AFAMRL-TR-84-007

THE USE OF PHYSARUM FOR TESTING OF TOXICITY/MUTAGENICITY

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APRIL 1984

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FOR THE COMMANDER

BRUCE O. STUART, PhD
Director Toxic Hazards Division
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SECURITY CLASSIFICATION OF THIS PAGE

REPORT DOCUMENTATION PAGE

1a. REPORT SECURITY CLASSIFICATION	1b. RESTRICTIVE MARKINGS
2a. SECURITY CLASSIFICATION AUTHORITY	3. DISTRIBUTION/AVAILABILITY OF REPORT
2b. DECLASSIFICATION/DOWNGRADING SCHEDULE	
4. PERFORMING ORGANIZATION REPORT NUMBER(S)	5. MONITORING ORGANIZATION REPORT NUMBER(S) AFAMRL-TR-84-007
6a. NAME OF PERFORMING ORGANIZATION Science Division Governors State University 6b. OFFICE SYMBOL *(If applicable)*	7a. NAME OF MONITORING ORGANIZATION AF Aerospace Medical Research Laboratory
6c. ADDRESS *(City, State and ZIP Code)* Park Forest South, IL 60466	7b. ADDRESS *(City, State and ZIP Code)* Aerospace Medical Division Air Force Systems Command Wright-Patterson Air Force Base, OH 45433
8a. NAME OF FUNDING/SPONSORING ORGANIZATION 8b. OFFICE SYMBOL *(If applicable)*	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER Contract Number F36615-82K-0514
8c. ADDRESS *(City, State and ZIP Code)*	10. SOURCE OF FUNDING NOS.

PROGRAM ELEMENT NO.	PROJECT NO.	TASK NO.	WORK UNIT NO.
62202 F	6302	04	24

11. TITLE *(Include Security Classification)*
The Use of Physarum for Testing of Toxicity/Mutagenicity

12. PERSONAL AUTHOR(S)
Joyce Mohberg, Ph.D.

13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT *(Yr., Mo., Day)*	15. PAGE COUNT
Final	FROM 5/1/82 TO 6/30/83	1984 April 19	40

16. SUPPLEMENTARY NOTATION

17. COSATI CODES			18. SUBJECT TERMS *(Continue on reverse if necessary and identify by block number)*
FIELD	GROUP	SUB. GR.	Key words: Physarum, toxicity testing, hydrazine, ethanol, hydrocarbons

19. ABSTRACT *(Continue on reverse if necessary and identify by block number)*

Growing and differentiating cultures of the true slime mold, Physarum polycephalum, have been investigated for possible use in testing for toxicity of several fuels--hydrazine, ethanol and hydrocarbons. Testing protocols were based on several already in the literature. Effects on growth were studied via inhibition of microplasmodial growth (Becker et al., Cancer Res. 23, 1910, 1963) and effects on differentiation by inhibition of (or interference with) plasmodial sporulation (Sauer et al., Exptl. Cell Res. 57, 319; 1969) and of amoebal flagellation (Mir and Wright, Microbios. Lett. 5, 39, 1978).

Effects on growth of microplasmodia were expressed as ED_{50} values, calculated from spectrophotometric measurements of whole culture protein, DNA or pigment. ED_{50}s (protein data) for hydrazine.2 HCl and ethanol were 40 ug/ml and 1.5% (v/v), respectively. Short, straight chain, even numbered hydrocarbons (C_6 - C_{10}) at 1% completely stopped microplasmodial growth. The C_{12} - C_{16} hydrocarbons at the same concentration did not inhibit

20. DISTRIBUTION/AVAILABILITY OF ABSTRACT	21. ABSTRACT SECURITY CLASSIFICATION
UNCLASSIFIED/UNLIMITED ☐ SAME AS RPT. ☐ DTIC USERS ☐	

22a. NAME OF RESPONSIBLE INDIVIDUAL	22b. TELEPHONE NUMBER *(Include Area Code)*	22c. OFFICE SYMBOL
Dr. Sheldon A. London	513-255-2703	AFAMRL/THB

DD FORM 1473, 83 APR EDITION OF 1 JAN 73 IS OBSOLETE.

SECURITY CLASSIFICATION OF THIS PAGE

growth and may have served as a carbon source, since they seemed to have a glucose-sparing effect.

Effects on formation of sporangia and spores were evaluated by microscopic observation and by counting spores per sporangium, measuring spore diameters and counting myxamoebae hatching from washed spores during incubation in water. The main effect of hydrazine on spore formation seemed to be to interfere with wall formation so that the number of spores per sporangium was severely depressed and spore walls were fragile. Ethanol had a somewhat similar effect and in addition blocked sporangial stalk formation if applied at the appropriate time. Both hydrazine and ethanol were active when added as long as 9 hr after the end of illumination, whereas cycloheximide and 5-bromo-2'-deoxyuridine were active only when added before or shortly after the end of the illumination period.

PREFACE

This is the Final Report on work performed under Air Force Contract F33615-82-K0514 and covers the period from May 1, 1982, to June 30, 1983. The project is entitled "The Use of Physarum polycephalum in testing for toxicity/mutagenicity" and was conducted at Governors State University, Park Forest South, Illinois.

Contract monitor was Dr. Sheldon A. London, Biochemical Toxicology Branch of Toxic Hazards Division, Air Force Aerospace Medical Research Laboratory, Wright-Patterson Air Force Base, Dayton, Ohio. Principal investigator was Joyce Mohberg, Science Division, College of Arts and Sciences, Governors State University.

The author wishes to thank Ms. Margaret Kelly for invaluable technical assistance.

INTRODUCTION

Among the environmental factors now suspected of contributing to the increased incidence of cancer and genetic defects in man are thousands of synthetic chemicals which have been released onto the market without prior testing for mutagenicity or carcinogenicity (Ames, 1979). Whole animal testing, as described in a recent paper by Weisburger et al. (1981), is generally accepted as giving results which are most validly extrapolated to humans. However, even studies with small rodents are extremely time-consuming and costly--about 3 years and $200,000 for a single dose level of a single substance--according to Ames (1979) and Heidelberger (1980). There is therefore a need for reliable, short-term tests for carcinogens, and several symposia have been devoted to the subject (Demopoulos and Mehlman, 1980; Montesano et al., 1980).

Pitot (1980) has tabulated many of the tests now in use, together with their endpoints. One of the most widely used is the Ames test, which involves reversion of histidine-requiring Salmonella mutants (Ames et al., 1975). However, since Salmonella is a prokaryote, some workers do not believe that the results have "any equivalency with the multistep, multifactorial process of carcinogenesis in eukaryotic organisms" (Sivak, 1976). Accordingly, several mammalian systems have been developed. Among them are the following: (1) the micronucleus test (Von Ledebur and Schmid, 1973), which involves treating mice with an agent and examining bone marrow smears for micronuclei (Howell-Jolly bodies); (2) the sperm head test, which consists of scoring sperm of the treated animal for morphological abnormalities (Wyrobek and Bruce, 1975); (3) host-mediated assays, which depend upon whole animals to provide the enzymes needed to convert test substances to their active forms; lymphoid cells from blood (Brewen et al., 1970) or from permanent culture (Huang, 1977) are implanted into the host animal, and, after an appropriate period of time, removed and examined for chromosome damage (Gautschi et al., 1972; Sans and Stich, 1975); (4) DNA repair or unscheduled DNA synthesis, which assesses the extent of damage to DNA from the size of single-stranded DNA in alkaline sucrose gradients; and (5) transformation of cells in culture, one of the most readily scored changes being the loss of contact inhibition so that cells begin to pile up and grow in plaques (Heidelberger, 1980).

Several lower eukaryotes have been exploited for testing for toxicity and mutagenicity. Both the yeast, Saccharomyces cerevisiae (Fahrig, 1971), and the mold, Neurospora crassa (Malling, 1972), have been used in host-mediated assays. After incubation in treated animals, the fungi were plated and screened for mutants. Das and Runeckes (1974) studied toxic and mutagenic effects of bisulfite in synchronous cultures of the flagellate, Chlorella pyrenoidosa. Dixon et al. (1979) have compared the sensitivities of several species of alga to hydrazine propellants.

The true slime mold, Physarum polycephalum, has been used in cancer research by H.P. Rusch and his co-workers for more than twenty years. Some of the same properties responsible for its selection for cancer research (Rusch, 1980) also give it potential for toxicity and mutagenicity testing. First, Physarum is a eukaryote with easily recognized chromosomes. Second, in the plasmodial stage of the life cycle, as well as several other points less well studied, there is naturally synchronous mitosis (Howard, 1931). It is also quite easily grown in a relatively simple medium consisting of tryptone, yeast extract, glucose, salts and hematin (Daniel and Baldwin, 1964). The plasmodium is uniquely suited for investigations of nucleic acid synthesis because DNA synthesis occurs during the

first third of the mitotic cycle (Nygaard et al., 1960), whereas RNA and protein synthesis continue throughout the mitotic cycle. (See Turnock, 1979, and Holt, 1980, for reviews).

Physarum has three distinct phases in its life cycle--the plasmodium, the spore and the myxamoeba--and each transition from one stage to the next provides a situation for the study of differentiation. Daniel and Rusch (1962) found that despite the complexity of the morphological changes occurring during the sporulation process (Guttes et al., 1961), the entire chain of events could be induced by simply starving a culture on a niacin-salts solution for several days and then exposing it to light. Sauer et al. (1969) found that once a plasmodium had been illuminated, the events in morphogenesis occurred at fixed times. Some of the most obvious changes were the appearance of sporangial buds at 7 hr, a synchronous nuclear division at 13 hr, spore cleavage at 13.5 hr and melanization at 14 hr after the end of illumination. Later work has shown that the nuclear division is mitotic, or at least nonreductive, and is followed immediately by DNA synthesis (Arescaldino, 1971). There are two meiotic divisions (Laane and Haugli, 1976), beginning at about 18 hours after spore cleavage and ending at about 48 hours after cleavage. After about 4 days of maturation, spores can be induced to hatch by washing and plating in distilled water. Close to the time of germination there is yet another synchronous nuclear division (Howard, 1931; Mohberg, 1977).

Amoebae which hatch from spores of heterothallic strains, such as the McArdle (M) isolates, are of two different mating types (Dee, 1960; 1982), and if they are cloned to separate the mating types, the amoebae can be cultivated indefinitely on Escherichia coli lawns on agar. Such amoebal cultures grow logarithmically, not synchronously, but the nuclear DNA content indicates that they are predominantly in haploid G_2-phase (Mohberg and Rusch, 1971; Turner et al., 1981), presumably because they have a comparatively short S-phase and no G_1-phase, like plasmodia. Both newly hatched amoebae and amoebae growing on bacteria can be induced to flagellate by washing and suspending in water or buffer (Aldrich, 1967; Mir and Wright, 1978).

When amoebae of different mating types are recombined, they mate to form a diploid zygote and this in turn develops into a diploid plasmodium. Amoebae of the Colonia strain (Wheals, 1970), however, have a single mating type and can form plasmodia within clones, a haploid amoeba developing into a haploid plasmodium (Cooke and Dee, 1974).

