6.2 for the control.

The mean time per generation, T/G, can be computed by dividing N into the time interval to be tested. Over the first 24 hours, the mean T/G for the test group is 5 hours, but for the control only 3.9 hours.

On the other hand, this does not represent a particularly high growth rate, even for the controls; hence not too much reliance should be placed on these data alone. From later experiments, for example, it will be seen that, in general, populations reach their maximum density in about 48 hours. That this group did not, but was still increasing slightly at 16 days, casts that much doubt on the validity of the results.

Experiment 2

As described in Section E., above, paper chromatograms of material taken from Experiment 1 showed, irregularly, spots which might be interpreted to be due to some contaminant which could not be readily identified. In addition to this, as also described, the growth rate of the control groups in this experiment did not seem to be very high.

The stock cultures of T. vorax PP were then held to be suspect, and steps were taken to insure the establishment of a pure culture.

An apparatus which appears to be something of a Rube Goldberg invention has been designed which in practice works quite well. It consists of a flask equipped with a large cotton plug through which a

glass tube ent@Fs, extending to the bottom of the flask. The glass tube has many spirals and angles; its other end terminates in an ordinary thistle tube. The entire apparatus is filled with culture medium and the level of liquid is adjusted by raising or lowering the height of the thistle tube on a ring stand. Then the apparatus is autoclaved.

Protozoa suspected of being contaminated with bacteria, mold, yeast or other protozoa are introduced into the thistle tube. They begin growing and migrating toward fresher medium. Eventually they either grow and/or swim into the flask which is carefully observed each day. When protozoa are first noted in the flask the tube is removed and the few protozoa which came over into the flask are allowed to grow up to provide new stocks.

The principle of the apparatus depends on these factors: 1) the protozoa are "driven" by the gradient of fresh medium as they begin to exhaust the medium where they are introduced; 2) bacteria usually exhibit either a positive or negative geotropism, thus tending to remain at one or another of the many angles or bends; 3) protozoa have immensely greater swimming speeds than bacteria; 4) non-motile organisms are quickly left behind; and 5) in the case of other protozoa, none other than _Tetrahymena_ have been found to have the ability to live in pure culture in the absence of bacteria, etc., or even to live upon _Tetrahymena_ alone.

Thus, for one of various reasons, if the apparatus is disconnected when the protozoa first appear in the collecting flask, success is practically 100%.

This apparatus was used to "decontaminate" the stocks of _T. vorax PP_.

Subcultures were prepared and when adequate stocks were established all old stocks were discarded.

The present experiment was prepared just as in Experiment 1, using cells growing in 1% yeast extract medium for the controls and cells growing in 1% yeast extract medium which contained a final concentration of 25 µg./ml. 8-MOP as the test.

The cell suspension used had 3750 cells/ ml. One ml of this was added to 9 ml. in the tubes, hence the initial inoculum was 375 cells/ ml. Samples were taken for counts at 24, 72, 120, and 360 hours.

The results, shown in Table 5, are quite comparable to the first experiment. N values for the first 24 hours are, for the test group 1.74, and for the controls, 6.55. T/G for the test group for this period is 15.5 hours, but for the control, only 3.64 hours. Again the control group reached a much greater maximum phase than the test group, and this maximum was higher than in the first experiment.

Later it will be shown, however, that these data are not sufficient to characterize well the growth of a population, and thus this same data will be dealt with in a different manner at the end of this section.

After 48 hours in the 8-MOP medium a sample was taken for inoculation into normal medium to see if the cells would continue to show the effects of 8-MOP. A sample of 1 ml. was taken having a count of 3750 cells/ml. and put into fresh tubes of 9 ml. of 1% yeast extract medium. As it happens, this gives a starting inoculum identical with that of the beginning of the experiment, 375 cells/ ml. Samples were then taken and counts made at 24 and 72 hours. This culture still contained, of course, 2.5 µg./ ml. of 8-MOP.

The results of this are shown in Table 6. The growth rate occupies

a position intermediate between that shown in the 25 µg./ ml. and that of the control, although it is closer to the control. For the first 24 hours, N = 4.55 and T/G = 5.3 hours.

It cannot really be determined from this data whether the culture in the presence of 8-MOP had damaged these cells in some way, resulting in a somewhat reduced growth rate, or whether the presence of 2.5 µg./ ml. 8-MOP was still sufficient to inhibit, slightly, their growth rate in the new medium.

Experiment 3

Subcultures of T. pyriformis E. taken from stocks which had been isolated from the decontamination apparatus were made one day prior to the beginning of this experiment. Samples were taken from a flask of rapidly growing cells and direct counts were made.

The tubes were prepared as previously described, such that the test group had 25 µg./ ml. 8-MOP in 1% yeast extract medium and the controls were grown in 1% yeast extract medium alone.

Each tube was inoculated with enough cells to give 2500/ ml. as a final suspension. Samples were taken and counts made at 24, 48, 72, 120, 144, 168, 192, and 216 hours.

The results, shown in Table 5 show some differences from the first two experiments, although the interpretation is the same. That is, the absolute values are somewhat different, but there are the same significant differences between the test and control groups.

N values are, for the control, 4.76; for the 8-MOP series, 2.17, during the first 24 hours. Mean T/G's are, respectively, 5.04 hours and 11.05 hours, for this period.

After 72 hours in the 8-MOP medium a sample from this series was taken for inoculation into normal medium, as was done in Exp. 2. Samples of 1 ml., having a count of 35,000 cells/ml. were put into tubes containing 9 ml. of normal medium, giving an inoculum of 3500 cells/ ml. as an initial suspension. Samples were taken and counts made at 48, 72, 96, and 144 hours.

Results are shown in Table 6. Since a sample was not taken at 24 hours it is only possible to compute N for the first 48 hours. This gives 5.9. The mean T/G for the whole 48 hours is 8.1 hours. It is clear that for the first 24 hours these values would be close to those of the control group.

Experiment 4

This experiment was similar to the above, except that T. pyriformis WH 14 was used. It was run concurrently with Experiment 3.

The inoculum used gave a final cell suspension in the tubes of 12,500 cells/ ml. Samples were taken and counts made at 24, 48, 72, 120, 144, 168, 192, and 216 hours.

The results are shown in Table 5. Some strain differences in growth characteristics are exhibited here. WH 14 seems to have a somewhat slower growth rate, but shows the ability to reach higher maximum populations.

For the first 24 hours N values are, for the control, 3.12, and for the test, 0.38. Mean T/G for this period are, respectively, 7.45 hours and 63 hours.

These figures are considerably different from those of Experiment 3. Nevertheless, it is of more importance that they are considerably

different from each other.

After 72 hours in the 8-MOP medium a sample from this series also was taken for inoculation into normal medium. Each sample of 1 ml., having a count of 30,000 cells/ml. was transferred to tubes containing 9 ml. of normal medium, giving an inoculum of 3000 cells/ ml. as an initial inoculum. Samples were taken and counts made at 48, 72, 96, and 144 hours.

Results are shown in Table 6. As above, samples were not taken at 24 hours, hence calculation for N and T/G were taken for the first 48 hour period. They are, respectively, 6.05 and 7.9 hours.

It may well be that this represents a significant difference between the growth in 2.5 µg./ml. 8-MOP following transfer from 25 µg./ml. 8-MOP as compared to those cells which grew only in normal medium. Although these data will be further analyzed, below, it is felt that no definite statement can be made regarding this point. That is, from this evidence, we cannot rule out the possibility that either the initial growth in inhibiting concentrations of 8-MOP, *per se*, or that some selective process operating, or some combination of these with other factors, may actually provide some stimulatory effect on subsequent growth. On the other hand, this one sub-experiment, without adequate controls, can scarcely be considered as very strong evidence for any such stimulatory effect, and it will be shown that there is more evidence to contradict than to support the hypothesis of stimulation following inhibition.

Experiment 5

Having determined the inhibitory effect of a 25 µg./ml. solution

of 8-MOP, it was next decided to test the effect of various concentrations.

The main value of Experiment 5, however, must lie in the realm of experience and working out of techniques, as the results were quite incomprehensible. Only two samples were taken, after which the experiment was abandoned when it became clear that the figures appeared to have been taken from a table of random digits. It was not even possible to prove contamination, which might otherwise well have explained the results.

The method of preparing a series of dilutions was as follows: A solution of 56 µg./ml. of 8-MOP was prepared, which is close to the limit of solubility of this substance in water. Then, a flask of 10% yeast extract was prepared. The addition of 9 ml. of the 8-MOP solution to 1 ml. of 10% yeast extract gives a solution which contains about 50 µg./ml. of 8-MOP and 1% yeast extract solution. These were prepared and autoclaved separately, in the dark, and mixed aseptically after cooling. Dilutions of this solution were made with 1% yeast extract by volumetric pipette into flasks. Then the tests were prepared by pipetting 9 ml. of each of these solutions into a series of tubes to which could be added 1 ml. of cell suspension.

Due to the fact that contamination was suspected in this experiment, in subsequent efforts these tubes would be placed in the dark several days to allow possible contaminants to grow up before the tubes were inoculated.

Another error in designing experiment 5 was in the simultaneous use of all five strains of _Tetrahymena_. In practice this becomes too

unwieldy since one is dealing with 8 different concentrations times 5 different strains times duplicate samples for each sample period; even without recounts on a particular sample, this means 80 operations for each time period. Each operation consists of taking a sample from the tube and transferring it to a killing flask, killing, taking a sample from this suspension and getting it to flow well under the cover-glass of the counting chamber, counting, recording the count, cleaning and drying the chamber and cleaning and drying the flask.

Even if each count is reduced to 3 minutes, it still takes on the order of 4 hours to make such a total count with the problems that 1) a perhaps not insignificant time period has elapsed between the beginning and end of the operation, and 2) the operator becomes fatigued to the point of making it very easy to make errors.

Experiment 6

With the lessons of the last experiment firmly in mind, this experiment was designed to study the effect of varying concentrations on a single strain.

A culture of T. vorax PP taken from a "decontamination" flask on Aug. 5th was prepared and subcultured prior to setting up this experiment on Aug. 11th.

A series of 8-MOP solutions was prepared as described above.

Inoculations were made such that each tube contained 750 cells/ml. initially. Samples were taken and counts made at 24, 48, 96 and 120 hours.

The results are shown in Table 7A. It becomes clearer in

retrospect that it would have been better to have more points during the first 48 hours since maxima are achieved in about this time, particularly in the control groups and lower concentrations.

This data will be analyzed more extensively below.

Experiment 7

This is almost identical in design to the experiment above. Cells of strain T. pyriformis E. were employed rather than T. vorax PP. They were collected from a "decontamination" flask which had been prepared Sept. 5th and had been through two subcultures prior to the beginning of this experiment, Sept. 26th.

