AQUATIC FUNGAL BIO-ASSAY METHODS FOR DETECTION OF CARCINOSTATIC AGENTS

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ABSTRACT

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DETECTION OF CARCINOSTATIC AGENTS

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Three aquatic Phycomycetes were selected for developing a rapid assay system for detecting antitumor agents. A preliminary test using Azaserine indicated that Achlya flagellata and A. flagellata with the tumor forming parasite, Olpidiopsis varians were very sensitive to minute quantities of this antitumor compound. The inhibition of zoospore development of A. flagellata could be detected at less than one gamma per milliliter of solution within six hours. In addition, the lack of any new tumor formations by O. varians in new hyphal tips of A. flagellata indicated inhibitory effect. These three fungi, A. flagellata, Olpidiopsis varians and Achlya sp. (13 SP), were selected to further test diverse chemicals for fungal morphological inhibitions.

The single test system with Achlya sp. and A. flagellata was tested with the tumor inhibiting chemicals. The results were based on the concentration of the chemical required to inhibit the zoospores. The double assay system with A. flagellata and O. varians which forms tumor-like swellings in the hyphal tips of the host was also tested with the tumor

inhibiting chemicals. The single system proved to be more easily handled in the laboratory and nearly as accurate.

Achlya sp. (13 SP) from Brazil was the most sensitive of any of the aquatic fungi tested. Olpidiopsis varians was more sensitive to the chemical than the host fungus, A. flagellata.

Compounds identified as alkylating agents required high levels of concentration for zoospore and hyphal inhibition corresponding to similar levels for mouse tumor regression. Amino acid antagonists also produced inhibitory action while antifolic agents and purine antagonists showed some inhibitory effect.

Many other chemicals with unknown modes of action also inhibited fungal growth or caused abnormalities. In some cases severity of the abnormalities increased as chemical concentrations increased. Water insolubility reduced the effectiveness of some chemicals since a freely diffusible condition must exist in order to have the fungal hyphae contact and absorb the test material.

Chemical inhibitory effects were observed by changes in Achlya zoospore numbers and rate of activity, hyphal abnormalities, oogonial and antheridial development, maturity rate, and Olpidiopsis tumor development.

Steroids and interthallic relationships were tested on heterothallic strains of Achlya. Variability was shown in the action of the Achlya hormone complex indicating varied concentrations of the steroid could control the maleness or femaleness of a strain.

A total of 36 antitumor agents were tested with the aquatic systems and found to be somewhat comparable in inhibitory effect to the results obtained in the various tumor assay systems in animals in a number of cases.

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INTRODUCTION

The most commonly used biological system for routine testing of inhibition levels of new antitumor compounds is the female Swiss albino mouse implanted with the Sarcoma 180 tumor. Such a system is time consuming and costly if all new chemicals are tested by this method. The tumors are implanted 24 hours before the assay is started. A period of one week is required for the assay to discover if the chemical is an antitumor agent. Injections on a rigid daily schedule are quite time consuming and costly particularly when many replicates are used for each test. An untested chemical must be checked at different dilutions in order to determine if there is inhibition of the tumor and any toxic reaction in the host. Numerous mice are thus needed to screen each chemical.

Certainly an assay method using mice and other animal screens is necessary for a clearer picture of chemical effectiveness of tumor control and toxic levels before tests are conducted at the human level. Regardless of the number of screening systems used before terminal patients receive the test chemical, the values of the chemical compound cannot be conclusively determined until tests are conducted at the human level. The more assay tests completed on increasingly

advanced biological systems, the greater is the picture of action and effective inhibition levels the chemical possesses before patients receive the medication.

Large numbers of new compounds are continually being made available as possible tumor-retarding agents. Testing countless chemicals using animal assays is practically prohibitive in time and cost except for large research centers or pharmaceutical companies. A number of assay systems using microorganisms have been developed with only partial correlations to the animal test systems (17, 20, 22, 29, 52).

Since it is well known that many of the aquatic fungi such as Achlya sp. are very sensitive to traces of chemicals in the water, the use of aquatic organisms as a test system was considered for these experiments. Species of aquatic fungi were selected, then subjected to serial dilutions of azaserine, a broad spectrum antitumor chemical used in earlier investigations. Fungal growth abnormalities and inhibitions with azaserine were recorded. Highly sensitive species of Achlya and Olpidiopsis were selected from the preliminary azaserine screening. These fungi were then used as the test organisms with other potential antitumor chemicals.