Although Physarum amoebae are quite resistant to mutagens, Haugli (1971) found that mutations could be obtained by treating log phase cells with N-methyl-N-nitro-N-nitrosoguanidine (NMG) or with ethyl-methane-sulfate (EMS). Mutations obtained thus far which are expressed in the amoebal stage include acquisition of resistance to cycloheximide (Haugli et al., 1972) and to bromodeoxyuridine (Haugli and Dove, 1972) and of sensitivity to elevated temperature, resulting in nucleolar vacuolization (K. Haugli et al., 1977), prolonged metaphase (Wheals et al., 1976) or inhibition of cytokinesis (Burland et al., 1981). Apogamic amoebae can also lose their capacity for clonal development of plasmodia (Anderson and Dee, 1977). Mutations which are expressed in the plasmodium include the acquisition of resistance to cycloheximide (Haugli et al., 1972) and to bromodeoxyuridine (Lunn et al., 1977; Mohberg et al., 1980) and of requirements for certain amino acids--valine (Dee et al., 1973) and leucine and lysine (Cooke and

Dee, 1975); and the loss of the yellow pigment (Anderson, 1977) with is characteristic of wild type strains.

Toxic substances have been used in many studies of Physarum, but in most cases both the substance and the test system were incidental to a larger project. A typical example is 5-fluoro-2'-deoxyuridine (FUDR), which has been widely used to "probe" nucleic acid biosynthetic pathways and to perturb the nuclear division cycle. (See Sachsenmaier and Rusch, 1964, and reviews by Turnock, 1979, Holt, 1980, and Tyson, 1982). A few laboratory groups have, however, focussed on the development and evaluation of the test systems themselves and some of their publications are summarized in the following section.

Becker et al. (1973) described a system for screening for anti-tumor activity by means of inhibition of growth of microplasmodia. Toxicity was measured in terms of the level needed to give 50 percent inhibition of growth, as estimated from pigment or protein content. Sachsenmaier used the same procedure to bracket concentrations to be tested with macroplasmodia (personal communication). He then determined effects of the agent on the timing of mitosis and on synthesis of DNA, RNA and protein in the synchronous plasmodium, where DNA synthesis is stepwise and RNA and protein synthesis essentially logarithmic (Sachsenmaier and Rusch, 1964).

Goodman et al. (1976) adopted the Physarum plasmodium for use in evaluating the long term effects on growth of exposure to extremely low frequency electromagnetic fields. Chin et al. (1978) has devised a system for studying the effects of subthreshold levels of heavy metals, using the plasmodium as test organism. Both groups score effects via mitotic delay. Cummins et al. (1976), on the other hand, used alkaline sucrose gradients to assess the effect of methyl mercury on plasmodial DNA.

Terayama et al. (1978) have taken yet another approach to evaluation of toxicity. They have measured effects on membrane potentials and on motive force of protoplasmic streaming and have found an essentially linear relationship between levels of heavy metals and of insecticides which are required to stop protoplasmic streaming and the LD_{50}'s of these substances in rodents. (For a review of the apparatus used by Terayama's group, see Ueda and Kobatke, 1982).

Sauer et al. (1969) chose the sporulating plasmodium for development of a model system for the study of differentiation because "it contains many of the characteristics of differentiation--competence, commitment...(and)...morphogenesis." Sauer and his co-workers used the Daniel and Rusch method for inducing fruiting and characterized the system by treating cultures with inhibitors of nucleic acid and protein synthesis and noting effects on fruiting efficiency and sporangial morphology. They found that periods of DNA and protein synthesis were entrained with the morphological events. (For a recent review, see Sauer and Pierron, 1983).

Wright's group (Mir and Wright, 1978; Mir et al., 1979) have been interested in agents which interfere with formation of microtubules and have devised a test system based on the myxamoeba-flagellate transition.

The present project was begun with the ultimate objective of devising test systems for screening chemicals for toxic and mutagenic effects using Physarum. Our progress to date has consisted of testing several of the published systems--

microplasmodial growth (Becker et al., 1963), sporulation (Sauer et al., 1969), and amoebal flagellation (Mir and Wright, 1978). We have been particularly interested in the sporulation system because, first, after the original work was done, it was found that some M_3 sublines can form sporangia without undergoing spore cleavage (Mohberg and Rusch, 1971; Mohberg and Babcock, 1982); and, second, we wanted to find quantifiable endpoints other than fruiting efficiency. This paper presents (1) data on performance and reproducibility of the original procedures; (2) modifications to improve reproducibility; (3) ED_{50}'s of hydrazine. 2 HCl, ethanol and C_6 through C_{16} straight chain, saturated hydrocarbons with growing microplasmodia; (4) preliminary results of attempts to use measurements of spore diameter and of hatchability to assess toxicity; and (5) effects of hydrazine and ethanol on the timing of mitosis in plasmodia and on morphogenesis of sporangia and spores.

MATERIALS AND METHODS

Isolation of Plasmodial Strains: M_3b"F2" was obtained by passing M_3b, a derivative of Wis 1, through the life cycle twice by mass plating of large numbers of spores on one-tenth strength plasmodial medium (Daniel and Baldwin, 1964) in 1% agar, as described by Mohberg et al. (1973). Nothing is known of the genetics of M_3b"F2" except that it is a diploid (Mohberg et al., 1980) and is presumably a mating type (mt) 1 x mt 2 cross, since these are the only mating types found in Wis 1 sublines (Dee, 1973).

LU647 x LU5001d was isolated at the University of Leicester, England, in 1974 in collaboration with D.J. Cooke. Parent amoebae were strain LU647, which carries the genetic markers of mt 1, plasmodial fusion types fus A_1 and fus B_1; and LU5001d, which carries the markers of mt h (which enables it to form plasmodia within clones) fus A_1 and B_1, and leu-, which causes it to require exogenous leucine. Both amoebal strains have the Colonia background. (For more details see Cooke and Dee, 1975). LU647 x LU5001d grows rapidly and spherulates and sporulates readily. It had been stored as spherules from the time it was isolated until this project was started.

Culture conditions: Stock cultures of microplasmodia were grown on a reciprocating shaker in 250-ml conical flasks, containing 15 ml of "N + C" medium (nutrient medium with citrate", Daniel and Baldwin, 1964), supplemented with bovine hematin.

Experimental microplasmodial cultures were grown in 25-ml conical flasks containing 5 ml of medium. They were shaken at 120 strokes per min (1-inch stroke length) on a Model R2 reciprocating shaker (New Brunswick Scientific Co.). In most experiments, Brewer's medium (Brewer, 1975) was used instead of N + C because it contains only tryptone, yeast extract, dextrose, $MgCl_2$ and citrate and no added trace metals and should therefore be less destructive to such test substances as hydrazine.

In earlier experiments inoculum was 0.1 ml per flask of a 1-day, 15-ml shaken culture, started from 5 ml of a 3-day stock culture. This was found to give inoculum levels which varied by as much as a factor of three (in terms of protein content) from one experiment to another. In later work packed microplasmodial volume was monitored. Inoculum cultures were centrifuged for 15 sec at about half speed in a table model, clinical centrifuge, as for preparation of plasmodia. Packed volume was noted and supernatant volume adjusted by removal of medium or addition of sterile water so that the supernatant: pellet volume ratio was about 12:1. The

mixture was then decanted into a 50-ml conical flask and swirled to resuspend microplasmodia. (It was essential to use the old medium for resuspension because its viscosity kept microplasmodia from settling during pipetting, as they did if suspended in water.) One tenth milliliter of inoculum suspension contained approximately 100 μg of microplasmodial protein.

Plasmodia were started in 9-cm pyrex petri dishes on hard-surfaced filter paper (Whatman #40), supported on stainless steel grids, using inoculum grown as described by Mohberg and Rusch (1969). Mitotic stage was determined by examining alcohol-fixed smears with a phase contrast microscope (Mohberg, 1982). For drug treatment, plasmodia which were still attached to filter papers were transferred to fresh petri dishes containing glass beads (5-mm) instead of grids (to eliminate possible effects of metals on test substances) and medium prepared with or without test agent.

Sporulation of Plasmodia: Replicate plasmodia were started on nutrient medium, either N + C or Brewer's, in petri dishes on filter papers, supported by stainless steel grids. When plasmodia had reached a diameter of about 8 cm (after 36 to 48 hours of growth), filters and plasmodia were transferred to dishes containing grids and niacin-salts-carbonate medium (Daniel and Baldwin, 1964). After incubation at 24°C in the dark for 3 or 4 days, cultures were illuminated for 4 hr in a Model 808 incubator (Precision Scientific Co.), equipped with "cool white" fluorescent lamps, mounted about 10 cm above the dishes. Temperature was kept between 24°C and 29°C. Cultures fruited during the 12 hours following illumination and were completely melanized by 14 hours, as observed by Sauer et al. (1969). Since melanization is completed within about 15 min and is an unmistakable end-point, drug exposure time periods were recorded in terms of hours of exposure prior to melanization.

Fuel treatment was accomplished by transferring starved, illuminated plasmodia, still attached to filter papers, to fresh niacin-salts-carbonate medium, made with or without test substances. Again, to avoid possible effects of metals in the stainless steel grids, filters were supported on glass beads. To improve reproducibility and reduce the amounts of substances used, plasmodia which were well-spread over filter paper were cut into thirds or fourths with sterile scissors. Segments were then put onto control and experimental media. Cultures were left on experimental media until analyzed (in 1 or 2 weeks) because it was assumed that once the sporangium and stalk have formed and hardened, no further uptake of materials in the medium is possible.

Preparation of Test Substances: Test agents used were of analytical grade and suppliers were as follows: ethanol, U.S. Industrial Co.; hydrazine dihydrochloride Fisher Chemical Co.; hydrocarbons, Alltech Co. and Theta Corp. Except for ethanol and hydrazine, which were assumed to be self-sterilizing, water-soluble chemicals were sterilized by filtration through Metricel TCM-450 (0.45 μm pore size). Hydrocarbons were filtered through Metricel GA-6 membranes and were suspended in medium by sonication for 10 min in a Mettler Electronic Ultrasonic cleaner. All test substances were added to medium immediately before inoculation and to plasmodia either immediately before mitosis II or at the beginning of G_2-phase (= 3 hr after MII).

Analytical methods: Hydrazine was analyzed by the method of Reynolds and Thomas (1964). Microplasmodia were analyzed for pigment by means of 415 nm absorbance of 5% trichloroacetic acid (TCA)-acetone extracts, as described by Becker et al. (1963). After pigment had been removed, cultures were extracted with hot 0.5 M perchloric acid (PCA) to remove nucleic acids, which were then analyzed for DNA and

RNA by the methods of Burton (1956) and of Ceriotti (1955), respectively. PCA-extracted residues were dissolved in 0.4 N NaOH and analyzed for protein according to Lowry et al. (1951). In every experiment duplicate 1.0- or 1.5-ml aliquots of inoculum were mixed with TCA-acetone and saved for analysis with the cultures. ED_{50}s of test substances were estimated from graphs of logarithms of dose vs. percent of control growth (=3-day test culture minus inoculum/control minus inoculum/100). Glucose in medium was quantitated by means of the hexokinase+glucose-6-phosphate dehydrogenase procedure, using a Sigma kit (Sigma Chemical Co.)

Counting and Sizing Spores: Estimates of spores per sporangium were made by picking ten sporangia from a culture with the aid of a low-power magnifying light and crushing them in 2 ml of distilled water by means of 3 or 4 strokes with a manually driven Potter-Elvehjem homogenizer with Teflon pestle. Spores were then counted in a hemacytometer. Well-spread plasmodia gave 100,000 to 250,000 spores per sporangium. Spore diameters were measured by photographing spores in a hemacytometer on the area with the 50- x 50-μm grid, then measuring spores and grid on a high-magnification print.