A series of 8-MOP solutions in yeast extract medium was prepared as usual.

Inoculations were made to give a cell suspension of 750/ml. final concentration. Samples were taken at 48, 96, 144, and 168 hours. As noted above, it would have been better to have earlier points.

The results are shown in Table 7B. They are rather poor, probably due to the fact that most of the later points are past the maximum stationary phase of growth and thus represent the wide fluctuations which may occur after this time. In the case of the tubes having a concentration of 1 µg./ml., however, there are probably some errors involved, either in counting or in the initial inoculum or in some other way, as this does not fit well in the general pattern.

As in experiment 6, this data will be analyzed more extensively below.

Experiment 8

Due in part to the somewhat erratic results of the previous experiment, it was decided to test a new technique for studying population growth. This technique was patterned after that which has been successful in measuring growth of some microorganisms, the densiometric method.

To this end a number of tubes were matched to give the same reading, with water, on the Beckman Model B Spectrophotometer.

The entire study of effect of concentration was run in two concurrent ways: by making a series of four tubes for each concentration of 8-MOP, and for the controls, and also water blanks. Cells (T. pyriformis E) were inoculated into these tubes to give an initial suspension of 1000 cells/ml. The second part consisted of a series of flasks containing the same concentrations of 8-MOP also with 1000 cells/ml.

Samples were taken for direct counts from the flasks at 24, 48, 72 and 96 hours. The tubes in quadruplicate for each concentration were read after shaking at time zero, 4, 8, 16, 24, 32, 40, 48, 64, 72, and 96 hours.

The averages of the four readings and the cells/ml. taken from direct count are shown in Table 8. It may be seen that though there is some increase in turbidity over the 96-hour period that most of the increase occurs between 40 and 48 hours. Since it will be seen more clearly later that some of the more interesting information concerns the period prior to this, one must conclude that this is not sufficiently sensitive for the purpose of measuring growth.

Experiment 9

It had appeared in Experiments 2, 3, and 4 that organisms that had first been cultured in the presence of 8-MOP and then transferred to a normal medium exhibited an increased growth rate over controls which had grown in normal medium alone for the same period of time. It was known, however, that merely transferring organisms to fresh medium had a stimulatory effect upon them, and these initial observations were not controlled.

In Experiment 9 a number of tubes of media were prepared, half of which had 50 µg./ml. 8-MOP. A flask of T. pyriformis E which had been subcultured 48 hours previously was selected for the inoculum. This inoculum was made with sufficient cells in 1 ml. to give a final concentration in the test tubes of about 1000 cells/ml.

At the end of 24, 48 and 96 hours, samples were taken from the top layers of unshaken tubes to get the largest number of cells and this sample was transferred to a tube of fresh medium. Counts were made on this material and appropriate amounts inoculated into flasks having a volume of 20 ml. of fresh medium.

This procedure enabled one to dilute the 8-MOP about 1:100, yet still obtain enough cells to make an inoculum. Also, by not stirring the tubes, and by taking the top layer, one selected the more vigorous swimming cells in this region so that the counts more truly represented the number of cells capable of continued growth and division.

Samples were then taken for direct counts an additional 24, 48 and 96 hours on cells growing in the flasks. These cells, then, either grew for a specified time in normal medium and then were transferred to fresh normal medium where they grew another specified

length of time, or else they grew for a specified time in an 8-MOP medium and then were transferred to normal medium where they grew another specified length of time.

Due to the fact that very few cells were to be found in the tubes containing 8-MOP at the end of the various periods, and to the fact that high dilution of the 8-MOP was necessary, a very small number of cells were in the inoculum to the test flasks. Since the tubes with normal medium always had numerous cells, and since the inocula were prepared in essentially the same manner, these inocula were larger. To make direct comparisons, however, it is only necessary to find the point at which the cells first grown in 8-MOP, the test group, reached the number equivalent to the original inoculum of cells first grown in normal medium, the control group, and compare the growth characteristics of each for a measured interval following this point.

Table 9 shows the number of cells counted at each test period. It will be seen that both sets attained a maximum density at about 48 hours. Those started from 1 cell per ml., however, had farther to go to attain this maximum. Calculations of N, or number of times the population has doubled, have been made for each 24-hour period. There are striking differences. The cells which had had prior treatment with 8-MOP have doubled better than 10 times in the first 24 hours while the control group doubled less than 7 times. In the next 24 hours, the test group still seems to double over 5 times, whereas the control group has doubled less than 2 times.

Mean times per generation for each 24-hour period were calculated by dividing doubling time into 24. Again it appears that the test

group had much shorter generation times.

It would have been pleasant to stop here and indulge in speculation about the nature of action of 8-MOP on inhibition of these cells. Hypotheses certainly might arise concerning the possibility that 8-MOP might inhibit some key process necessary for division, while allowing the accumulation of elements necessary for subsequent division when the "restraint" was removed. Or again, that such an experimental procedure had resulted in the selection of a more vigorous population, etc., etc.

However, another analysis remains to be done. Suppose we plot the densities of these populations, or rather the logarithm of such numbers, against time. Then suppose we locate the intercept on the curve of "test" groups at a value equal to that of the value of the inoculum of the "control" groups. An examination and comparison of the two sets of curves from this point to the point of maximum density should give a critical value: the slope of these curves. If the "test" group is growing at a higher rate, then this value should be significantly higher.

Such data are presented in Fig. 5. Attention is called to the following points concerning this figure: at a time not later than 48 hours all groups reached a maximum. Fluctuations after this time do not have significance. The interesting period, then, is in the first 48 hours. The "test" group reached 1000 cells/ml. (Log = 3.0) in about 20 hours. In the next 23.2 hours this curve intercepts the line (Log = 5.0) In the remaining 4.8 hours it attains the line (Log = 5.4) which is the maximum. But the "control" group took 24 hours to go from (3.0) to (5.0). And if one makes the not unreasonable

assumption that this group also increased from here to (5.4) in about 4.8 hours (the hypothetical extension of this curve), then the groups are as nearly identical in growth characteristics as experimental variation would permit!

Such an analysis gives, geometrically, an almost perfect parallelogram with both slides having the same slope, indicating that in this experiment there is evidence that the growth rate of the two groups is almost identical.

It was thought instructive, though obviously not conclusive, to plot similar data from Experiments 2, 3 and 4. Since these were originally done in a rather casual fashion, merely being at this stage designed to see whether the cells were still capable of growth after having been in 8-MOP, a real "control" group consisting of transfers from normal to normal media were not set up at this time. Still, if one may consider that the original inocula in each experiment when made into normal media represent a close approximation to such a specific control, then comparisons may be made to Experiment 9. Such plots are shown, but without extensive analysis, in Fig. 6.

It seems fairly clear that cells of the <u>WH 14</u> strain, as well as a different species, <u>T. vorax PP</u>, give comparable results, that is, there is no demonstrable stimulatory effect from being relieved from 8-MOP inhibition, but on the other hand, it seems certain that once the inhibition is relieved these strains studied are capable of resuming normal growth.

It would be of obvious advantage in a study such as this to obtain a single value which would represent the growth performance

culture time. For convenience in comparison, the logarithm of this number was taken. This number is, then, the log. organism-hours per hour, which can be termed LOH.

For the periods measured here, this number provides an excellent means of comparison, especially of the effects of various concentrations of 8-MOP which were employed in Experiments 6 and 7.

Fig. 7 has been prepared to demonstrate these differences. A particular relationship is apparent: with but two exceptions LOH is smaller for each increase in 8-MOP. One exception is the LOH value for 25 gamma 8-MOP in Experiment 6 which is actually slightly higher than the preceding value for 15 gamma 8-MOP. The other is for 2.5 gamma 8-MOP in Experiment 7. In general, however, the decrease in LOH with increasing concentration of 8-MOP over the entire range is quite clear and easy to read.

Data for these calculations are shown in Tables 10, 11 and 12.

There remains but one observation to be recorded. On Jan. 3rd, 1959, a culture of cells was noted which had been used the preceding Dec. 27th in preparing Experiment 9. These cells had been in 8-MOP medium (50 μg./ml.) for over 168 hours. A drop of this culture was examined and a few cells were found to be swimming very slowly. One ml. of this suspension was inoculated into a flask containing 20 ml. of fresh medium.

The following day (24 hours) there were too few cells to be counted in the hemacytometer. A drop of approximately 0.05 ml. from a WBC pipette was placed on a slide and this drop was surveyed under low power. One cell, swimming at normal speed, was observed. To the extent that this was a "good" sample, this implies that there

were on the order of 20 cells/ml. at this time.

The day following this (48 hours), a sample again was taken from this flask. At this time the count was 86,000 cells/ml!

Assuming the 24-hour count to be valid, this yields a doubling time of 1.92 hours, or that the population doubled 12.5 times within this 24-hour period, surely a remarkable rate for a cell of this complexity.

Both from personal observations of cell division with phase contrast microscopy and from conversations with cellular physiologists, there seems to be fairly close agreement that the actual mitotic time is probably rather constant, on the order of 30 minutes, and that variations in generation time are due to variation in the intermitotic interval.

Table 5. The Effect of 25 μg./ ml. 8-Methoxypsoralen on the Growth of Various Strains of Tetrahymena.

Experiment 1. Inoculum: 160 cells/ ml.
T. vorax PP

Time(hrs) of Sample	Cells/ ml. 8-MOP	Control
24	4,400	11,600
48	22,500	60,000
120	26,000	64,000
360	55,000	87,500
N_{24}*	4.8	6.2
T_{24}/G**	5 hours	3.9 hours

Experiment 2. Inoculum: 375 cells/ ml.
T. vorax PP

Time(hrs) of Sample	Cells/ ml. 8-MOP	Control
24	1,250	35,000
48	3,750	49,400
72	5,000	73,750
120	16,250	57,500
144	32,500	66,700
168	18,750	82,500
192	37,500	104,750
N_{24}	1.74	6.55
T_{24}/G	15.5 hours	3.64 hours

Experiment 3. Inoculum: 2,500 cells/ ml.
T. pyriformis E

Time(hrs) of Sample	Cells/ ml. 8-MOP	Control
24	11,250	67,500
48	30,000	116,250
72	35,000	120,000
120	53,750	122,500
144	75,000	61,250
168	35,000	95,000
192	50,000	68,750
216	27,500	40,000
N_{24}	2.17	4.76
T_{24}/G	11.05 hours	5.04 hours

(cont.)

Table 5 (cont.) The effect of 25 µg./ ml. 8-Methoxypsoralen on the Growth of Various Strains of *Tetrahymena*.