LITERATURE REVIEW

Diverse Microbiological Screening Systems

Cancer is an uncontrolled growth and reproduction of undifferentiated individual cells. Generally normal cells do not complete differentiation before neoplastic growth begins. Suddenly, for still unknown reasons, body tissues begin to produce avasive cells that serve no function. The abnormal growth generally spreads swiftly through the infected tissue and invades adjacent tissues or organs. Anabolism in tumor tissue is appreciably greater than catabolic processes due to immaturity of the cancer cells. If the growth is not reduced, the terminal result is death to the patient. "It is thought that an alternation in one of the nucleic acids causes a cancer cell to be produced" (29). The altered DNA is transmitted from parent cancer cells to daughter cancer cells.

The most common means presently being used for arresting cancerous growth is surgical removal of the infected area and some adjacent healthy tissue. Of course many times this is not possible when visceral areas are concerned. When non-removable organs are infected, irradiation and chemical chemotherapy are used. A combination of two or all three of these

cancer eradication methods may be used. The field of cancer chemotherapy has increased by the greatest proportions while advancements in irradiation and surgery have been less notable. Radiation rays can be more accurately and specifically directed to individual tumors and cells with the aid of more complex machines as well as tracers. Although surgery has broadened its techniques, the capabilities of chemotherapy seem to be greater. The number of new chemicals, extracts, filtrates and other compounds have steadily increased until at the present time over 50,000 new materials are produced annually as possible tumor inhibitors (19, 25). Eventually these new materials will be screened through various assay systems to determine their antitumor possibilities. Compounds are being created faster than they can be tested through biological systems. According to Gelhorn (25), the total number of compounds which have been examined by empirical screening procedures during the past 15 years probably exceeds 300,000.

The aim of any assay method is to detect a broad chemical spectrum of tumor inhibitors. Initial screens should be capable of testing large numbers of different chemicals in short time periods. Few false positives and no eliminations of potential inhibitors on the human level are also desired goals. A minimal requirement is that the screen should have a very

small chance of rejecting any material known to be effective in animals and man. With each succeeding, more biologically complex screen, false positive and false negative materials are eliminated as possible tumor inhibitors. Identifying positive bacterial or fungal inhibitors does not necessarily correlate with the results in animal tumor inhibitor tests. Preclinical and human pharmacological tests will also eliminate many materials selected with animal screens. Extensive controlled clinical programs can only prove the value of promising tumor inhibitors. However, even in clinical programs, no evaluation picture can be complete since the drugs are used in only late stages of the cancer. Negative inhibition of tumors may occur in the treatment of terminal cases, however by using the drug on younger tumors, positive inhibition could result.

Expense and complexity of cell and tissue culture techniques favor more simple initial biological screen systems. In an advanced mammalia screening system using mouse leukemia L1210 Venditti (66) found two factors which have limited the success of chemotherapy due to increased tissue complexities: the host toxicity to the tumor inhibitor, and the capacity of the tumor to develop resistance to the treatment. He also noted that environmental and genetic uniformity are necessary

considerations in distinguishing different susceptibilities to the cancer as well as chemical tolerance and effect. As the test system becomes more physically advanced, increased screening problems develop.

At times other assay programs not considered in this literature review have been devised for clinical screening specific effects in order to obtain clues to the biological properties of particular chemicals. Potential mass screening programs should eliminate a large percentage of compounds tested leaving only a small minority as possible tumor retarding agents. This mass screen involves simple biological systems where results can be read within a short time period. As noted previously, further tests should be concerned with experimental animals, biological tests, and human investigations of nonterminal patients.

In the opinion of Armitage (2), the optimal goal for any stage of a screening program depends also on the relative frequence increase of effective materials. More precision may be desired such as retaining all materials with a biological activity beyond a certain level. Armitage states some screening programs are devised with a goal of returning a fixed proportion of all materials submitted, or to screen out

a fixed number each year in order to equal the capacity of workers to whom the positive materials are passed.