Determination of Spore Viability: Spore viability was assessed by (1) incubating washed spore suspensions at room temperature for 5 to 18 hours and counting myxamoebae and flagellates in a hemacytometer; or (2) plating known numbers of spores (50 to 5000) with a slow-growing strain of E. coli (a gift from the McArdle Laboratory, University of Wisconsin) on liver infusion agar (LIA), according to Dee and Poulter (1970), and counting amoebal plaques after 4 to 8 days of incubation at 24°C. Density of the E. coli lawns was controlled by suspending bacteria (from a 100-mm, 24-hr nutrient agar plate) in sufficient water (about 8 ml) so that 0.2 ml, diluted with 5 ml of water, gave an absorbance of 0.2 to 0.25 at 600 nm in 13-mm cuvettes in a Bausch and Lomb Spectronic 20 spectrophotometer. Bacteria were then mixed with an equal volume of counted spores and 0.2-ml aliquots were spread on plates, using a glass spreader.

Estimation of Degree of Flagellation: The test organism for this section was isolated by seeding LU647 x LU5001d spores on LIA, cloning several plaques, and selecting the clone which gave the most vigorous growth. Amoebae to be used for testing were grown on E. coli lawns (prepared as for plating spores) until late log phase, by which time there were 5 to 6 million cells per plate. Cells were scraped from agar by means of a glass spreader, washed free of bacteria by suspending in water and centrifuging (400 g, 10 min, 0 to 4°C). Washed cells were finally suspended in sufficient phosphate buffer (KH_2PO_4/K_2HPO_4, 0.02 M, pH 6.2) to give 2 to 4 x 10^5 cells per ml, and 5-ml portions were dispensed into 25-ml conical flasks. Test substance (25 μl of solution) was added and flasks were put on a shaker and shaken at slow speed for 2 hr. At 15, 30, 60, 90 and 120 min a 0.2-ml aliquot was removed and mixed with 50 μl of Lugol's iodine solution to kill cells and stain flagella. After all samples had been collected, each was examined in a hemacytometer. In early experiments, differential counts were performed by scanning the slide until 100 cells had been examined, but in later work we simply counted the myxamoebae, flagellates, encysted cells and "other" (usually cell fragments and unidentified particles) per 0.1 mm^3.

RESULTS AND DISCUSSION

TESTING WITH GROWING CULTURES

Effects of Growth Conditions: Becker et al. (1963) tested anti-tumor agents against microplasmodia, growing in 10 ml N + C medium in 25-mm diameter test tubes on a rotary shaker. Under their conditions an inoculum of about 0.25 mg protein per ml reached 3 mg per ml in 90 to 96 hours. When we tried test tubes on a reciprocating shaker, we obtained no growth, even with 5 ml of medium in slanted tubes, apparently because agitation was not sufficient to keep microplasmodia suspended and aerated. However, when we changed to 25-ml conical flasks, containing 5 ml of medium, an inoculum of 0.04 to 0.06 mg per ml reached 1.2 to 2.4 mg in 3 days (Table 1). (Note that data in tables are in terms of μg protein per 5 ml of culture.)

In the experiments shown in Table 1, inoculum was defined only in terms of volume and age of the culture (0.1 ml of 24-hr culture.) This gave considerable variability from one experiment to another. Since some of the substances tested were toxic only to low levels of inoculum, in later experiments packed microplasmodial volume was monitored, as described in Materials and Methods, and the inoculum suspension adjusted so that 0.1 ml contained about 0.1 mg of protein.

M_3b"F2" microplasmodia, at least in short term experiments, seemed to grow as well in Brewer's as in N + C medium with either hematin or hemoglobin supplementation (Table 1). Replication of cultures was poor (Experiment 1) if cultures which had been freshly grown from spherules were used as inoculum before they had had several passages in shaken culture to disperse clumps. However, once a microplasmodial culture was established, replication was within ± 10% (Experiments 2 and 3). Effects of aging should have been negligible, since fresh cultures were grown up from spherules at roughly 3-month intervals.

Effects of Hydrazine: Addition of hydrazine. 2 HCl (Hz) to different media gave the data shown in Table 2. Tentative conclusions, based on the 90 μg Hz cultures, were that (1) hemoglobin may have had a protective effect, particularly in Experiment 2, where the increase in protein is 63% in N + C + Hb and 34% in N + C + H; and (2) none of the three microplasmodial components which were measured seemed specifically inhibited by hydrazine, although in three of the five tests DNA synthesis was inhibited 15 to 20 % more than were synthesis of protein and pigment.

When Hz.2 HCl concentrations in the range of 15 to 45 μg per ml were tested (Figure 1), both M_3b"F2" and LU647 x LU5001d had ED_{50}'s of about 40 μg per ml. Inhibition reached a maximum of about 80% at 90 μg per ml and did not increase further with doses up to 300 μg per ml. Another experiment showed that 450 μg per ml bleached the inoculum and apparently lysed it, since the 3-day protein value was lower than that of the inoculum.

Chemical analyses (Reynolds and Thomas, 1964) of spent medium from the experiment of Table 2 showed that an initial concentration of 30 μg per ml had dropped below the level of detection after 3 days of growth, and 90 μg per ml had fallen to about 10 μg. From these results it appeared that either the analytical procedure did not give valid data in the presence of Physarum medium or that hydrazine was being degraded by microplasmodia or by a component of the medium. The

TABLE 1. GROWTH OF MICROPLASMODIA IN DIFFERENT MEDIA.

M_3b"F2" microplasmodia were grown in 25-ml flasks in 5 ml of medium, "N + C" or Brewer's (B), supplemented with hematin (H) or hemoglobin (Hb). Each flask was inoculated with 0.1 ml of 24-hr suspension culture. Growth was measured by Lowry analysis of inoculum and of microplasmodia at 3 days. "Increase" = total 3-day protein minus inoculum, and "Fold Increase" = increase in protein/inoculum protein.

Expt.	Medium	Inoculum	3-Day Microplasmodia	Increase	Fold Increase
		μg protein per 5-ml culture			
1	N + C + Hb	162 185 174 ± 11.5	5,747 4,689 7,218 5,885 ± 1,037	5,711	32.8
2	N + C + H	273 339 309 ± 30	13,230 11,464 13,200 12,631 ± 825	12,322	38.6
2	N + C + Hb	Same as above	11,464 12,044 13,812 12,440 ± 996	12,131	39.3
3	B + H	381 384 382.5 ± 1.5	8,594 9,328 9,271 ± 284	8,888	23.2
3	B + Hb	Same as above	7,643 7,930 8,659 8,077 ± 428	7,694	20.0
3	N + C + H	Same as above	7,625 9,641 9,469 8,912 ± 912	8,529	22.3

TABLE 2. EFFECTS OF HYDRAZINE DIHYDROCHLORIDE ON PROTEIN, PIGMENT AND DNA OF M_3b"F2" MICROPLASMODIA

In Experiments 2 and 3 of Table 1, duplicate flasks were prepared with 30 or with 90 µg per ml Hz.2 HCl in both N + C + H and N + C + Hb. After 3 days of growth, microplasmodia were analyzed for protein, pigment and DNA. Results are expressed as percent of the increase of the appropriate controls.

Medium	Protein		Pigment		DNA	
	Expt. 2	Expt. 3	Expt. 2	Expt. 3	Expt. 2	Expt. 3
	% of control increase in 3 days					
N + C + H:						
Control	100	100	100	100	100	100
+ 30 µg/ml Hz	64.9	67.0	62.3	73.4	62.7	79.5
+ 90 "	33.6	52.8	21.3	64.9	33.9	60.9
N + C + Hb:						
Control	100	N. D.[a]	100	N. D.	100	N. D.
+ 30 µg/ml Hz	73.6	"	88.5	"	75.3	"
+ 90 "	63.4	"	58.6	"	48.3	"
B + H:						
Control	N. D.	100	N. D.	100	N. D.	100
+ 30 µg/ml Hz	"	81.5	"	85.5	"	79.0
+ 90 "	"	53.8	"	51.8	"	41.5
B + Hb:						
Control	N. D.	100	N. D.	100	N. D.	100
+ 30 µg/ml Hz	"	106	"	106	"	91.6
+ 90 "	"	64.3	"	64.3	"	53.5

[a] Not done.

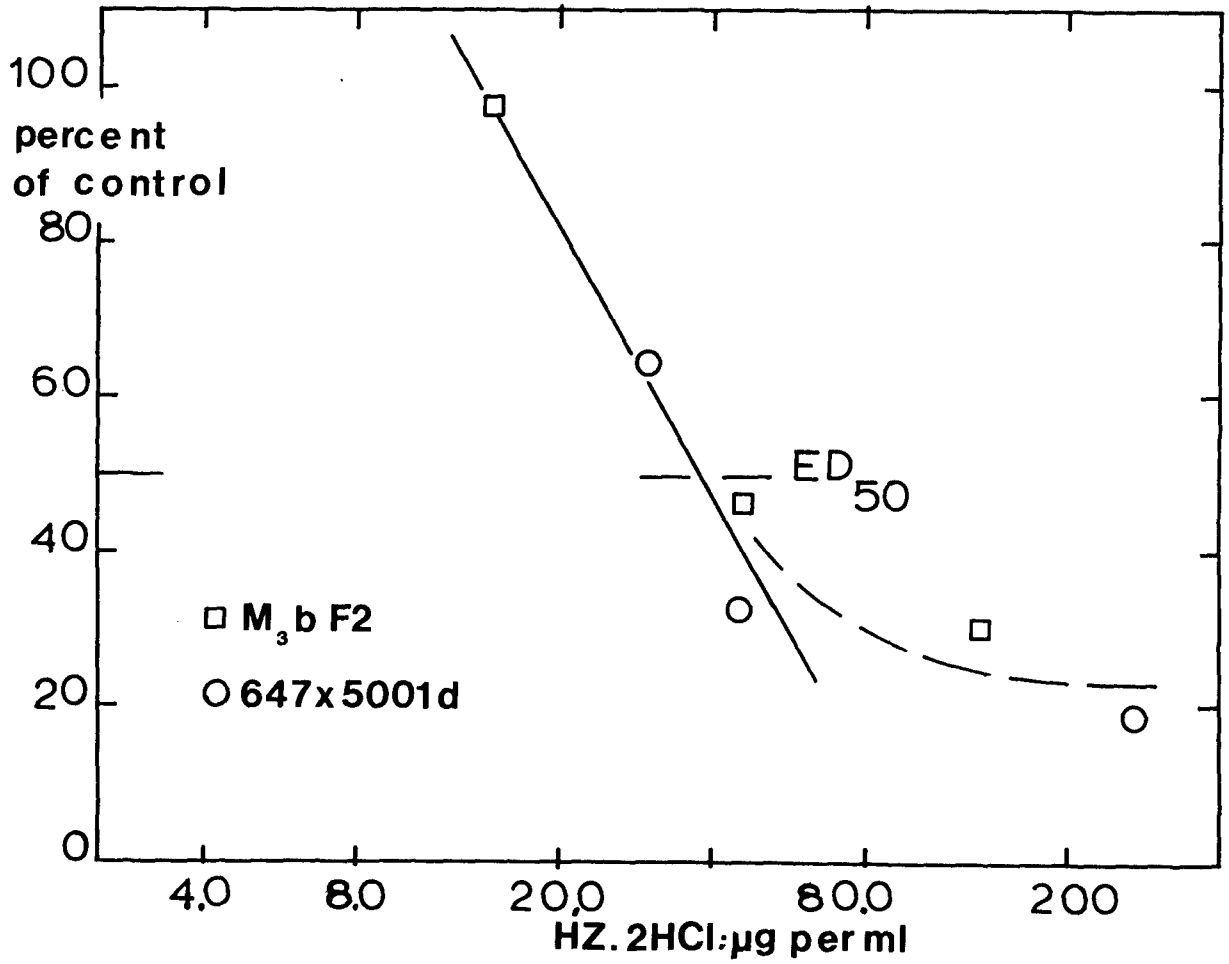

Figure 1. Log dosage curve for hydrazine dihydrochloride vs. M_3b"F2" and LU647 x LU5001d microplasmodia.