Experiment 4. Inoculum: 12,500 cells/ ml.
T. pyriformis WH 14

Time(hrs) of Sample	Cells/ ml. 8-MOP	Control
24	16,250	108,750
48	17,500	102,500
72	30,000	176,250
120	20,000	125,000
144	25,000	200,000
168	35,000	206,250
192	31,250	206,250
216	43,750	167,500
N_{24}	0.38	3.12
T_{24}/G	63 hours	7.45 hours

*N_{24} = Number of times the population doubles in 24 hours.

**T_{24}/G = Mean time per generation during the first 24 hours.

Table 6. The Effect on Growth of Transferring Species of _Tetrahymena_ from Solutions Containing 25 µg./ml. 8-Methoxypsoralen to Fresh Medium Containing 2.5 µg./ml. 8-Methoxypsoralen.

Experiment 2
Species: vorax PP
Inoculum: 375 cells/ml.
Time of growth in 25 gamma 8-MOP: 48 hours

Time (hrs) of sample	Cells/ml.	N_{24}*	T_{24}/G**
24	8,750	4.55	5.3 hours
72	51,250		
96	42,500		

Experiment 3
Species: pyriformis E
Inoculum: 3,500 cells/ml.
Time of growth in 25 gamma 8-MOP: 72 hours

Time (hrs) of sample	Cells/ml.	N_{48}	T_{48}/G
48	131,250	5.9	8.1 hours
72	112,500		
96	117,250		
144	107,500		

Experiment 4
Species: pyriformis WH 14
Inoculum: 3,000 cells/ml.
Time of growth in 25 gamma 8-MOP: 72 hours

Time (hrs) of sample	Cells/ml.	N_{48}	T_{48}/G
48	107,500	6.05	7.9 hours
72	110,000		
96	222,500		
144	312,500		

* N_{24} and N_{48} = Number of times the population doubles in 24 and 48 hours, respectively.

** T_{24}/G and T_{48}/G = Mean time per generation during the first 24 and 48 hours, respectively.

Table 7A. The Effect of Various Concentrations of 8-Methoxypsoralen on the Growth of T. vorax PP.

Concentration of 8-MOP in µg./ml. | Time of Sample (hours)	Zero	24	48	96	120
Zero (control)	750	15,700	81,500	96,000	71,300
0.5	750	11,880	63,000	87,500	71,800
1	750	15,300	60,200	75,700	64,200
2.5	750	20,440	63,000	50,500	90,000
5	750	13,300	46,200	74,000	50,000
15	750	12,700	43,000	60,800	60,700
25	750	9,550	40,200	78,800	42,400
50	750	3,440	12,600	16,500	20,000

Table 7B. The Effect of Various Concentrations of 8-Methoxypsoralen on the Growth of T. pyriformis E.

Concentration of 8-MOP in µg./ml. | Time of Sample (hours)	Zero	48	96	144	168
Zero (control)	750	115,000	93,600	56,000	100,000
0.5	750	53,000	104,000	60,000	45,000
1	750	60,000	119,000	35,000	35,000
2.5	750	83,000	89,500	32,000	67,000
5	750	70,000	89,300	46,000	67,000
15	750	47,000	51,400	56,000	69,500
25	750	27,000	45,000	55,000	72,000
50	750	16,000	22,800	35,000	36,700

Table 8. The Effect of Various Concentrations of 8-Methoxypsoralen on the Growth of *T. pyriformis* E as Determined by Direct Counts[1] and Densiometric Measurements[2]

Concentration, µg./ml. ↓ / Time, hrs. →	Zero	4	8	16	24	32	40	48	64	72	96
Control	1000 .125	.144	.144	.154	25,000 .150	.153	.171	72,000 .235	.319	137,000 .330	.405
0.5	1000 .123	.143	.149	.155	16,000 .145	.156	.170	57,800 .228	.316	98,000 .334	.394
1	1000 .124	.156	.150	.158	14,000 .156	.161	.175	57,000 .222	.313	79,000 .328	.398
2.5	1000 .126	.141	.144	.153	14,000 .152	.156	.175	64,000 .230	.304	61,000 .325	.400
5	1000 .135	.158	.154	.155	11,100 .160	.154	.175	62,000 .221	.307	62,000 .334	.396
15	1000 .151	.159	.148	.159	3,300 .154	.156	.164	40,000 .190	.233	44,000 .283	.325
25	1000 .150	.159	.149	.157	3,300 .158	.155	.156	20,000 .179	.205	34,500 .217	.278
50	1000 .148	.160	.142	.145	1,000 .143	.145	.145	6,000 .158	.159	18,000 .165	.207

[1] Cells/ml.

[2] Spectrophotometric readings

Table 9. The Effect of Transferring T. *pyriformis* E from Cultures Containing 50 µg./ ml. 8-Methoxypsoralen to Normal Medium

A. Cells Grown in Normal Medium.

Hrs. before subculture into normal medium	Cells/ml. inoculated	Count in Cells/ ml. at specified times			
		24	48	72	96
24	1000	101,000	263,000	249,000	—
48	1000	110,000	244,000	216,000	--
96	1000	118,000	—	—	202,000

B. Cells Grown in 50 µg./ ml. 8-MOP.

Hrs. before subculture into normal medium	Cells/ml. inoculated	Count in Cells/ ml. at specified times			
		24	48	72	96
24	1	5,000	245,000	221,000	—
48	1	5,000	207,500	223,000	—
96	3	7,500	—	—	236,000

Calculations:

Number of doubling times in interval specified

	N_{24}	N_{48}	N_{96}
Control 24	6.6	1.38	—
" 48	6.78	1.15	—
" 96	6.88	—	1.11
Test 24	10.35	5.61	—
" 48	10.35	5.37	—
" 96	11.28	—	4.97

Mean Time per Generation in interval specified

	T_{24}/G	T_{48}/G	T_{96}/G
Control 24	3.64	17.4	—
" 48	3.54	20.8	—
" 96	3.49	—	21.6
Test 24	2.32	4.3	—
" 48	2.32	4.5	—
" 96	2.12	—	4.8

Table 10. Analysis of Data of Experiment 2 to Compute LOH.

Interval	Control	8-MOP (25 ug./ml.)
1	7500	3000
2	14000	3000
3	20000	2000
4	27000	1000
5	34000	1000
6	37000	1000
7	40000	2000
8	43000	2000
9	46000	2500
10	49000	3000
11	53000	3500
12	58000	4000
13	63000	4500
14	68000	5000
15	73000	5000
16	71000	6000
17	69000	7500
18	68000	8500
19	66000	10000
20	64000	11000
21	63000	12000
22	61000	13000
23	59000	14000
24	58000	15000
25	58000	18000
26	60000	21000
27	62000	24000
28	64000	27000
29	66000	31000
30	69000	30000
31	72000	27000
32	75000	24000
33	79000	24000
34	82000	18000
35	87000	21000
36	92000	25000
37	97000	28000
38	101000	34000
	2275500	488200
	x5	x5
	11377500	2441000

Log (11,377,500/192) = 4.771 = LOH (control)

Log (2,441,000/192) = 4.105 = LOH (test)

Table 11. Analysis of Data of Experiment 6 to Compute LOH.

Interval	Concentrations of 8-MOP							
	Control	0.5	1.0	2.5	5.0	15	25	50
1.	3500	3000	3500	4500	3000	3000	2500	1000
2.	6500	5000	6500	7000	5000	5500	4500	2000
3.	9500	7500	9000	1200	8000	7500	6000	2000
4.	12000	9500	12000	16000	10500	10000	7500	3000
5.	15000	11500	15000	20000	13000	12000	9000	3500
6.	26500	20000	23000	27500	18500	17000	14000	4500
7.	40000	30000	37500	36000	26000	23500	20500	6500
8.	54000	40000	41000	45000	32500	29500	26500	8500
9.	68500	50000	50000	54000	39000	36000	33500	10500
10.	81500	61000	59000	62000	46000	42000	39000	12000
11.	83000	65500	61500	62000	49000	45000	44000	12500
12.	84500	68000	63500	60500	52000	46500	47500	13000
13.	86000	70500	65000	59500	55000	47500	52000	13500
14.	87500	73000	66500	58000	57500	50500	55500	14000
15.	89000	75500	68500	56500	61000	52500	60000	14500
16.	90500	78000	70000	55500	63000	54000	63000	15000
17.	92000	80500	72000	54000	66500	56000	67500	15500
18.	93500	82500	73500	53000	69000	57000	71500	15500
19.	95000	86000	75000	51000	72500	60000	76000	16000
20.	95000	87000	75000	55000	72500	61000	76000	16500
21.	89000	83500	73000	63000	67500	61000	68500	17000
22.	84000	80000	70000	70500	63000	61000	61500	17500
23.	79000	76500	68000	79000	57500	61000	53500	18500
24.	74000	73500	65500	87000	53000	61000	47000	19500

Control: Σ intervals x 5 = 7695000; Log (7695000/120) = 4.806

0.5 : Σ " x 5 = 6935500; Log (6935500/120) = 4.761

1 : Σ " x 5 = 6117500; Log (6117500/120) = 4.706

2.5 : Σ " x 5 = 5792500; Log (5792500/120) = 4.684

5 : Σ " x 5 = 5305000; Log (5305000/120) = 4.646

15 : Σ " x 5 = 4800000; Log (4800000/120) = 4.602

25 : Σ " x 5 = 5042500; Log (5042500/120) = 4.623

50 : Σ " x 5 = 1360000; Log (1360000/120) = 4.047

Table 12. Analysis of Data of Experiment 7 to Compute LOH.