Speculations on possible cancer-causing agents, such as virus or bacteria, and symbiotic relationships have lead to developing some of the screening techniques. According to Hirschberg (29), viruses may be considered as causative agents of cancers since viruses are known to cause a variety of neoplastic conditions by alterations of a nucleic acid (DNA). Another accepted idea concerns the somatic mutation theory of cells. On this basis, research in bacterial genetics and mutations could provide contributions to cancer causes. The effects of chemical carcinogens on bacteria, fungi and protozoa are areas followed in broadening cancer studies. Biological systems have been selected as screens because of elaborate studies on biochemical components and tumor functions. Microbial screens are among the most promising possible test systems, according to Snell (61), as the limited successes achieved to date in cancer chemotherapy have been with compounds (for example, antimetabolites related to such substances as folic acid and purine bases) first studied because of their effect on bacterial growth. Blocking of biochemical reactions by an agent which closely resembles a naturally occurring chemical found in the maturation process

has lead to the use of a wide variety of chemicals that have been tested as potential cell division inhibitors.

Lasagna (38) was quite critical in his evaluations on screening techniques. "There is a great tendency to carry on scientific love affairs with pet screening techniques, learning with time to disregard blemishes and faults and developing a paradoxically increasing contentment as vitality and promise wane. A screen must be judged only on its pragmatic usefulness for discovering compounds that inhibit abnormal mitotic sequences in man."

In reviewing the assay systems, the most widely used test is with the female Swiss albino mouse inoculated with a sarcoma or carcinoma. Trocar injections of the tumor are made followed by special inoculations of the screening material at various concentrations. Clinical usefulness of the chemical is determined by subscission of the tumor.

Another common screening agent has been Escherichia coli (34, 42, 68). A great number of effective carcinostatic agents were suggested for screening against tumors on the basis of their antibacterial activity. The principle assay techniques include inoculated liquid media that contains different test compound concentrations, agar streak plates, soaked filter paper discs on inoculated agar plates, and the

cylinder agar plate method. In the screening systems devised by various investigators, E. coli was used for more assays than any other bacteria. Hirschberg (29) cites several early examples of bacterial chemical antimetabolite relationships, first tested around 1947. Escherichia coli seems to screen inhibiting agents of amino acid synthesis at the indole and serine level. Antimetabolites for pteroylglutamic acid (Folic acid essential for growth) such as 2, -6- diamino-purine, thioguanine, 2-4- diamino -5- (3,' 4' dichlorophenyl) -6- ethylpyrimidine, and 6- mercaptopurine resulted in growth inhibition of Lactobacillus casei and Streptococcus faecalis. These were the first important compounds used in acute leukemia treatment when an acceleration of leukemia was noted following treatment with pteroyldi and triglutamic acid. Only temporary retardation has been registered. The two bacteria, L. casei and S. faecalis are more sensitive for screening folic acid and nucleic acid derivatives.

Drug resistance and drug sensitive strains of Escherichia coli and Streptococcus faecalis were selected by growing the organisms in increasing concentrations of inhibitors then used to detect new potential agents for drug resistant cancer cells. Metabolic processes in all living cells, bacterial as well as neoplastic cells, were considered similar. Schabel

(57) showed that S. faecalis and Leukemia 1210 convert 8-azaguanine to 8- azaguanylic acid, while the 8- azaguanine resistant S. faecalis and Leukemia 1210 do not convert the antagonistic purine to the ribotide acid. Drug resistant cells fail to metabolize the natural purines (hypoxanthine and guanine) to the corresponding ribotides and they fail to metabolize non-occurring purines (8- azaguanine and 6- mercaptopurine) to the corresponding ribotides while the drug sensitive cells carry out these metabolic conversions.

Bacteria can be used to select new antitumor chemicals as well as to study the modes of action. A group of Japanese (49) tested 68 substituted halogenated azaindolizines on Lactobaccillus casei and S. faecalis for growth inhibiting properties. One azaindolizine (7- methyl -1 -5, 6- dichloro -1, 3, 8, - traazaindolizine) inhibited animal tumors as well as the bacteria.

Escherichia coli strains (68) made resistant to nitrogen mustard and derivatives were also found resistant to radiomimetic anticancer agents (1-methyl-3-nitro-1-nitro-1-nitrosoguanidine, mitomycin C, azaserine) and U-V radiation but not nonradiomimetic compounds. Woody (68) indicated genetic differences in organisms existed between radio-resistance and chemoresistance and that each anticancer agent would have two

or more cytotoxic mechanisms. When E. coli was used to test several antitumor compounds in the presence of varied amounts of oxygen, the chemical toxicity varied according to oxygen availability in the screens (39). E. coli mutagens were used extensively in a screening teehnique to determine potential antitumor chemicals by the common paper disc method. The varying degrees of chemical mutagenicity also registered similar results with experimental animal cancers (64). Chemicals tested included antibiotics, purines, pyrimidines, nitrogen mustards, ethylenimine derivatives and miscellaneous chemicals similar to those in this investigation.