Microplasmodial cultures were exposed to different levels of hydrazine. 2 HCl and analyzed for protein after 3 days of growth. Data are plotted as percent of control increase vs. $\log_{10}$ of the dosage of Hz.

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following experiments were done to clarify this point:

(1). Analysis of hydrazine in the presence of medium. Brewer's medium gave a slight color with the dimethylaminobenzaldehyde color reagent which was used to quantitate hydrazine but it did not interfere with the reaction with hydrazine, for standards were identical in the presence and absence of medium, provided that the appropriate medium blank was subtracted. Not surprisingly, spent control medium contained material which gave a color reaction with dimethylaminobenzaldehyde. This reading was equivalent to 1 to 2 µg of Hz.2 HCl and was subtracted from the values for Hz media in preparing Table 3.

(2). Preincubation of medium with hydrazine. In order to learn whether the

medium was destroying Hz, media containing 0 to 50 μg per ml Hz.2 HCl were analyzed for Hz at 0, 1, 2, 3 and 4 hours after preparation. At these same times, shaken cultures (5 ml medium with 80 μg inoculum protein per flask) were started. After 3 days of growth, media were analyzed again for Hz. Analyses of uninoculated media at 0 to 4 hours after preparation showed no systematic loss of Hz, for the five media read 42.9, 49.4, 49.4, 48.7 and 46.9 μg per ml. Likewise, there were no significant differences among the media after 3 days of microplasmodial growth, for the Hz.2 HCl level in all flasks was between 19.1 and 23.7 μg per ml and the protein increase was 53 to 58%, relative to control growth.

(3). Use of higher levels of inoculum. Results in Table 3 were obtained when media containing 0, 30 and 90 μg per ml of Hz.2 HCl were inoculated with three levels of inoculum. An initial concentration of Hz of 90 μg per ml decreased to about 12 μg in 3 days when the inoculum was equivalent to 0.11 mg of protein and the final protein yield was 2.5 mg per flask, and to 7 μg per ml when the inoculum was 0.33 mg and the final yield was 10 mg per flask. Why both 30 and 90 μg per ml Hz.2 HCl reached the same final value of 6.5 μg per ml is not known.

Increasing the amount of inoculum not only caused a greater loss of Hz but also raised the ED_{50}, which was about 60 μg per ml with 0.11 mg protein, 120 with 0.22 mg and 200 with 0.33 mg. Presumably, the poor agreement between the value for 0.11 mg in this experiment and the data for the same level of inoculum in Figure 1 is due to the fact that 90 μg is beyond the range of linearity in the log dosage curve.

From the above results it appears that microplasmodia destroy hydrazine, but we have not followed the time course of the degradation to determine whether the inoculum "titrates" Hz at t_0, or whether a fraction of the microplasmodia is constantly reacting with Hz as the culture grows.

Since data for Hz-treated microplasmodia suggested that DNA synthesis was slightly more sensitive to Hz than were protein and pigment synthesis, Hz was tested with plasmodia. When the agent was added immediately before mitosis, so that it was present throughout the S-phase, 6 hours of treatment resulted in a small (15 percent) inhibition of protein and RNA synthesis, whereas DNA synthesis was not affected (Table 4). When Hz was added at 3 hours after mitosis, by which time the bulk of DNA synthesis should have occurred, 6 hours of exposure had little effect (8 percent or less) on any of the three polymers.

It has been shown with mice (Back and Thomas, 1964) that arginine and ornithine protected against hydrazine toxicity. However, when arginine was added to Physarum medium in 1.5:1 molar ratio to hydrazine, results were equivocal, with arginine having no effect in one experiment and improving growth of both control and Hz cultures in another, so that the ED_{50} was the same in the presence and absence of arginine. We also tried adding NH_4Cl in equimolar amounts to the NH_3 that would be released by 15, 45 and 135 μg Hz.2 HCl per ml, assuming that every molecule of Hz broke down to give two molecules of NH_3. All NH_4^+-treated cultures showed a slight (10%) depression in growth, suggesting that the toxicity of Hz could be only partially due to NH_3 formation.

Effects of NDFDA: Microplasmodia were quite sensitive to NDFDA nonadecafluorodecanoic acid), and 18 μg per ml gave a 50% inhibition of growth (Figure 2).

TABLE 3. EFFECT OF INOCULUM SIZE ON STABILITY OF HYDRAZINE*

Treatment	Inoculum level; mg					
	0.11		0.22		0.33	
	Hz µg/ml	protein mg	Hz µg/ml	protein mg	Hz µg/ml	protein mg
Control	1.3	6.24	1.5	9.97	1.5	14.32
30 µg/ml Hz	7.1	4.28	6.6	9.19	6.5	12.62
90 µg/ml	12.3	2.53	8.9	5.83	6.7	9.54

*Brewer's medium was prepared with 0, 30 and 90 µg per ml Hz.2 HCl, six flasks with each medium. Two flasks of each medium were inoculated with 0.1 ml (=0.11 mg protein), two with 0.2 ml and two with 0.3 ml of inoculum. After 3 days of growth, cultures were harvested and all media were analyzed for hydrazine. "Apparent" hydrazine of controls was subtracted from raw data for Hz cultures in order to correct for non-hydrazine, dimethylaminobenzaldehyde-positive substances released by growing cultures.

However, when the compound was tested with macroplasmodia, growing on filters on liquid medium, 100 µg per ml added as long as 4 hours before mitosis had no effect on the timing of the next mitosis. It also had no effect on growth, as judged from increase in culture diameter, for at least 24 hours. After that time, the plasmodium ceased to expand but showed no visible signs of damage. NDFDA at a concentration of 400 µg per ml had no effect on sporangial or spore formation, as described later. We now suspect that this lack of activity against stationary cultures may have been due to precipitation of NDFDA, possibly as a Ca^{+2} salt, for NDFDA was very toxic to plasmodia when it was incorporated into Brewer's medium, which was then solidified with agar.

Effects of Ethanol: Daniel and Baldwin (1964) showed that P. polycephalum could use ethanol (ETOH) as a carbon source when glucose was not available. Growth was slower than with glucose, however, and in the presence of 1% (v/v) ETOH, growth at 157 hours was about 90% of that obtained in 72 hours with glucose. When we found that ETOH interfered with spore cleavage, as shown in a later section, we rechecked the effect on microplasmodia and obtained the results shown in Figure 3. With an inoculum of 0.1 mg protein, the ED_{50} was about 1.5% (v/v), whereas with an inoculum of 0.3 mg, it was about 4%. Furthermore, ETOH at 1 and 1.5% appeared to improve growth slightly, suggesting that it was being used as an auxiliary carbon source. We therefore tried to induce greater ETOH utilization by giving microplasmodia four passages in medium containing 2% ETOH and the usual 1% glucose. Cultures were then inoculated into media containing ETOH and decreasing amounts of glucose. Apparently no induction was achieved, for there was no improvement in the ability of the culture to grow with or without ETOH in the presence of glucose, or to grow on ETOH in the absence of glucose.

TABLE 4. EFFECT OF HYDRAZINE ON SYNCHRONOUS CULTURES (PLASMODIA)

Replicate M_3b"F2" plasmodia (16) were grown on nutrient medium until Mitosis II minus 30 min. They were then transferred to fresh medium, containing 0 or 150 µg per ml Hz.2 HCl. Duplicate cultures were harvested at MII + 2, 4 and 6 hours and analyzed for protein, RNA and DNA. Results are expressed both as ug and as % of the appropriate control.

Treatment	Protein		RNA		DNA	
	µg	%	µg	%	µg	%
CONTROLS						
M - 0.5 hr	5,552		968		78.0	
	5,256		1,074		74.0	
	5,404	---	1,021	---	76.0	---
M + 2 hr	6,942		1,353		130.6	
	6,176		1,210		123.3	
	6,559	100	1,281	100	127	100
M + 4 hr	7,802		1,482		147.9	
	7,720		1,478		146.9	
	7,761	100	1,480	100	147	100
M + 6 hr	7,775		1,770		134.8	
	8,486		2,060		157.7	
	8,131	100	1,915	100	146	100
+ HYDRAZINE						
M + 2 hr	6,658		1,211		128.8	
	6,723		1,270		131.9	
	6,691	102	1,241	97	130	102
M + 4 hr	6,689		1,468		144.4	
	6,848		1,561		146.3	
	6,764	87	1,515	102	145	99
M + 6 hr	7,090		1,726		144.8	
	6,899		1,491		146.0	
	6,995	86	1,608	84	145	100

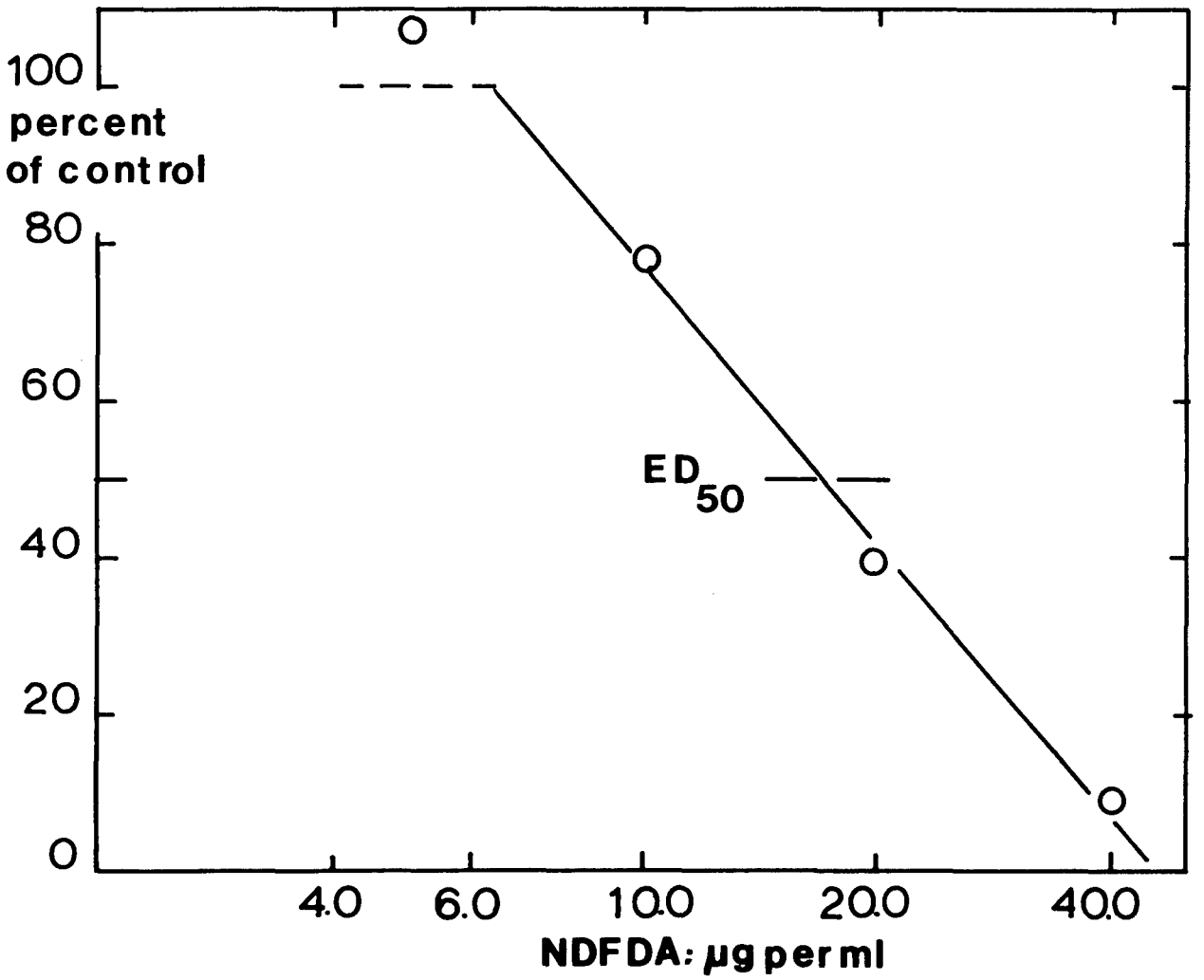

Figure 2. Effect of nonadecafluorodecanoic acid on growth of M_3b"F2" microplasmodia.