Interval	Concentrations of 8-MOP							
	Control	0.5	1.0	2.5	5	15	25	50
1.	3000	3000	5000	5000	5000	3600	2200	1400
2.	18000	8200	11200	16000	11200	7800	4800	3000
3.	30000	14000	16800	22000	18800	12600	8600	4600
4.	42000	19000	22500	30800	26000	17500	10400	6200
5.	54000	25000	29000	39600	33800	22400	13000	8000
6.	66000	30200	35000	48000	40800	27200	13700	9400
7.	78000	36000	41000	56800	48000	32000	18400	11000
8.	90000	41000	47000	65200	55000	36600	21000	12600
9.	110000	46000	53600	74000	62000	42000	23800	14200
10.	114000	52600	60000	82000	69200	46600	26400	15800
11.	113000	58000	66000	83600	72000	47400	28500	16600
12.	111000	63000	72000	84400	74000	47800	30200	17200
13.	109000	68600	78000	85200	76000	48300	32300	18100
14.	107000	74000	84000	86000	78000	48700	34100	18800
15.	105000	79400	90000	86000	80000	49200	36100	19400
16.	103000	84600	96000	87400	82000	49600	37900	20100
17.	101000	90000	102000	88000	84000	50100	39900	20900
18.	99000	95200	108000	88800	86000	50600	41600	21600
19.	97000	100000	114000	89600	88000	51000	43600	22300
20.	94000	100000	120000	88800	88000	51500	44200	23100
21.	90000	96000	108000	82400	83200	52000	46300	24500
22.	86000	91400	100000	76400	79000	52500	47300	25800
23.	82000	87000	90800	70400	74000	53000	48300	27000
24.	78000	82800	82000	64400	68000	53500	49400	28300
25.	73000	78000	72000	58000	65200	54000	50500	29600
26.	69000	74400	64000	52000	60400	54300	51500	30800
27.	65000	69800	54800	45200	56000	54800	52600	32100
28.	61000	64800	46400	39600	51800	55400	53700	33300
29.	56000	61000	36000	33200	47000	55800	54700	34600
30.	62000	58200	35000	37000	49200	57800	57600	35200
31.	72000	55000	35000	45600	53800	60700	61100	35600
32.	80000	52400	35000	51600	58000	63500	64400	35900
33.	90000	49200	35000	60000	62000	66300	68000	36300
34.	99000	46400	35000	66000	66000	69100	71600	36600

Control: Σ intervals x 5 = 13585000; Log (13585000/168) = 4.907

0.5 : Σ " x 5 = 10772000; Log (10772000/168) = 4.806

1 : Σ " x 5 = 10400500; Log (10400500/168) = 4.792

2.5 : Σ " x 5 = 10448000; Log (10448000/168) = 4.794

5 : Σ " x 5 = 10257000; Log (10257000/168) = 4.786

15 : Σ " x 5 = 7726000; Log (7726000/168) = 4.663

25 : Σ " x 5 = 6383500; Log (6383500/168) = 4.579

50 : Σ " x 5 = 3699500; Log (3699500/168) = 4.342

GENERAL DISCUSSION

In studies of both malignant human cells and protista the action of 8-methoxypsoralen has been shown to inhibit cell reproduction. At the same time it has been demonstrated that, at least with the protozoa, the inhibition only takes place during actual contact with the compound; upon reduction of the concentration inhibition is reduced, and when the compound is sufficiently diluted cell reproduction again commences at rates which must be considered normal, immediately after dilution.

There is no evidence of toxicity in concentrations great enough to produce complete inhibition, as measured by subsequent growth patterns or as evidenced by observable changes in morphology, either in cytoplasmic or nuclear detail. These cell populations behave, in fact, during contact with 8-MOP, as if they were hibernating, if one can use such an analogy.

A number of considerations suggest the possible significance of these findings.

Since Ehrlich first applied his "Lock and Key" analogy to the theory of structural placement--or displacement--in the field of immunology, there has developed what might be called the metabolic analogue approach to biological research. In general this involves studies of enzymatic inhibition by chemicals structurally related to the natural substrate.

The elucidation of the relationship of p-aminobenzoic acid, sulfonamides, and later folic acid are classical examples of the profit of such an approach, from Woods' (62) identification of p-aminobenzoic

acid, PABA, as being the sulfonamide-inhibitor which Green (17) had isolated from yeast. Reviews of this relationship are given by Henry (18) and Northey(36).

Thousands of metabolic analogues have been prepared and tested on a variety of systems, although it must be confessed that most of these have not, to this date, proven useful. Utility, however, may depend on the purpose: whether to find specific chemotherapeutic agents which will act against disease or whether one is trying to understand certain biochemical and physiological functions.

It should be mentioned at this point that _Tetrahymena geleii_ has been used as a test organism by Kidder and Dewey (26,27) in testing analogues of some of the purines and pyrimidines.

The rational approach to studies in biological antagonism is, of course, to produce alterations in molecular structure of certain crucial metabolites, especially vitamins, or nucleoproteins, which are thought to enter critical reactions as parts of coenzymes or enzyme systems.

In the present study, however, we are confronted with a kind of accidental discovery, not based on predicted behavior from knowledge of chemical structure. Nevertheless, the implications should not be overlooked that the action of 8-MOP, structurally not very similar to any known metabolite, produces a result which is not inconsistent with competitive inhibition, and therefore just may indicate hitherto unsuspected metabolic reactions of great importance. The indications that it functions similarly, that is, to the same end result, in certain plant tissues, lend support to this hypothesis.

Of great importance also might be the relations of the present findings to the Osgood (38,41) concept concerning the etiology of leukemia, etc.

It is desirable to summarize briefly the salient features of this concept in order to clarify the presumed relationship.

After an embryonic stage of growth, only the immature cells of multicellular organisms divide. After such a division, only one of the daughter cells would normally differentiate, leaving the other cell immature and still capable of subsequent division. This type of cell reproduction may be termed arithmetic division since it leads to arithmetic changes in the cell number. Since only a few exponential divisions can mathematically account for the total number of cells in the adult, it is probable that in postnatal life only four or five such divisions actually occur. If this is the case, each series of cells of any type for which there is a dynamic equilibrium established requires regulators of a more or less specific nature to maintain this equilibrium.

Osgood's stimulating concept is that with the majority of cell series the homeostatic regulator of arithmetic division might reasonably be produced by the most mature cells of that series. Further, somewhat earlier there must be produced an inhibitor of the logarithmic divisions of which the most primitive cells are potentially capable.

The type of cell reproduction occurring in various systems may be clarified by reference to a plot of this growth on different types of graph paper. That is, arithmetic division, which characterizes the type of change going on over moderate periods of time

in the adult organism, would give a straight line on ordinary arithmetic paper. The total growth of an organism, e.g., in the growth of a human from periods of infancy to adulthood, in which the intervals between logarithmic divisions become progressively longer, would give a straight line plot on log-log paper. Finally, protista, in a suitable environment, grow at a rate in which doubling times are equal and the growth data can be illustrated by a straight line on semi-log paper.

Osgood postulates that "the fundamental alteration (to produce leukemias, etc.), is <u>any</u> genetic change leading to lack of any enzyme system necessary for a full life span of the differentiating cell of a series." Thus any such change which resulted in early cell death, that is, shortening of the life span of a mature cell, might result in a quantitative decrease in the amount of inhibitor which it is capable of producing, with the result that the next more immature cell which is capable of division begins to divide at a faster rate. Since each division carries the finite risk of more damaging genetic changes, such a faster rate of division increases the risk of further alterations, of these immature cells being also affected in such a way that they no longer produce inhibitor for more immature cells, etc., as seems to happen, for example, in the terminal acute phase of a long-standing chronic leukemia.

It is possible to look on a cancer cell as if it were a foreign invading organism of no relation to the patient, or the tissue in which it grows, as if it were a parasite. Such a view leads to questions as to how best to rid the host of the "parasite"--by surgery, by chemotherapy, by radiation, or other techniques designed

to kill or remove this invader. But it is possible to look at a cancer cell as a symptom of a process which continues to go on in an organism for any number of reasons, which process may potentially go on no matter how many cancer cells are removed or destroyed. It is true that in a relatively blind attack on cancer cells it may be that at times we also accidentally, as it were, do alter this process. Of this we cannot be sure; it lacks predictability. If it did not, then we should be able to cite a "sure cure" for cancer, leukemia, etc. But this approach may not be the most fruitful if the development of malignancy is basically the absence of some agent, rather than the presence of some supposed carcinogen. (This is not to deny that carcinogens potentially produce cancer; it would simply imply that they do so only by interference and alteration of the normal growth process.) Still, it is rather like an attempt to eliminate automobile accidents by sending out wrecking crews of one kind or another to remove all autos which have been damaged in wrecks.

Rose (54) has found evidence that fresh, adult tissues produce alterations in the same tissues of developing embryos, and Saetren (55) has demonstrated a specific inhibitory effect on the regeneration of liver cells with extracts of liver cells.

It is possible that the transient benefits of exchange transfusion in the treatment of leukemia is due to the introduction of normal inhibitor substances.

The partial success in the administration of irradiation and chemotherapeutic agents, including inhibitors of mitotic division, in the treatment of leukemic patients has been conceived of by

Osgood as acting as substitutes for the absent inhibitor in such a way that they tend to keep the leucocyte count maintained and thus reduce the total number of cell divisions with the reduction in the attendant risk of further genetic change that might lead to a still further shortening of the life span of the cell. Unfortunately, both irradiation and effective mitotic inhibitors so far discovered are mutagens, a property which the normal inhibitor or inhibitors probably do not have.

In studying Oregon J 96 in which the cell populations have shifted to logarithmic growth, probably by a change in distribution of the ratio of immature to mature cells, we have found in 8-MOP an inhibitor for logarithmic division. Going further, to a study of unicellular organisms which are certainly capable of logarithmic growth, we have shown that 8-MOP is quite an effective inhibitor here, again of logarithmic division. The probability is that certain groups of cells of the rapidly growing onion root tip and wheat coleoptile, being embryonic tissues, also exhibit logarithmic growth, and 8-MOP has been shown to be an effective inhibitor here.

It would be apparently worth investigating if 8-MOP has any mutagenic properties. The fact that psoralens in general are normal constituents of the pericarp of certain seeds suggests that, for these plants at any rate, psoralens are probably not mutagenic. The absence of any observable morphological changes in Oregon J 96 grown in the presence of 8-MOP does not, at least, give any evidence that it is mutagenic for this type of cell. Tetrahymena would presumably not be a good test system to use to study this point, since they have a very low natural mutation rate, and have resisted

attempts by means of radiation, etc., to increase this rate. Protozoa grown in 8-MOP exhibited no morphological change but showed reduced swimming speeds.

In any event, it might seem that 8-MOP fulfills the requirements for the role of an inhibitor such as is normally produced. It does not destroy the cells which are capable of logarithmic division rates, but merely prevents them from dividing. If the Osgood concept is correct, the destruction of these cells is not a desirable end in itself, since there would be nothing left to produce inhibitor. On the other hand, it might be that if these cells were kept alive without dividing, some differentiation might take place with the subsequent production of normal inhibitor. Even if this were not the case, the stoppage of production of new cancer cells might be desirable from the view of the energetics of the organism as a whole. Since it can be postulated that this production of leukemia has certain feed-back characteristics where each damaging alteration increases the probability for further damaging alterations, etc., then the mere act of breaking into this circuit should increase survival time.

If it could be shown that 8-MOP, or some related compound, is sufficiently non-toxic and free of too many unfavorable side effects, then it is even possible to speculate that, even if it fails to reverse the process entirely, it could nevertheless be administered in doses designed to maintain a certain level of inhibition which might result in greatly increased survival times of patients suffering from malignancy diseases.

This line of thought approaches again, from a different direction,

the notion that 8-MOP may be structurally or at least functionally similar to some normal metabolite.

It would be tempting to use the term "mitotic poison" in regard to this action but for the difficulty, pointed out by Biesele (2), in the use of this term in view of the failure to point out the precise function poisoned.