Hitchings (31, 32) derived a screening test method using Lactobacillus casei on a basal medium lacking folic acid, purine and pyrimidine bases but otherwise nutritionally adequate. When test chemicals were added, modes of action could then be identified according to their ability to replace or antagonize the nucleic acids. Folic acid is needed by L. casei for its nutrition, but the purine and pyrimidine can be substituted. Test chemical affect to the bacterial nucleic acid biosynthesis therefore can be detected.

Streptococcus faecalis and Lactobacillus arabinosus were used in screening amino acid derivatives and compared with Sarcoma 180 tests in animals. The later system gave higher positive inhibitory results (44).

Anaerobic bacteria have been used in assay screens for antitumor chemicals. Bradner (10, 11, 12) and his associates used the agar-plate penassay disc procedure with Clostridium feseri. This anaerobe screen was comparable to results when the mouse mammary adenocarcinoma was used for 755 chemical screens, however, other bacteria (Bacteroides limosus, B. pseudoremosus, B. tumidus, B. rettgeri; Closteridium acidi, C. cylindrosporum; Micrococcus aerogenes; Propionibacterian freudenreichii, P. shermanii) did not give the same correlative results. C. feseri does not register purine analogues, however good inhibition results were registered with steroids, alkylating agents, glucose analogues and all antitumor antibiotics. Inhibition screening levels of the anaerobes also gave a forecast of animal toxicity although some negative results were recorded here as well as in the antitumor screens.

Fungi have been included with other microorganisms as screening systems. Of many organisms tested by Coffey et. al. (14), E. coli and one yeast, Kloeckera brevis were found to be a good potential assay system similar to the in vivo control test of Crocker mouse Sarcoma 180 when using azaserine, a wide antitumor chemical. A wide screening program using a combination of yeast cells, Staphylococci and Bacillus

mycoides, all mutagens with impaired oxidation due to high temperatures, U.V. light and urethan. Unnamed "anticancer preparations" (23) were selected on the basis that the mutagens responded similar to the cancer cells inasmuch as a defective mitochondria respiratory apparatus is characteristic of both. It is quite well known that oxygen consumption in cancer cells could easily decrease to half the amount registered for normal cells. Gause (24) also used the impaired oxidation theory in selecting test chemicals. Mutagens of the yeast, Saccharomyces cerevisae as well as Staphylococcus aureus were used to screen the actinomycete chemical extracts before any further antitumor tests were considered. Ten cultures produced chemical substances that inhibited growth of the mutants and inhibited in vitro cells of ascites mice tumors.

Common to all wide screens using bacteria and fungi, particularly yeasts, oxygen tension experiments are noted. Another such program (17) incorporated anaerobic Clostridium tetani and related strains, respiratory mutants of Saccharomyces cerevisiae, and an airborne strain of Rhodotorula. Known antitumor agents registered negative results by the criterion that the compounds should inhibit a respiratory mutant but not the normal parent, however no inactive compounds registered as false positives.

Foley and co-workers (20, 21, 22) selected several *in vitro* test systems using 89 tumor active compounds, 91 tumor negative compounds and rated them according to their effectiveness in selecting known positive and negative chemical agents. Used singly, the microorganisms (*Escherichia coli*, *Lactobacillus arabinosus*, *L. fermenti*, *L. casei*, *Pediococcus cerevisiae*, *Streptococcus faecalis*, *Neurospora crassa*, *Candida albicans*, *Saccharomyces carlsbergensis*) did not average above 75 percent effective with the average about 55 percent. Using the two highest positive screening organisms (*Streptococcus faecalis* and *Neurospora crassa*), positive identification of tumor inhibitors registered 95 percent and above, results equal to S180, Ca755 and L1210 *in vivo* mouse tumor screens. Variations in percentages were recorded due to changes in substrate media, microorganism strains, differing experimental conditions such as the addition of a variety of supplements including vitamins, amino acids, inorganic salts, and natural products, false positives, false negatives, sensitivity of an *in vitro* screen in relation to an *in vivo* system, the failure to detect direct or indirect modes of action, and the stability of the compounds in relation to the organism.