A stock solution of NDFDA was made by dissolving 8 mg in 1 ml absolute ethanol (ETOH). This was further diluted 1 + 1, 1 + 3 and 1 + 7 with ETOH. Aliquots of 25 µl were added to 5-ml portions of medium, giving 0.5% (v/v) ETOH. in all flasks and 40, 20, 10 and 5 µg/ml NDFDA. Two sets of triplicate controls were made, one with 0 and one with 25 µl ETOH. Inoculum was 165 µg protein per flask and the mean 3-day control protein values were 9220 (without ETOH) and 8550 µg (with ETOH).

Effects of Hydrocarbons: Exposure to hydrocarbons was tested with the expectations that, first, they might be toxic to low levels of inoculum but serve as a carbon source to higher levels; and, second, that they would be metabolized as 2-C fragments, if they were indeed utilized. We therefore chose straight chain hydrocarbons with even numbers of carbons and tested them vs. two levels of inoculum. Table 5 gives the results. Some of the general trends were that (1) toxicity decreased with chain length, 1% octane and 1% hexane (not shown) completely

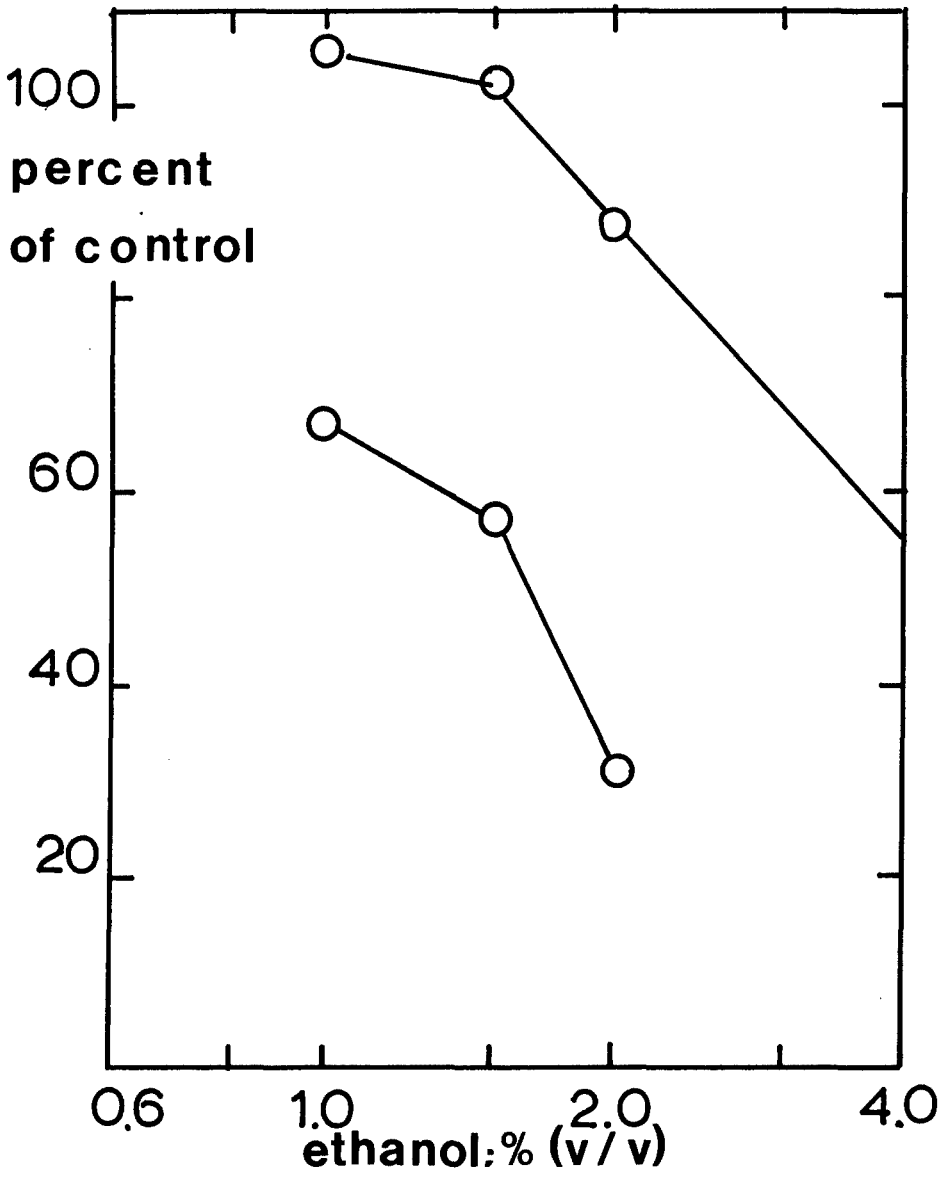

Figure 3. Log dosage curves for ethanol vs. two levels of M_3b"F2" inoculum.

Quadruplicate flasks were prepared with 0, 1.0, 1.5 and 2.0% (v/v) ETOH. Two flasks of each set received 0.1 ml inoculum (= 0.10 mg protein) and two received 0.3 ml (= 0.30 mg). The increase in protein in 3 days, as % of the appropriate control, is plotted vs. the log of the ethanol concentration.

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destroying an inoculum of about 0.3 mg protein, and do-, tetra- and hexadecane allowing essentially normal growth; (2) the growth of replicates could vary widely when dosages were close to the toxic level, as with 0.5% dodecane and the high inoculum, for example; (3) an increase in the amount of inoculum gave little protection against octane and decane, but was quite helpful against the C_{12}

TABLE 5. EFFECTS OF STRAIGHT CHAIN HYDROCARBONS ON GROWTH OF MICROPLASMODIA

Hydrocarbons, sterilized by filtration through Metricel GA-6 membranes, were added to 25-ml culture flasks, containing 5 ml of Brewer's medium. Four flasks were prepared with each level of hydrocarbon. After flasks had been sonified to suspend hydrocarbons, two flasks of each set were inoculated with 0.1 ml of inoculum suspension (= low inoculum) and two with 0.3 ml (= high inoculum). Triplicate controls, which received no hydrocarbon but were sonicated for 10 min, were started with each of the two levels of inoculum. Flasks were then shaken at 23° for 3 days. Protein content of the inocula and 3-day cultures was estimated by the Lowry method. Data below are from four different experiments.

Culture/Treatment	Increase in Protein in 3 Days			
	low inoculum		high inoculum	
	µg protein	%	µg protein	%
OCTANE: (Inoculum = 140 and 280 µg protein per 5 ml culture)				
Controls	4,230	100	6,190	100
0.01% (v/v)	3,720	88	7,400	119
0.1%	4,025	95	5,980	97
1.0%	-40	---	-150	---
DECANE and DODECANE: (Inoculum = 165 and 490 µg protein)				
Controls	6,340	100	10,645	100
0.2% decane	700 & 3,865	11 & 61	6,833	64
0.5% "	-144	---	700 & 3,560	6.6 & 33
1.0% "	79	0.6	310	1.5
0.2% dodecane	5,035	79	10,085	95
0.5% "	5,880	93	9,400	88
1.0% "	4,870	77	11,050	104
DODECANE, TETRADECANE and HEXADECANE (Inoculum = 115 and 345 µg protein)				
Controls	5,440	100	6,840	100
1.0% dodecane	4,410	81	8,790	129
0.2% tetradecane	4,050	74	7,640	116
0.5% "	4,500	83	8,160	119
1.0% "	4,750	87	8,080	118
0.2% hexadecane	4,730	87	6,880	100
0.5% "	4,690	86	6,400	94
1.0% "	3,660	67	6,400	94

through C_{16} hydrocarbons; (4) 1% do- and tetradecane slightly increased the yield from a large inoculum; and (5) the uneven numbered hydrocarbons--tri- and pentadecane--were somewhat toxic to a small inoculum but essentially inactive toward a larger one (Table 6).

Since some of the hydrocarbons, particularly tetradecane, gave a slight increase in yield, it seemed possible that they might have a glucose-sparing effect, and glucose analyses were therefore done on used media containing C_{12}, C_{14} and C_{16} hydrocarbons. When the ratio of mg glucose used per 5-ml culture to mg protein produced per culture was calculated (Table 7), controls were found to use about 2.3 times as much glucose by weight as protein produced. In the presence of dodecane, the glucose utilization was only about half as great. Both tetra- and hexadecane had a glucose-sparing effect when the inoculum (and final yield of culture) was large.

ETOH and Hydrocarbon Utilization: Direct measurements of ETOH and hydrocarbon uptake have been attempted but have thus far been unsuccessful. ETOH and the shorter hydrocarbons are readily measured in fresh medium by gas chromatography. However, in exhausted medium there is a substance which co-migrates with ETOH, making it appear that ETOH increases rather than decreases as growth progresses. In the case of the hydrocarbons, the problem is that they separate from the medium during growth and are trapped as droplets among the microplasmodia so that analyses of medium are pointless. (It is also possible that the droplets are actually inclusions within microplasmodia which are expressed by centrifugation. This remains to be investigated.)

No attempt has been made to work with media which have been saturated with hydrocarbons and the excess removed before inoculation on the theory that this would not provide enough carbon to support growth, unless some sort of "slow feed" method could be devised so that hydrocarbon could be added in small amounts throughout the growth period.

TABLE 6. EFFECTS OF TRI- AND PENTADECANE ON GROWTH OF MICROPLASMODIA*

Culture/Treatment	Increase in Protein in 3 Days			
	low inoculum		high inoculum	
	µg protein	%	µg protein	%
TRIDECANE and PENTADECANE: (Inoculum = 220 and 660 µg protein per 5 ml)				
Controls	9,890	100	15,800	100
0.2% tridecane	9,375	95	15,270	97
0.5% "	9,725	98	15,365	97
1.0% "	8,735	88	13,500	85
0.2% pentadecane	8,155	83	15,535	98
0.5% "	7,565	77	14,740	93
1.0% "	7,865	80	14,175	90

* Protocol as for Table 5.