On the other hand, Hughes (25) believes the term can be used to describe the delay or interruption of mitosis by "treatment of a cell with drugs at a concentration which has no visible effect on the cell during interphase. In this respect, natural cell constituents can act as 'mitotic poisons.'"

To elucidate in chemical terms the structures and functions of such inhibitors and to test them in biological systems may well be rewarding. It may not only result in the production of new and useful chemotherapeutic agents, but also broaden our understanding of cancer, wound healing, aging and growth in general.

The approach to many of these problems has been reflected by the question, "How do cells grow?" It may well be worth asking, "Why do they stop growing?" That is, as the study of biological systems has been simplified from the standpoint of organization complexes, to single cells or populations of single or closely related cell types, provided with a suitable environment, growth and reproduction increase. Even some cells which rarely divide in the adult, such as certain nerve cells, or bone cells, can be shown to grow and divide in tissue culture, under the proper conditions. Fascinating though the question may be of what stimulates these cells to grow, it is possible to start with the axiomatic assumption

that the structure and components of the cell favor growth and examine what components of the biological system delimit growth. It is easy to list a few: exhaustible supply of metabolites, temperature, accumulation of wastes, need for respiratory gases, etc. Rashevsky (51) has used biomathematical theorems to describe the limitation in size of a single cell, under certain conditions. It is possible to extend these concepts to include multicellular organisms or even whole populations.

Volterra (61), a mathematician interested in population dynamics, has described mathematical models for closed ecological systems, i.e., ideal systems, in which several species are competing with each other. It might be possible to develop analogous mathematical models to describe the development of different types of cells within a multicellular organism.

The experimental techniques used in the present study are subject to grave limitations of accurate mathematical relations because of the physical construction of the system. Even with an adequate number of samples and sampling intervals, we can only plot the familiar growth curve and subsequent analysis must be based upon that. Much general information may be derived, but some of the crucial questions of the dynamics of the system which might provide information as to the basis of homeostatic regulation remain elusive.

Such a growth curve is the continuous algebraic summation of two curves: the rate of production of new cells, which we might think of as "birth rate," and the rate of destruction or disintegration of cells, which may fairly be called the "death rate." An

examination of Fig. 1, for example, will make clear the following assumptions: during the lag phase the population consists of a certain number of cells which, though still alive, have, in the old culture from which they came, passed a critical point such that certain irreversible changes have already begun to take place. They are destined to die, even in the new, superior environment. The cells capable of division are synthesizing materials necessary for division. Here, death rate slightly exceeds birth rate, but both are very low. During the phase of exponential growth, all these old cells carried from the old culture have died and all cells present are dividing at the maximum rate of speed possible under these growth conditions. Here, death rate is negligible, or zero, and the curve is due almost entirely to birth rate. During the maximum stationary phase, birth rate probably drops to near zero; as more and more energy is spent in seeking food, less is available for division; death rate, however, is also near zero. Then, toward the end of the curve, death rate gradually begins to rise.

In such a system it is extremely difficult to study an agent which has an effect on "birth rate" alone, whether that agent causes "stimulation" of birth rate, such as a vitamin, or whether it causes depression of the birth rate, such as we believe 8-MOP is doing, simply because there is no way to rule out that that which apparently increases birth rate could be, either in addition to or independently, decreasing death rate.

If we assume that the events of cell reproduction involve the synthesis of a great variety of proteins, nucleoproteins, etc., involved in enzyme systems, plus a host of metabolites which the

daughter cell will carry with it, plus the duplication of certain structures which may be initially single (as of mouth parts in *Tetrahymena*), whereas death must involve the wearing out or irreversible blocking of certain enzyme systems essential to metabolism, then in order to understand the molecular events of cell inhibition it is necessary to separate these events for analytical purposes.

Tetrahymena is uniquely suited to such a study. It is sufficiently complex biologically and biochemically to present biochemical systems homologous to higher organisms, and physiological systems analogous to higher organisms. It is free-living and may be cultured in synthetic medium. An apparatus specifically designed for stabilizing the environment of *Tetrahymena* in order to permit careful studies of population responses to measured stimuli has been described by Browning (7).

The principal advantage of this type of apparatus is to hold a culture at an equilibrium population. The dynamics of the system are not the "natural" dynamics of a growing culture in which death rate accounts for the removal of organisms from the population, but rather as a synthetic, controllable device for removing cells from the population before they die and at a rate just equal to birth rate. It is the ideal system with which to study "exfoliative" growth, defined by Hoffman (22) as the resultant growth of a population in which cells are removed as fast as they are produced.

This is accomplished by permitting a flow-through rate against a certain volume in the growth chamber to carry in fresh medium of a given nutritive content while at the same time it is washing out a certain fraction of the population. As the density of the population

increases, given a constant volume fraction being removed during each time interval, an increasing number of cells will be washed out. By adjusting the flow-through rate for a given set of conditions, it is possible to stabilize the population, and more importantly, it can be stabilized at a level such that the time per generation is minimized. For this population, also, death rate is minimized, and is probably zero.

Under these conditions it is thus possible to devise an extremely sensitive system which will respond to very slight changes in any environmental factor. The addition to this system of a test substance will give information which can be strictly related to the rate of cell division, since toxicity effects could be tested by further analysis of samples collected.

Reference to Fig. 9 will suggest a method whereby, with the proper arrangements of drive shafts operated in a constant ratio off a central drive shaft of a motor which can be moved perpendicular to the axis of its shaft at a constant speed, media from two different flasks might be delivered in a constantly changing and predetermined ratio. This would allow for measurements of all concentrations of a test substance, by means of delivering a complete range of mixtures of two substances. In the Fig. 9, for example, if the distance between the two drive wheels for feeder a and feeder b were equal to the radius of the central drive wheel, then when "a" is at the edge of the wheel, all medium being delivered comes from reservoir A. As the motor moves such that drive wheels a and b are equidistant from the center of the shaft, then equal amounts of media are being delivered from each reservoir.

Instead of testing discrete concentrations of a substance at

arbitrary intervals, this allows for a continuous test for every concentration over the entire range.

At the outflow of the growth chamber it might be desirable to introduce a photocell-photomultiplier or resistance grid-amplifier which could be led to a continuous recording device. Additionally, it might be desirable to devise a collection apparatus on the design of a fraction collector which would collect a given volume in a certain time period into each of several tubes from which further studies could be made.

The author has assisted in the design and fabrication of such a chemostat. The growth curve obtained from one set of conditions is much like that shown in Fig. 8. It will be seen that the curve resembles a damped sine wave around an hypothetical stable population level.

It could be more quickly stabilized, prior to a test run, by using information from a continuous recording device as to the number of cells in the outflow to modify continuously the flow rate, although this should not be necessary, in general.

It would seem that with this system, a chemostat in which are growing Tetrahymena and in which chemically defined medium was used, information regarding regulation of growth should become more readily accessible. It would be possible, for example, to measure the volume of protoplasm being produced against the reduction or diminution of any factor or factors being used in the medium. This could readily be used in conjunction with radioisotopes. It should be possible, by the elimination of factors or fractions of factors in the input medium to a point just greater than that at which growth is affected, to

obtain finally the minimum quantities of each individual substance necessary to permit growth under given conditions. With this information to be compared to the quantity and variety of new factors present in both the intra- and extra-cellular compartments of the outflow medium, it is conceivable that virtually the entire energetics of the system might be worked out.

Much of the above discussion is highly speculative, but it should be pointed out that this does at least furnish a means of obtaining some quite valuable information. It is possible to get an accurate dose-response curve, giving a starting point for testing the effect of a particular agent on a multicellular organism. It is possible to test whether a response is due to depression of the mitotic rate, *per se*, the rate of synthesis of metabolites in general, or perhaps DNA in particular, or to the blockage of some enzyme system which might result in the accumulation of a metabolite, etc.

If a given cell inhibitor produces chromosomal aberrations, or morphological changes, or nuclear/cytoplasmic ratio changes, etc., it would be possible to test the concentration necessary to produce these changes. The mutagenic rate of a cell inhibitor could easily be measured.

It would seem worthwhile at this time to test the action of 8-MOP in experimental animals in which tumors occur or have been produced. The "leukemic" strain of AKR mice would seem to provide an excellent test animal for this compound, to determine if it can increase the survival time in those mice which "spontaneously" develop leukemia.

Since 8-MOP is being tested in photodynamic systems on a

variety of laboratory animals and is currently being used clinically for certain purposes, generally in relation to the effect produced by subsequent irradiation with visible light (sun) or with pure U-V light, it should be emphasized again that the reactions reported here are dark reactions, not dependent on subsequent irradiation.

SUMMARY

Evidence has been presented to show that 8-methoxypsoralen, a naturally-occurring chemical compound of the furocoumarin class, elicits an inhibitory effect on the rate of cell division in two complex, but widely separated biological systems. This evidence has been obtained experimentally by enumerating populations of cells or organisms grown without or with various concentrations of this drug under otherwise similar culture conditions.

The data have been analyzed in several ways in an attempt to illustrate these findings in as clear a way as possible.

No biochemical studies of the action of this drug have been undertaken; the mechanisms of its action upon the cell as to metabolic or morphologic loci, etc., remain unknown. Comparisons have been made, however, to its possible role as an antimetabolite and also a comparison has been drawn between its action and that which would be expected of an inhibitor substance produced by cells which have to do with homeostatic auto-regulation.

Suggestions and recommendations are made in regard to further investigations. In particular a special growth apparatus is discussed, the use of which should permit a high degree of quantitative biological analysis of growth phenomena.

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Fig. 1. Ideal growth curve. For explanation see p. 10 of the text.

Fig. 1

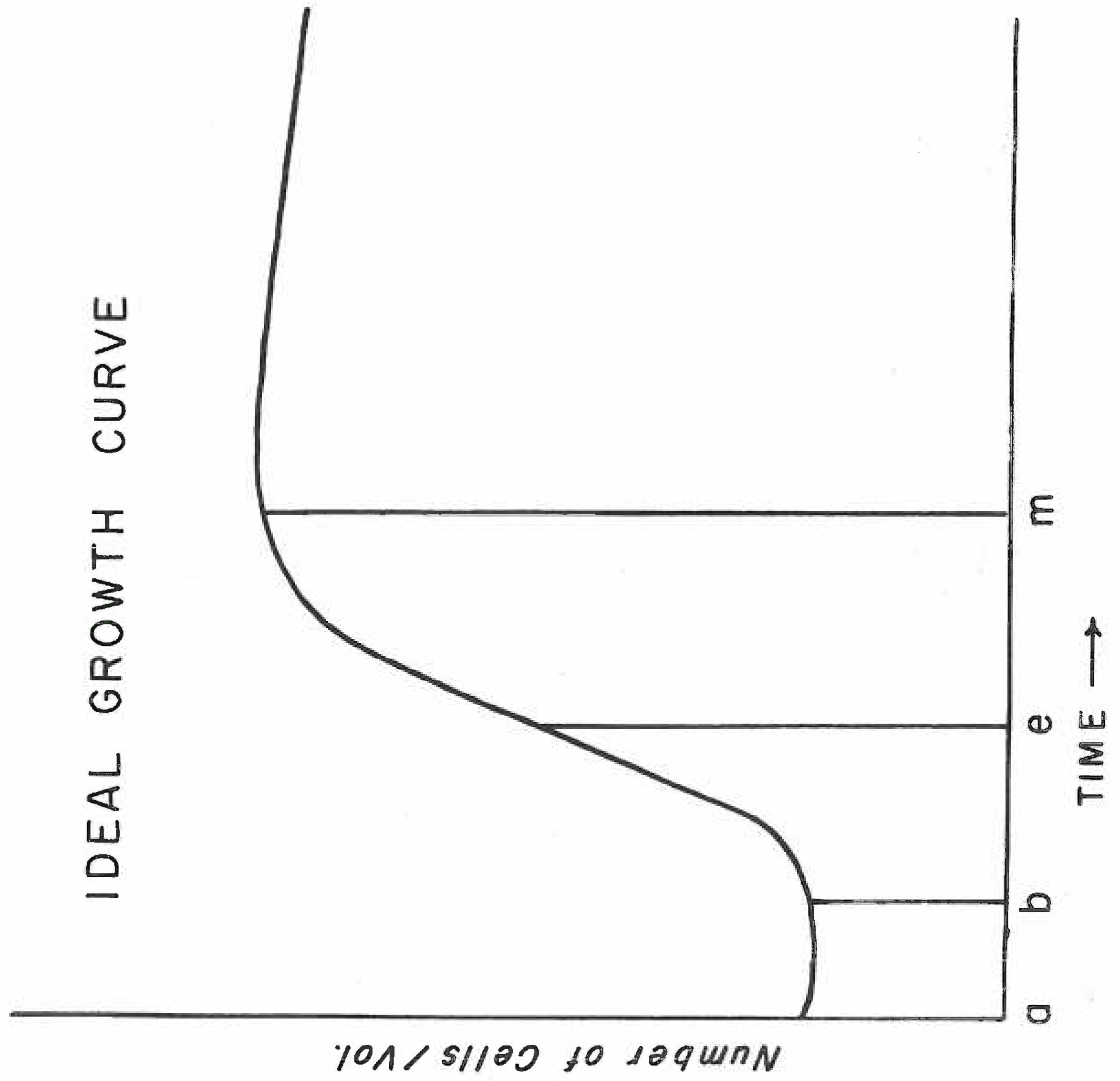

Fig. 2. Slide-cap Culture. A Kahn antigen mixing vial is affixed to a microscope slide with beeswax and inverted. Cells grow on surface of slide.

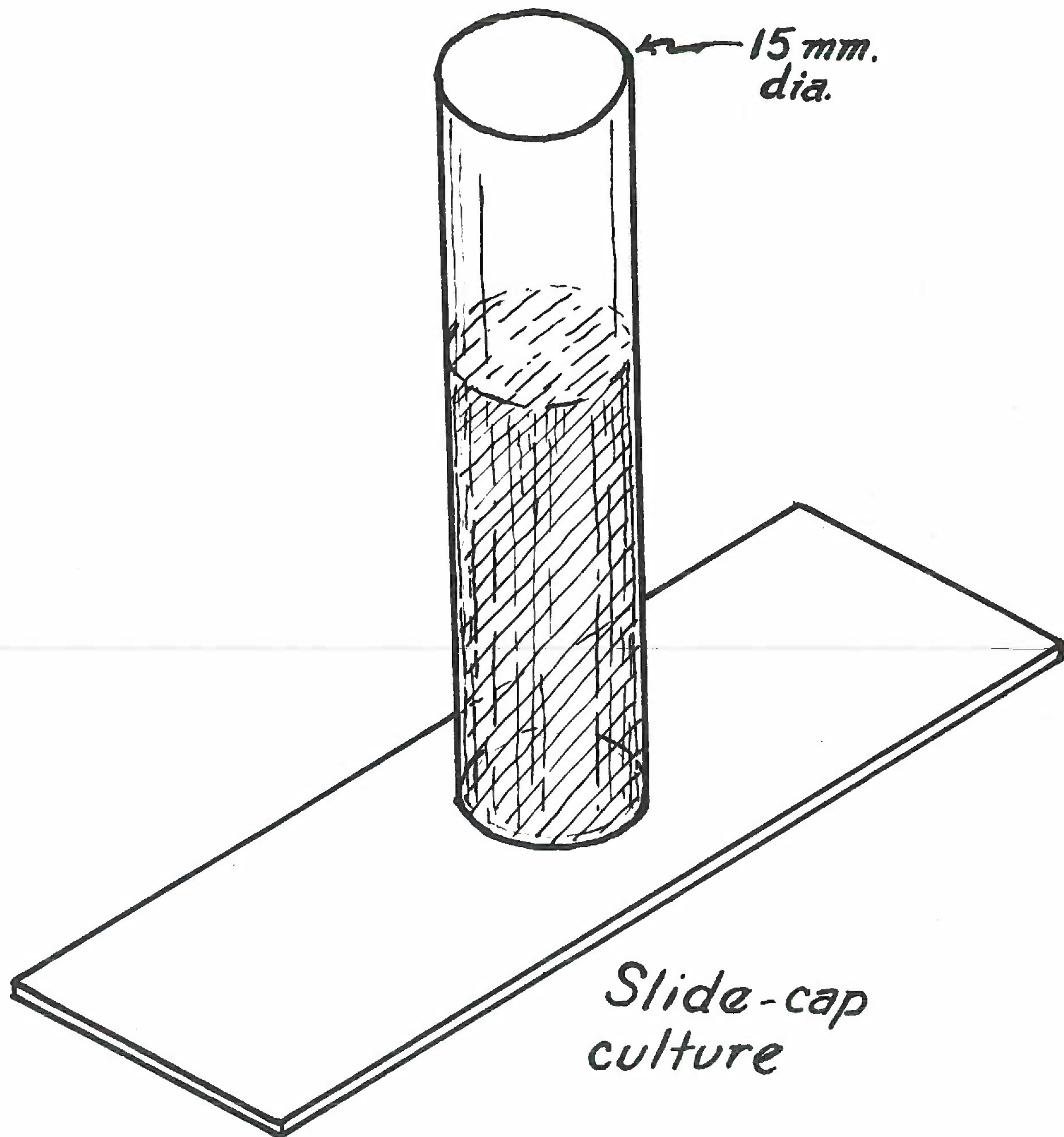

FIG. 2

Fig. 3A. Early stages of slide-cap culture of Oregon J 96. Cells resembling fibroblasts, reticulum cells, and giant cells may be seen.

FIG. 3A

Fig. 3B. Later stages of slide-cap culture of Oregon J 96. Reticulum cells predominate. Mitoses are present and multinucleate giant cells may be noted.

FIG. 3B

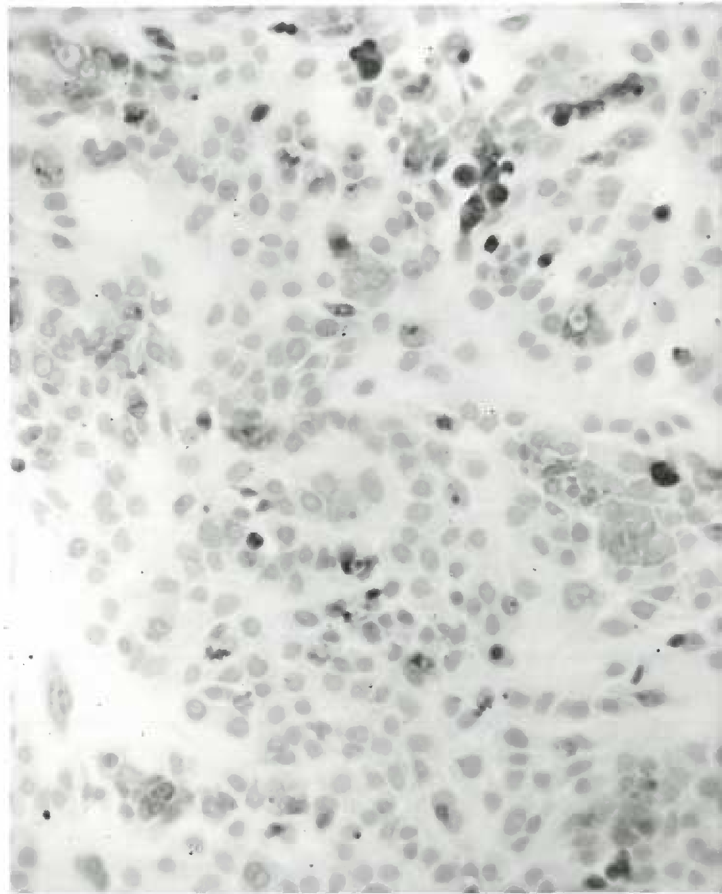

Fig. 3C. A photomicrograph under oil immersion of Oregon J 96. Note large nuclear masses with several nucleoli. The cell to the center, left, is binucleate.

FIG 3C

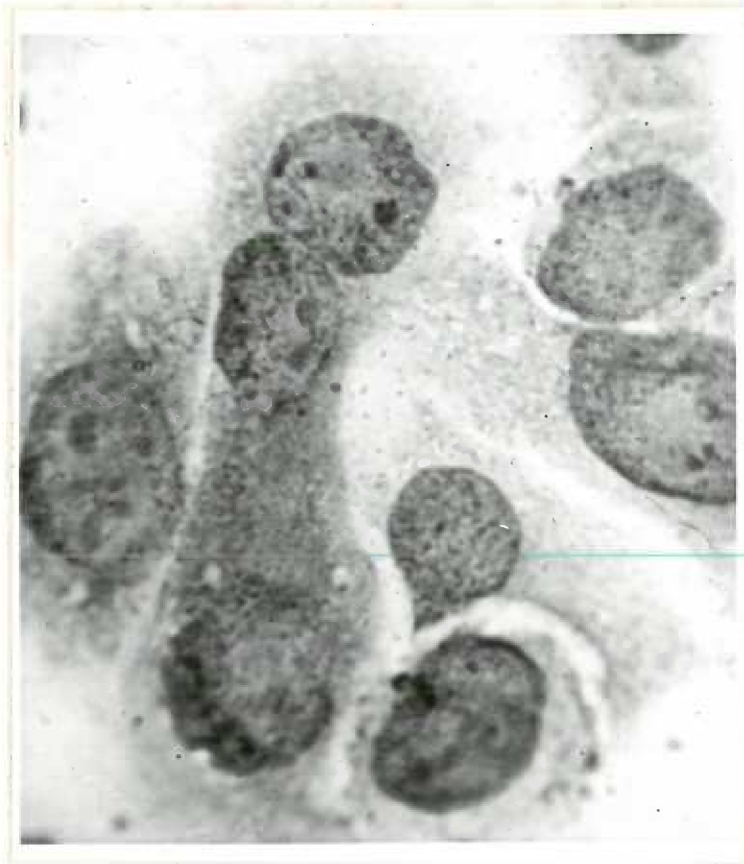

Fig. 4. A Dose-response curve for 8-MOP on
Oregon J 96, at 120 hours. See
also Tables 1-4A and text, p.23
and following for discussion.