TABLE 7. EFFECTS OF HYDROCARBONS ON GLUCOSE UTILIZATION BY MICROPLASMODIA*

Culture/Treatment	glucose uptake	protein increase	glucose uptake / protein increase
	mg per culture		
CONTROL			
low inoculum	12.78	5.44	2.34
high "	15.84	6.84	2.27
DODECANE; 1%			
low inoculum	3.89	4.05	0.96
high "	8.15	8.87	0.92
TETRADECANE; 1%			
low inoculum	14.26	4.75	3.00
high "	14.25	8.08	1.76
TETRADECANE; 0.5%			
low inoculum	7.78	4.05	1.92
high "	12.59	8.16	1.54
HEXADECANE: 1%			
low inoculum	5.74	3.66	1.56
high "	11.11	6.40	1.74

*Used media from an experiment with C_{12} - C_{16} hydrocarbons (Table 5) were analyzed for glucose. Glucose consumption per 5 ml of culture medium was calculated and divided by the increase in protein in 3 days to give data in the column on the far right. Low inoculum = 115 µg protein; high = 345 µg.

TESTING WITH DIFFERENTIATING CULTURES (FRUITING PLASMODIA)

Effects of Culture Conditions on Sporulation: It was known from earlier work that LU647 x LU5001d (Mohberg, 1977) and M_3b"F2" (Mohberg, unpublished) gave 10 to 30% fruiting when plasmodia were kept on half-strength growth medium for 10 to 20 days and were then illuminated with fluorescent light. Since this was obviously unacceptable for the present study, attempts were made to find a better method for inducing fruiting. Daniel and Rusch (1962) and Sauer et al. (1969) sporulated "M" sublines by coalescing large amounts of microplasmodial slurry on filter paper, incubating on niacin-salts-carbonate medium for several days, and illuminating with "cool white" fluorescent lamps. In our hands (Mohberg et al., 1973), however, this protocol caused lysis of some strains and was not uniformly successful, even with "M" sublines, presumably because microplasmodia were not always in late log phase when plated (Arescaldino, 1968; Chapman and Coote, 1982). We therefore tried a modification of the niacin-salts method (Haugli et al., 1972) in which plasmodia were started on nutrient medium and transferred to niacin-salts after 2 or 3 days of growth. This procedure gave almost quantitative fruiting with both LU647 x LU5001d and M_3b"F2", provided that (1) the plasmodium exceeded approximately 5 mg of total protein at the time of transfer to salts medium, and (2) cultures were starved for at least 3 days before illumination. These results agree with those of Daniel and Rusch (1962),

of Hosoda (1981) and of Hosoda and Kaneka (1981). Chapman and Coote (1982) also found that the minimal starvation period was 3 days, but they did not find a threshold for plasmodial size.

Measurement of Spore Diameter: Some batches of normal LU647 x LU5001d spores showed considerable variability in size (Figure 4A). M_3b"F2" tended to be more uniform (Figures 4B and C) and slightly smaller, averaging about 10 μm, as compared to 12 in LU647 x LU5001d.

When M_3b"F2" spores with the size distribution shown in Figure 4D were suspended in 0.9% NaCl and counted in a Coulter Counter, fitted with 100-μm aperture tube, they gave a peak at a window of 20 to 24 (Figure 5). Since latex beads with an average diameter of 10 to 12 μm peaked at about 36 with the same counter settings, the apparent spore diameter was 22 x 11/36 or 6.7 μm, which is much less than the 10 to 12 μm calculated from photos. Spores thus appear to behave like nuclei in that they cause much less of an interruption of aperture current than expected for their volume (Mohberg, unpublished data).

Enumeration of Spores per Sporangium: Counts of spores in normal sporangia of M_3b"F2" and LU647 x LU5001d showed that in both strains there could be from 100,000 to 600,000 spores in a sporangium, depending upon whether or not the plasmodium had been well spread at the time it fruited. However, variability of counts within and among well-spread plasmodia of the same batch was much less, as shown by these data for duplicate segments (A and B) of two control plates (1 and 2) of M_3b"F2": Segment 1A, 287,500 ± s. d. 21,400; 1B, 224,500 ± 11,600; 2A, 242,500 ± 9,300; 2B, 269,000 ± 39,000. We therefore adopted the practice of using only well-spread cultures and of comparing treated segments with control segments of the same culture, as described in Materials and Methods.

Determination of Spore Viability: Since the most commonly used method of evaluating spore viability is to plate spores on bacterial lawns and to count plaques after 3 or 4 days (Dee and Poulter, 1970), we tried to do that in this laboratory. However, we found that plaque formation with LU647 x LU5001d was much slower (7 or 8 days, rather than 3 or 4) and less efficient (10 to 20%, instead of 60%) than seen in earlier work with this strain in England (Mohberg, 1977). The performance of M_3b"F2" was even worse, for as many as 10,000 spores were plated in one dish in some experiments without producing a single plaque after incubation for as long as 2 weeks. In no instance was plating efficiency higher than 2 plaques per 100 spores. Interestingly, by the time that M_3b"F2" amoebal plaques were finally visible, about half of them already contained tiny plasmodia. This suggests that M_3b"F2" might produce spores which are apogamic or heterozygous for mating type (see Dee, 1982, for review), and this needs further investigation.

With a view to improving plating efficiency of spores, we tested different bacterial lawns, including the *E. coli* used by the *Physarum* genetics laboratories at the University of Wisconsin and at Case Western Reserve University, as well as HB101, a weakened *E. coli* strain used in genetic engineering. We also tried formalin-killed Case Western strain, and these modifications of the basic protocol: (1) varying age of spores (5 to 70 days) and temperature of storage (4° C or room temperature); (2) overlaying seeded plates with agar, gelatin or Redigel (Conviron Inc., Goshen, Ind.) and incubating in plastic bags to increase/retain humidity; (3) reducing the concentration of liver infusion in the LIA to decrease

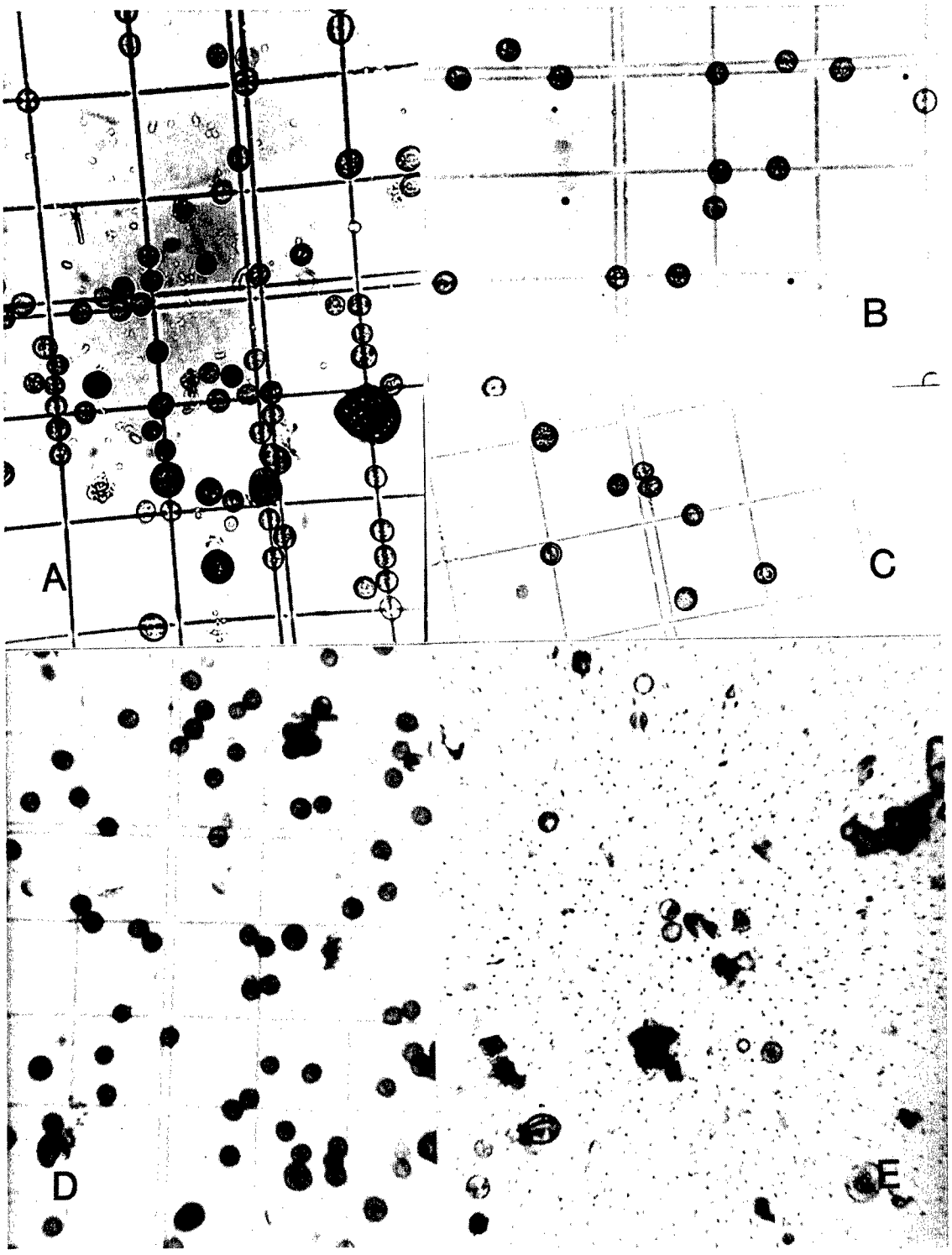

Figure 4. Photomicrographs of spores in hemacytometers. Washed spores were suspended in water and photographed in hemacytometers on areas with 50-μm grids. A. LU647 x LU5001d, normal; B and C, two fields of M_3b"F2", normal; D. M_3b"F2" suspension used for sizing with Coulter counter (Figure 5); E. LU647 x LU5001d, exposed to 1.5% (v/v) ethanol for 5 hr prior to melanization. Shows spores, wall shards of various sizes, and E. coli (granular background material).