FIG. 4

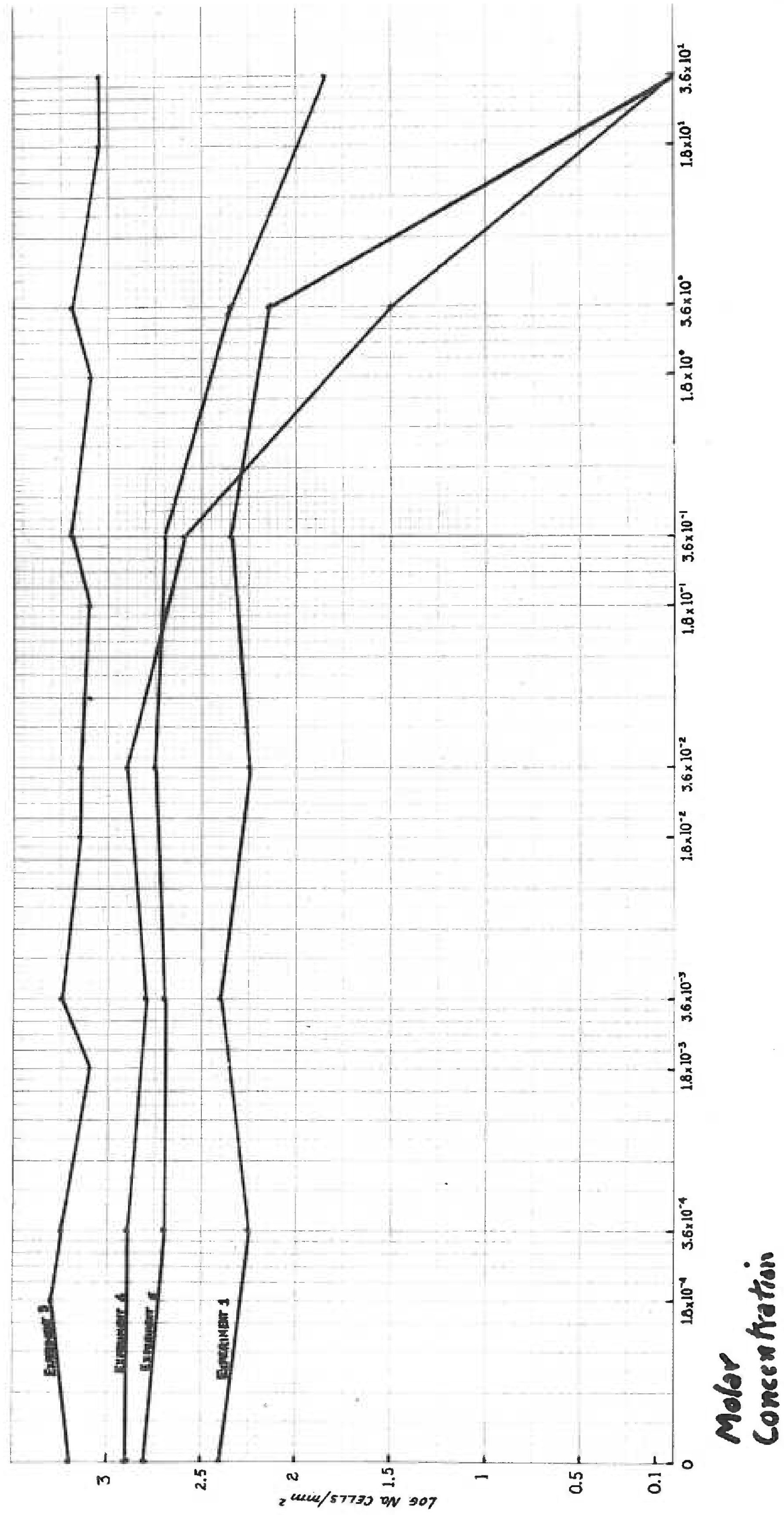

Fig. 5. A comparison of cultures of *Tetrahymena* transferred from 8-MOP medium to normal versus those transferred at the same time from normal to normal media.

FIG. 5

EFFECT ON GROWTH RATE ON TETRAHYMENA TRANSFERRED FROM 8-MOP TO NORMAL MEDIUM

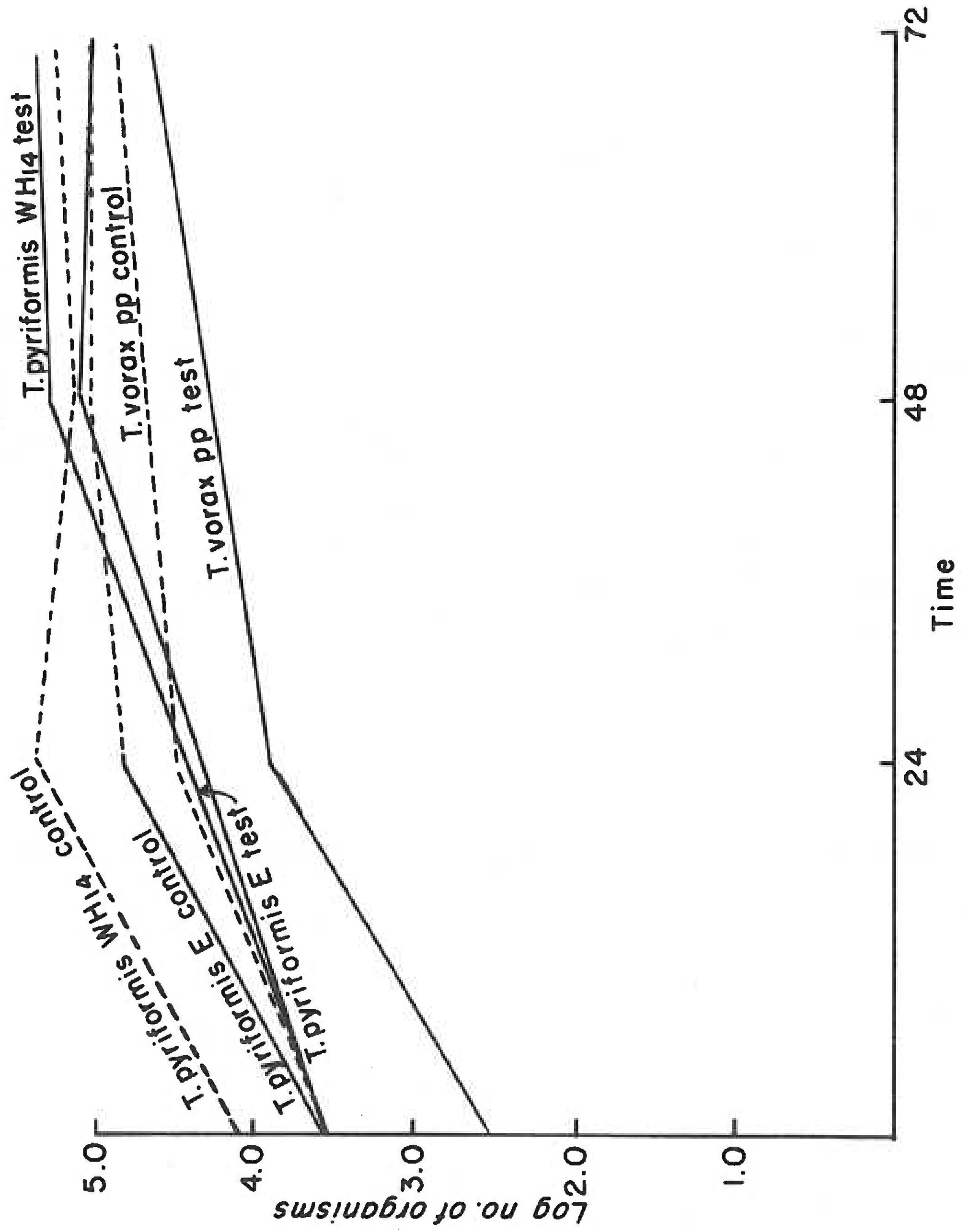

Fig. 6. An analysis of Experiment 9. See also Table 9 and text, p.62. This analysis strongly suggests that there is no change in growth rate in *Tetrahymena* after the inhibition effects of 8-MOP are relieved by transfer to normal medium.

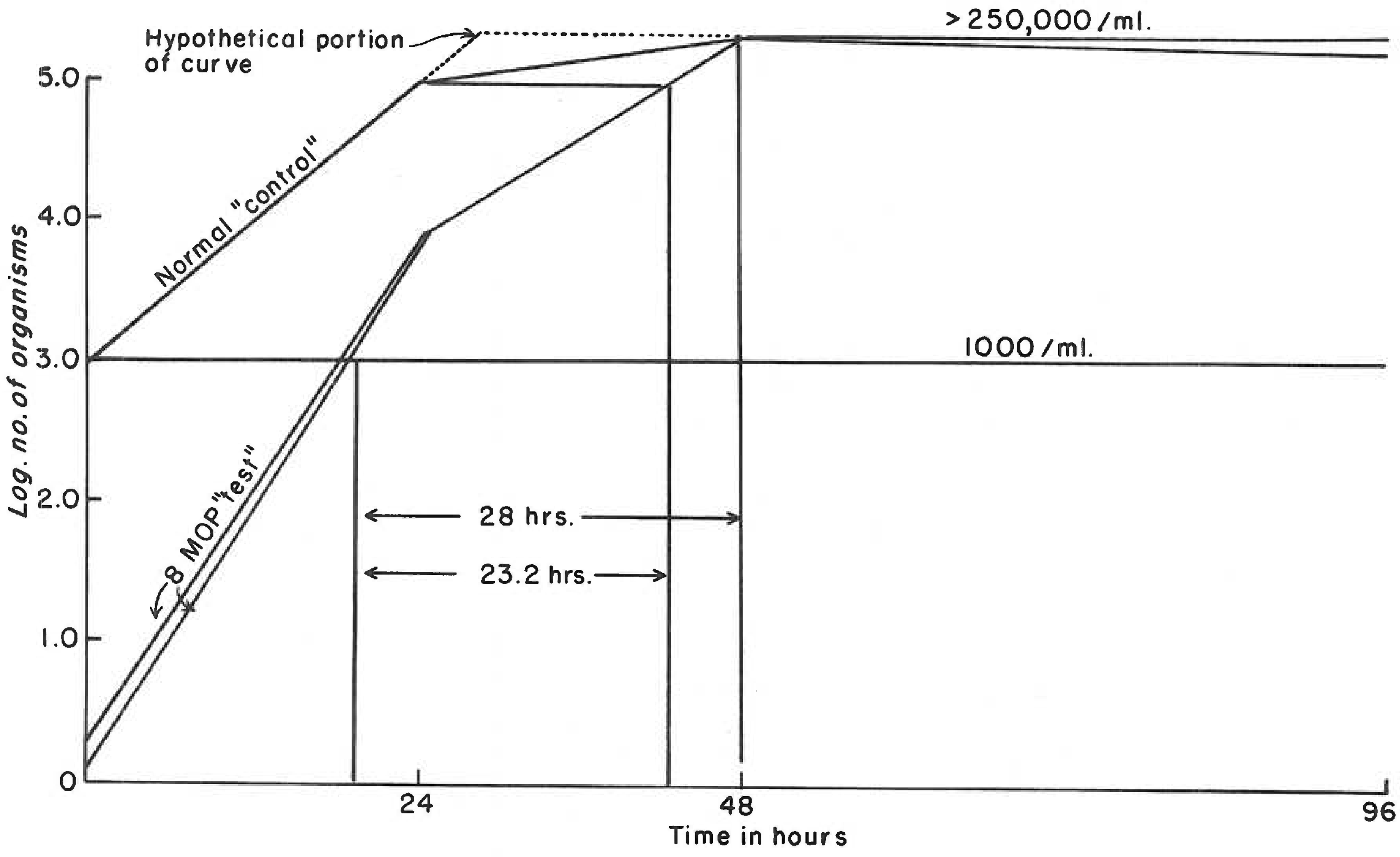

FIG. 6

Fig. 7. Log Organism-Hours plotted against concentration of 8-MOP. Each point is obtained by integrating the area under the curve of a growth curve of *Tetrahymena* cultured in the indicated concentration of 8-MOP. See also Tables 10, 11, and 12, and text, p.64.

FIG. 7

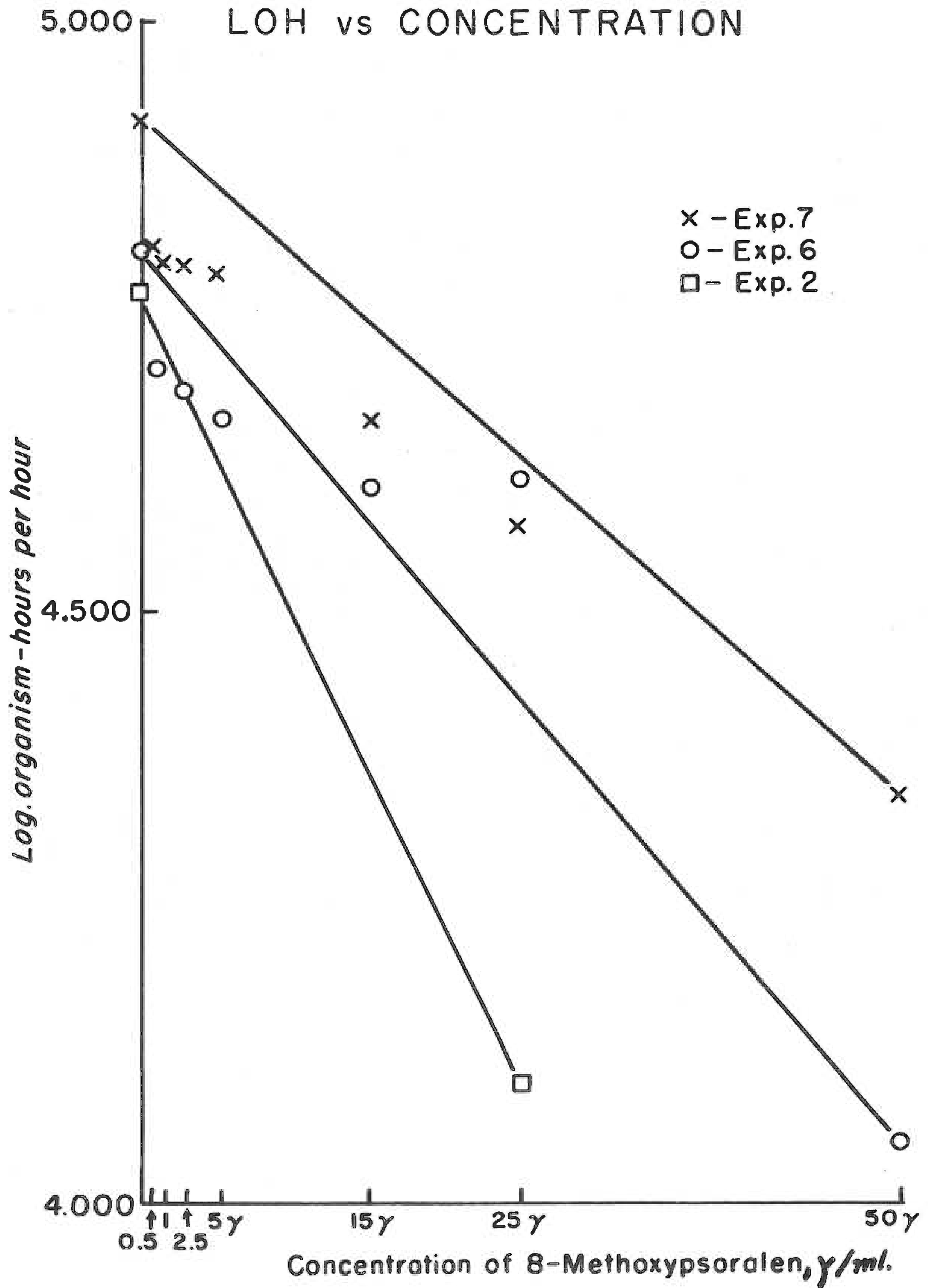

Fig. 8. Idealized growth-curve for free-living organisms in a chemostat. Fresh medium is added and a fraction of the population is being removed at a set rate.
See also text, p. 86.

FIG. 8

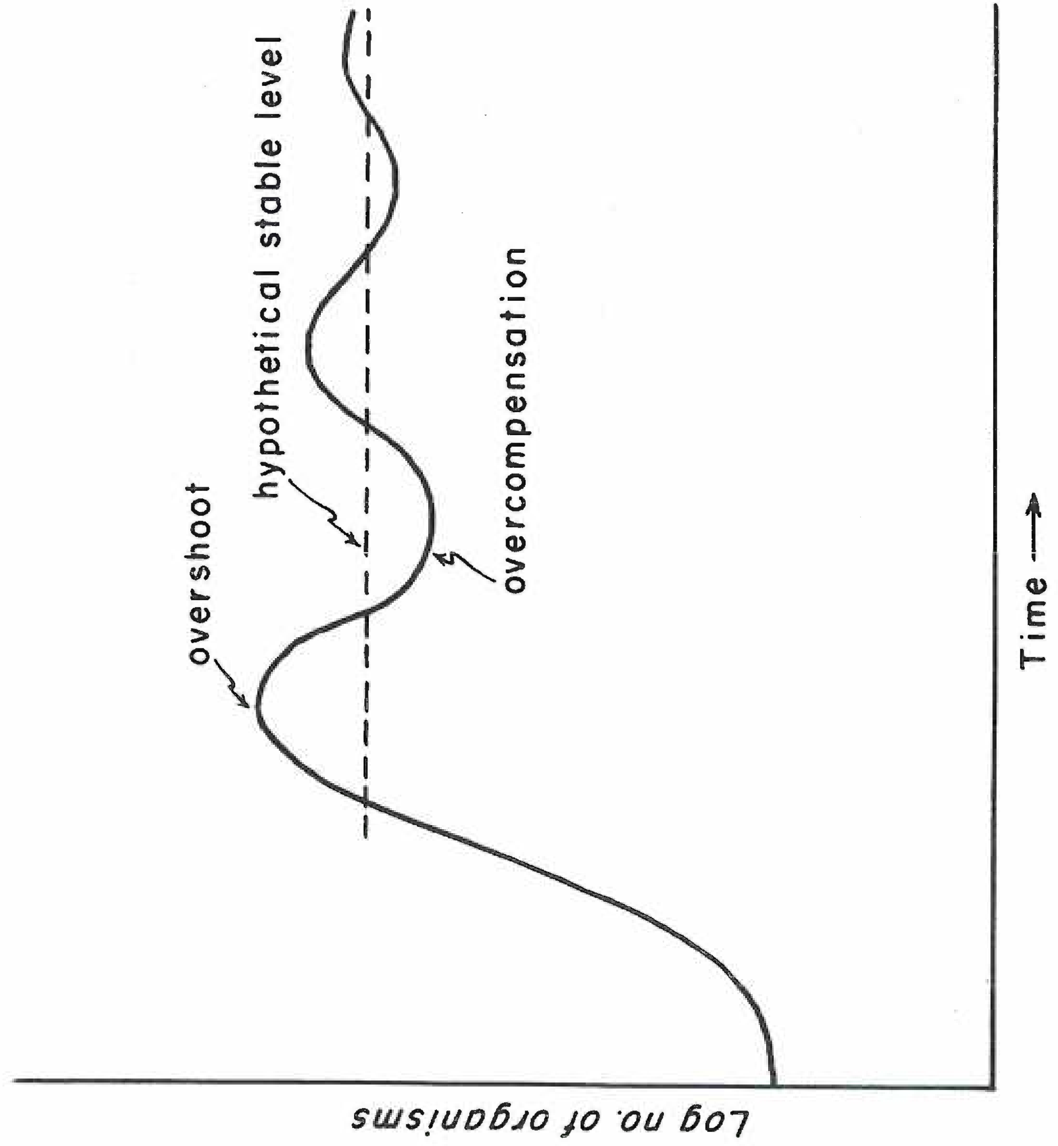

Fig. 9. Proposed design for an apparatus to be used in conjunction with a chemostat for testing a continuous range of concentrations of a given substance. See text, p. 87.

FIG. 9

APPARATUS TO INTEGRATE FEEDING OF TWO DIFFERENT MEDIA TO CONTINOUS GROWTH CHAMBER