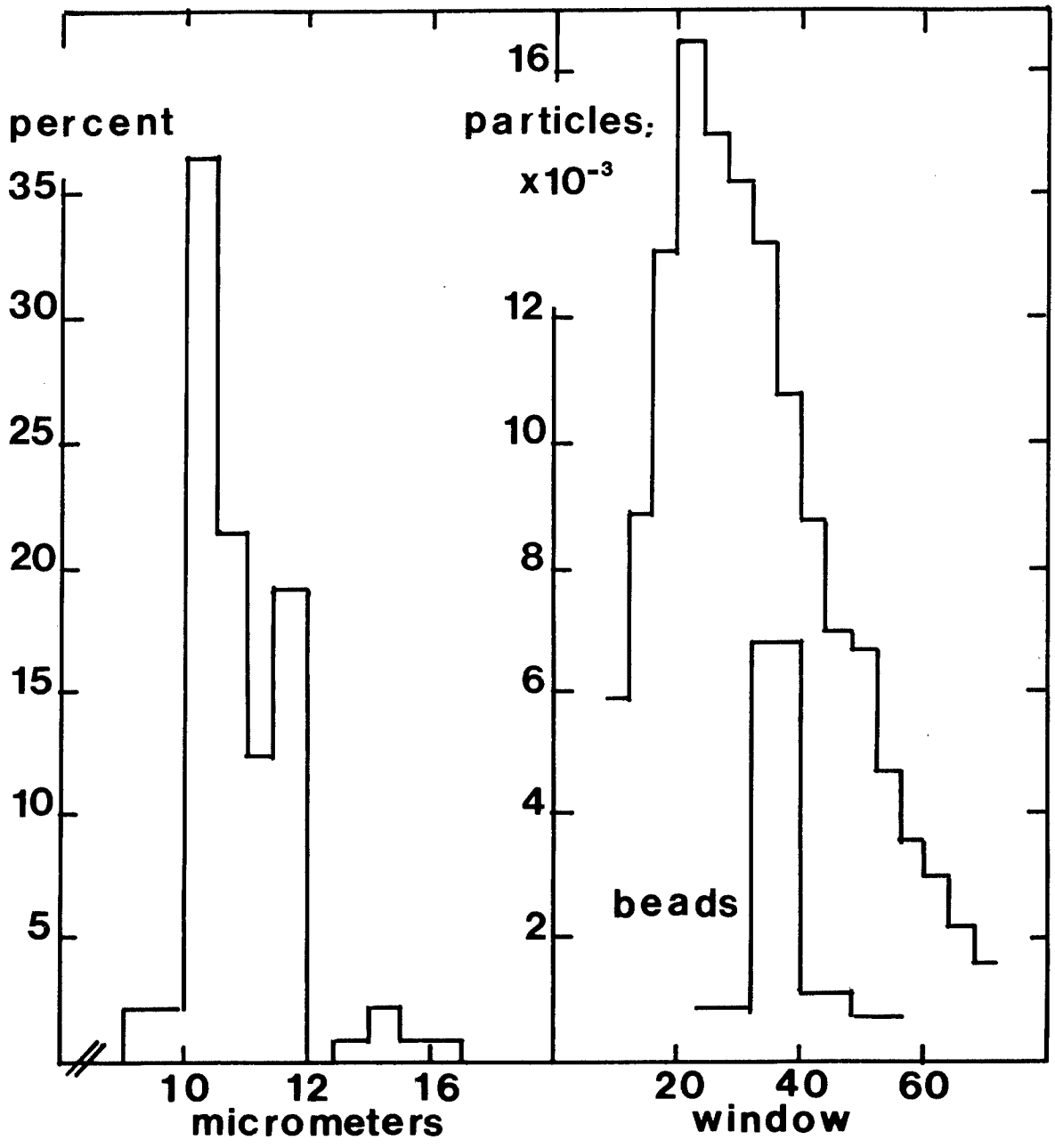

Figure 5. Sizing of spores with the Coulter counter.

M_3b"F2" sporangia from one entire culture were crushed in a Potter-Elvehjem homogenizer, stirred in 60 ml of water in a Waring blendor and passed through milk filter to remove stalks. Spores were pelleted by centrifuging for 5 min at about 2500 g and were resuspended by blending in 0.9% NaCl to give a final spore count of approximately 400,000 per ml. The suspension was then sized with a Model B Coulter counter with 100-µm aperture tube, aperture current "1" (= 0.5 mamps) and an amplification of "2". Coulter data are presented on the right of the figure and photographic data for the same batch of spores are given on the left.

the density of the bacterial lawn; and (4) supplementing LIA with hematin, thiamine and yeast extract or replacing liver infusion powder with N + C/10 so that amoebae would grow better (McCullough et al., 1978). None of the changes helped and the modifications which gave denser bacterial lawns depressed plaque formation even further. We therefore checked the feasibility of scoring viability by counting myxamoebae and flagellates which hatched from spores during several hours of incubation in water.

Counting newly hatched amoebae proved far less laborious than plating spores on agar, but as later data show, results were extremely variable. Part of the problem was almost certainly that a variable number of myxamoebae were being missed in counting because either they were so flattened that they were not seen with bright field optics, or they were attached to spore wall shards or to the surface of the dish. Whatever the reason, hatching data obtained thus far are probably only qualitatively correct.

Effects of Test Substances on Sporangia and Spores: Since ethanol is one of the most commonly used solvents for test substances, it was tested for its effects on sporangia. Exposure of M3b"F2" sporangia to 1.5% (v/v) ethanol, a level which did not inhibit microplasmodial growth (Figure 3), for 7 hours prior to melanization reduced the spore count to about 10% of that of the control (Table 8). Although shorter exposures were less harmful, even two hours of treatment decreased the spore count to about half that of the controls.

LU647 x LU5001d seemed to be still more sensitive to ethanol, for a 5-hour exposure reduced the spore count to less than 4% of the control. The few spores that did form were light in color and appeared to be fragile, judging from the large amount of broken wall material seen in suspensions (Figure 4E). In addition, the spores were slightly larger in diameter than controls, averaging 11.73 $\pm$ 1.3 μm, as compared to 10.43 $\pm$ 1.0 in controls. ETOH treatment also caused abnormalities in the whole sporangium, as shown in Figure 6. Stalks, which were a conspicuous part of sporangia of control (Figure 6A) and cycloheximide- and BUDR-treated cultures (not shown), were completely missing from the 5-hour ETOH sporangia. Spore hatchability was drastically reduced in all cultures exposed to ETOH, even 1 hour of treatment causing a 90% reduction in hatchability.

Effects of hydrazine were somewhat similar to those of ethanol, for the number of spores per sporangium was reduced and spores were light in color and fragile. However, measurement of the few spores obtained from a culture exposed to 450 μg per ml Hz.2 HCl for 5, 3 and 1 hour indicated that spore diameter was not affected. Diameters of the Hz samples were 9.76 $\pm$ 1.23, 10.33 $\pm$ 1.35, and 10.86 $\pm$ 0.84, respectively, whereas controls for the same time points had diameters of 10.14 $\pm$ 0.88, 9.65 $\pm$ 1.96, and 10.88 $\pm$ 0.59 μm.

As expected from the work of Sauer et al. (1969), neither BUDR at 300 μg per ml nor cycloheximide at 10 μg per ml interfered with sporulation when added 5 hours or less before melanization. There may have been a depression in the spore count per sporangium, but spore viability actually appeared to have increased, for BUDR and cycloheximide spores yielded 90^{+} amoebae per 100, as compared to 43 for controls (Table 9). Although, as already mentioned, hatching data obtained to date are not very reliable, it would appear from the data for 5-hour samples (Table 9) that the percent of spores which hatched in water was 3 to 5 times higher than the percent which formed plaques on agar. If the ratio

TABLE 8. EFFECTS OF AGENTS ON SPORE FORMATION.

In Experiments I and II M3b"F2" sporangia were exposed to ethanol (1.5% v/v) or to hydrazine dihydrochloride (50 to 450 µg per ml) for varying lengths of time prior to melanization. After storage at room temperature for 1 to 2 weeks, ten sporangia were picked from each culture, crushed in 2 ml of water and counted, as described in Materials and Methods.

In Experiment III LU647 x LU5001d sporangia were exposed to ethanol (1.5% v/v), cycloheximide (10 µg per ml), or to 5-bromo-2'-deoxyuridine (300 µg per ml).

Culture/Treatment	Hours of Exposure before Melanization			
	6-7	4-5	2-3	0.5-1
	spores per sporangium			
Experiment I				
Control	151,000	N. D.[a]	228,000	189,000
+ ethanol	18,000	N. D.	88,000	72,000
Experiment II				
Control	N. D.[a]	280,000	190,000	200,000
+ 50 µg/ml Hz.2 HCl	N. D.	130,000	N. D.	176,000
+ 150 " "	" "	79,000	87,000	98,000
+ 450 " "	" "	50,000	97,000	171,000
Experiment III				
Control	N. D.	150,000	190,000	97,000
+ ethanol	" "	5,500	19,000	53,000
+ cycloheximide	" "	84,000	169,000	132,000
+ 5-bromo-2'-deoxyuridine	" "	82,000	201,000	109,000

[a] N. D., not determined

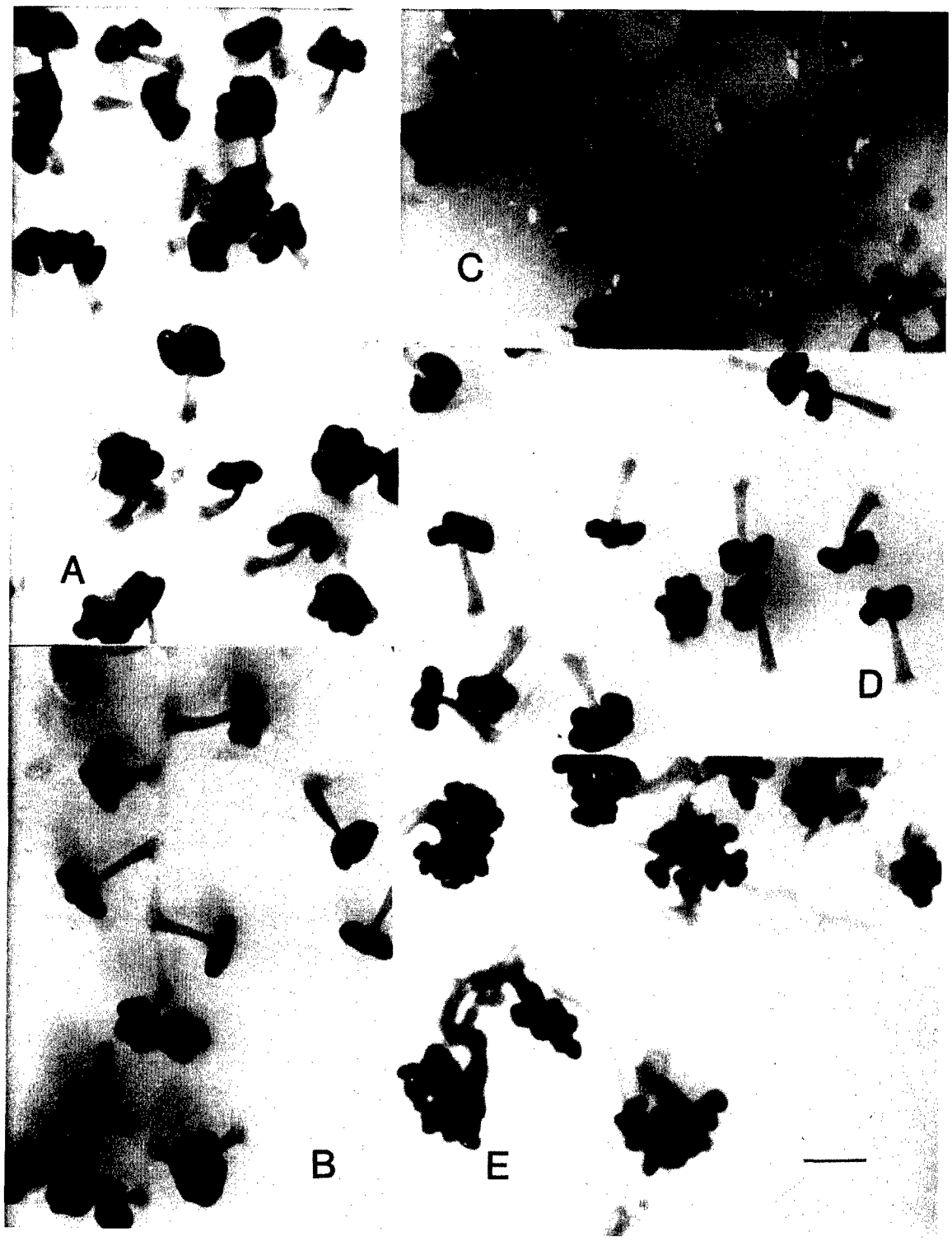

Figure 6. Effect of ethanol and cycloheximide on sporangial morphology. A-D, LU647 x LU5001d sporangia. Starved, illuminated cultures were transferred to niacin-salts-carbonate medium, containing 0 and 1.5% ethanol at 5 hr (A and C) and at 3 hr (B and D) before melanization. E. M_3b"F2" sporangia, exposed to 10 µg per ml cycloheximide, beginning at the end of illumination (= 14 hr before melanization). Sporangia were photographed through a dissecting microscope. Bar in E (on 5-mm bead) = 1 mm.

TABLE 9. EFFECTS OF AGENTS ON SPORE GERMINATION.

LU647 x LU5001d spore suspensions (same as used for Table 8) were incubated at room temperature for 3 to 5 hours and the amoebae and flagellates were counted. Data are presented as amoebae + flagellates found per 100 spores present at zero time. Viability of spores from cultures treated with toxicants for 5 hr before melanization was also assessed by plating spores immediately after washing on LIA and counting plaques at 5 days.

	spores per ml	amoebae	amoebae per 100 spores	plaques
5 hr exposure before melanization				
Control	1,500,000	645,000	43.0	12.6
+ ethanol	55,000	0	0	0
+ cyloheximide	840,000	785,000	93.4	19.3
+ bromo deoxy-uridine	820,000	815,000	99.4	20.7
3 hr exposure before melanization				
Control	950,000	500,000	52.6	25.0
+ ethanol A[a]	100.000	0	0	N. D.
" B	90,000	0	0	N. D.
+ cycloheximide	845,000	330,000	39.0	N. D.
+ bromo deoxy-uridine	1,005,000	230,000	22.9	N. D.
1 hr exposure before melanization				
Control	485,000	430,000	88.7	N. D.
+ ethanol	265,000	10,000	3.8	N. D.
+ cycloheximide	660,000	150,000	22.7	N. D.
+ bromo deoxy-uridine	545,000	175,000	32.1	N. D.

[a] A and B were segments from duplicate cultures.

were exactly 4:1, it could mean that 75% of the spores which germinated failed to start plaques if each spore hatched to give a single amoeba, or that 50% failed if each spore hatched to give two amoebae, as chromosome data suggest (Mohberg, 1977). An attempt was made to settle this question by counting both myxamoebae (or flagellates) and ungerminated spores at the end of the incubation period and calculating the ratio of amoebae hatched to spores germinated. However, this proved impossible because empty spore cases could not be distinguished from ungerminated spores, even after iodine staining.

As observed by Sauer and his colleagues, exposure of cultures to cycloheximide, starting at the end of illumination, completely blocked sporulation (Table 10). Under the same conditions, BUDR and ETOH (1%, instead of the 1.5% used earlier) had no noticeable effect on formation of sporangia, but ETOH depressed spore count. Addition of cycloheximide at 4 to 6 hours after the end of illumination did not prevent fruiting but it caused a peculiar, nodular shape to the sporangia (Figure 6E). In contrast to the results with short-term exposure to 1.5% ETOH, sporangia exposed to 1% ETOH for 12 or 14 hours before melanization gave spores which, at least in this experiment, hatched better than controls, yielding 20 to 40 amoebae per 100 spores, as compared to 0.5 to 2 in controls. This may be the result of the ETOH-treated sporangia forming spores with much thinner walls so that they hatch faster, although not necessarily with a higher efficiency.

TABLE 10. EFFECTS OF LONG-TERM EXPOSURE TO TOXIC AGENTS ON SPORE COUNTS AND HATCHABILITY*

Treatment	Hours after Illumination 0		2	
	spores per sporangium	amoebae per 100 spores	spores per sporangium	amoebae per 100 spores
Control	287,000	2	237,000	0.4
+ ethanol	109,000	36	153,000	20
+ cycloheximide	did not fruit	--	216,000	1.0
+ bromodeoxyuridine	184,000	9	192,000	2.0

*M_3b"F2" cultures were transferred to plates containing ethanol (1% v/v), cycloheximide (10 µg/ml) or BUDR (300 µg/ml), at 0 and 2 hours after the end of the illumination period. This corresponded to exposures of 14 and 12 hours, respectively, prior to melanization. Spores and amoebae were counted as for Tables 8 and 9.

TESTING WITH DIFFERENTIATING CULTURES (FLAGELLATING MYXAMOEBAE)

Culture Conditions: Aldrich (1967) showed that amoebae could be induced to flagellate simply by scraping cells from agar, washing and suspending in water. However, when we tried this technique for producing cells on the scale needed for

toxicity testing (Mir and Wright, 1978), we encountered several problems. Among them was that, first, cultures often contained a substantial proportion (up to half) encysted cells and/or structures resembling ghosts by the time they had reached peak growth. In addition, there were losses of as many as 90% of the cells during the exhaustive washing procedure. We found that a much more uniform cell population could be obtained by using a strict 48-hr transfer schedule with an inoculum of 4×10^5 cells and an E. coli food supply of 0.1 ml of suspension, prepared as described in Materials and Methods. With this regimen the population ranged between 5 and 6 million at the end of 2 days and the fraction of encysted cells and ghosts was negligible. Nevertheless, as an added precaution we harvested each plate separately and examined it with a microscope before adding it to the pool, in order that any encysted plates could be discarded. We also have improved cell recovery by reducing the number of washes from the five specified by Mir and Wright to two. This should not affect purity of the final cell preparation because microscopic examination showed that the ratio of bacteria to amoebae did not change during the last three washings. To prevent flagellation from beginning during the washing procedure, cells were harvested by scraping into ice-water and were kept at 0^o to 4^o C until they were suspended in buffer for plating. From this point on they were kept at 22^o to 24^o C.

Induction of Flagellation of Amoebae: Although Kerr (1972) and Wright's group used KH_2PO_4/K_2HPO_4 buffer to suspend cells for exposure to test agents, neither group offered any data to support the choice of potassium over sodium phosphates. Our first experiments with flagellation were therefore done to check the effects of 0.02 M, pH 6.2 Na/Na_2-, Na/K_2-, K/K_2-, and K/Na_2-phosphate buffers on the time course of flagellation. In two experiments with Na/Na_2 and Na_2/K_2 flagellation ranged from 70 to 80%, and in two with K/K_2 and K/Na_2 it ranged from 60 to 70%. It thus appeared that the nature of the monovalent cation was not critical, and K/K_2 buffer was used in subsequent experiments.

Effects of Hydrazine on Flagellation: Table 11 presents results of incubating amoebae in the presence of 50 µg/ml Hz.2 HCl or 7.5 µg/ml cycloheximide. Flagellation occurred in about 60% of the control cells in 2 hr and in only about 20% of the Hz-treated cells. Cycloheximide gave complete inhibition of flagellation, as expected from data for another true slime mold, Didymium nigripes (Kerr, 1972). Both the control and Hz-treated cells appeared to contain about 8% encysted cells at the end of the incubation period, but this might have been an artifact, resulting from our inability to distinguish between cells which had encysted and those which had rounded up for some reason.

CONCLUSIONS

All of the published Physarum test systems which we tried in the course of this investigation appeared to have potential for use in general toxicity testing, and each procedure had its own particular advantages and limitations. The microplasmodial growth inhibition system (Becker et al., 1963) was convenient because it gave results in terms of ED_{50}'s which could be compared with similar data for other microorganisms. This test was also the easiest way to determine dosages for further testing with macroplasmodia (Sachsenmaier and Rusch, 1964) which have naturally synchronous mitosis and discrete S- and G_2-phases, so that effects on DNA, RNA and protein synthesis can be studied separately. However,

TABLE 11. FLAGELLATION OF AMOEBAE IN THE PRESENCE OF CYCLOHEXIMIDE AND HYDRAZINE*

	Myxamoebae	Flagellates	Cysts	"Other"
CONTROLS	Percent of total cells counted			
0 min	97.6	0.0	2.1	0.0
30 "	75.0	25.0	0.0	0.0
60 "	77.1	22.9	0.0	0.0
90 "	41.7	58.3	0.0	0.0
120 "	35.9	58.7	8.7	2.2
CYCLOHEXIMIDE				
0 min	100.0	0.0	0.0	0.0
30 "	95.4	3.1	0.0	1.5
60 "	93.3	1.7	5.0	0.0
90 "	96.6	1.7	0.0	1.7
120 "	100.0	0.0	0.0	0.0
HYDRAZINE.2 HCl				
0 min	98.3	0.8	0.0	0.8
30 "	82.1	1.4	3.6	0.0
60 "	42.9	42.9	0.0	1.4
90 "	61.1	30.6	5.5	2.8
120 "	68.2	20.0	8.2	3.5

*Washed cells were suspended in 5 ml of phosphate buffer (control) or in phosphate buffer containing 7.5 µg per ml cycloheximide or 50 µg per ml hydrazine. 2 HCl. At zero time and at 30-min intervals thereafter, 0.2-ml aliquots were removed and mixed with 50 µl of Lugol's iodine reagent. Suspensions were put in a hemacytometer and cells were scored as indicated above.

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the microplasmodial system gave "false negatives" if the inoculum was too large, and we found that for reproducible results it was necessary to monitor the packed microplasmodial volume of the inoculum before inoculation and to reduce the inoculum to one fifth to one half of the amount originally recommended. The test was also relatively insensitive, at least in regard to hydrazine, for the ED_{50} was 40 µg of Hz.2 HCl (= 13 µg free Hz) per ml, whereas soil bacteria are strongly inhibited by 1 to 2 µg of free Hz per ml (London, 1979) and walled and walless algae by 1.6 and 0.4 µg per liter, respectively (Dixon et al., 1979). This was perhaps to be expected, since Becker and her group found Physarum was much more resistant to a number of inhibitors of nucleic acid and protein synthesis than were KB tissue culture cells.

The sporulation system of Sauer et al. (1969) had several attractive features, among them being that it provided a means of studying effects of chemicals on differentiation and that there were a number of endpoints for assessing

toxicity, such as fruiting efficiency, number of spores per sporangium, spore diameter, and spore viability. The spore count seemed particularly useful, for it responded to treatment regimens which had no effect on fruiting efficiency or sporangial morphology, such as exposure to ethanol shortly before spore melanization.

Induction of amoebal flagellation was perhaps the quickest test, since final results could be obtained in 2 days or less. However, we had to do considerable experimentation before we could produce log phase amoebae in the quantities needed for starting material for this test. Another undesirable aspect was that amoebae of the strain used here cannot be grown axenically, and while amoebae can be washed free of uningested bacteria, material already in food vacuoles quite probably affects responses to toxicants.

Although all of the test systems seemed to give valid data, some would be easier to set up in the routine microbiological laboratory than would others. Provided that the experiment is properly planned and 24-hr inoculum cultures and a 3-day block of time for growth are available, the microplasmodial growth test is easily fit into a 40-hr week, 8-hr day schedule, for preparation of experimental media and inoculum can be done in 2 or 3 hours and harvested samples can be saved for later processing, once they have been suspended in trichloroacetic acid-acetone. The same is also true for exposure of macroplasmodia to toxic agents during the S- and G_2- phases of one mitosis, say MII or MIII, although the day of treatment with test substances will probably be 10 to 12 hours long. However, if a substance is being evaluated via mitotic delay in macroplasmodia, it is almost unavoidable to work overtime or to use two shifts of observers, for the normal interphase time is 8 $\pm$ 1 hr and will obviously be longer in inhibited cultures.

Sporulation is more complicated to set up than growth systems because cultures have to grow for 2 days and starve for 3 before illumination, but from this point it is simply a matter of setting the illuminator timer for a convenient 4-hr interval, transferring cultures to experimental media and watching for melanization.

None of the procedures used in this work requires any special expertise beyond knowledge of aseptic technique and basic instrumentation, but success in making plasmodia and mitotic smears, for example, depends upon having the proper mix of speed and benign neglect. Furthermore, Physarum microplasmodia are much more fragile than are bacteria and fungi and are easily damaged by pipetting and centrifuging. We therefore recommend that new workers spend a day or two either visiting an established Physarum laboratory or observing an experienced consultant after they have tried the basic techniques themselves.

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