Christine Reilly (51) also developed a wide range screen procedure incorporating two gram negative bacteria (*Escherichia coli* 351, *Serratia marcescens* 23), two gram positive

bacteria (Bacillus subtilis 352, Staphylococcus aureus), two acid fast bacteria (Mycobacterium smegmatis 374, M. phlei 375), four yeasts (Torulopsis utilis 356, Saccharomyces cerevisiae 358, S. cerevisiae 376, Kloeckera brevis 366), two filamentous fungi (Penicillium notatum 229, Aspergillus fumigatus 943), and actinomycetes (Streptomyces griseus 945, S. antibioticus 955). Results indicated a highly specific growth inhibition according to the microorganisms and antitumor chemical used. Extracts of over 1,000 soil fungi and actiomycetes produced no relationship between antibacterial and antitumor effects. Miss Reilly, Dr. Ehrlich and associates (37) initiated their vast screening program by selecting the Kloeckera brevis yeast as their first highly sensitive species. The agar diffusion assay method was developed to screen shake culture filtrates of Streptomyces from which azaserine, the earliest antitumor agent was later obtained.

The slime mold assay system (30) using Dictyostelium discoideum was not successful in chemical selection since no correlation was noted by Hirschberg between the inhibitory concentration of an agent. The technique included inhibition readings on the aggregation of myxamoeba and the culmination of plasmodia into a stalk and sorus. Physarella oblonga was also tested.

It might be noted that even before many microorganisms were used in anticancer screenings, some were used directly in cancer therapy as early as 1868 (52). Live cultures of Streptococci, Bacillus prodigiosus, Sporosarcina ureae, Spirochete infections; filtrates and spores of Clostridium histolyticum, Bacterium coli, and extracts of several fungi (Agaricus rufus, Aspergillus niger, A. fumigatus, Fusella olivacea, Saccharomyces cerevisiae, Penicillium spinulosum, Calvatia gigantea) were all used with some positive inhibition results on various tumors. Many others were used (52) with less but some positive success (Fusarium, Gliocladium, Helminthosporium, Trichoderma, Trichothecium, and Stemphylium sp. and Boletus edulus).

Other diverse assay systems initiated due to cancer chemotherapy interests should be briefly mentioned at this time. No correlative results could be selected with chemical screens using bacterial viruses in a paper disc phagebacteria agar plate method (3). The phages had no obvious response classification even using one bacterial host.

Chemicals of known antitumor activity and culture filtrates of several Streptomyces sp. were used to evaluate the Onion Test screening method (1). Two day old onion sets were exposed in darkness to diluted test filtrates causing readable effects such as frequency of mitotic activity, spindle

disturbances, scattering effects, chromosome fragmentation and bridge formation. Negative results occurred due to effects created by non antitumor active chemicals such as inorganic salts and streptomycin. Pea and Trillium also gave the same results.

The developing embryo of the frog (9) and chick (35) as chemical assay methods of rapid cellular division test organisms recorded sensitivity toward antagonists of nucleic acids. Transplantable rodent tumors, first successfully completed in 1885 by two German doctors Hanau and Jensen, (28), are today the most common screening systems. However, no single transplantable carcinogen has evolved that can predict clinical usefulness of chemotherapeutic agents. In large governmental screening programs, several transplantable tumors are used frequently with the same test compounds without combining the separated data. Other problems in transplanted tumors include bacterial contamination, foreign environmental relations, intra tumor contamination, differences within an animal strain, and morphological, biochemical or biological changes in transferring tumors (36). Many of these problems developing from mature animal test systems were eliminated by McKenna and associates (43) by introducing the tumor tissue into young undifferentiated in vivo cell tissue. Yolk sacs

of young embryonic eggs were inoculated with tumor tissue through the air sac on the fourth day of incubation.

According to the tumor screen initiator, tumor type or sequence, the more common transplantable tumors include Bashford, Crocker, Ehrlich, Yoshida, Walker, Guerin and Flexner-Jobling neoplastic sarcomas or carcinomas, leukemia and He La cells. The leading in vivo screening technique is the mouse Sarcoma 180 test using female Swiss albino mice by I.P. injection described by Clarke (16). Marked inhibition of the tumor growth was measured following timed injection of graded test substances in saline solutions. Reilly (53) popularized this screening technique when she screened over 5,000 culture filtrates taken from species and strains of 411 Streptomyces and 657 filamentous fungi. Approximately 15 percent of the filtrates showed moderate inhibition of tumor growth.

Other large scale screening includes suspensions of He La or other human cell cultures as a cytotoxic test (65). An accepted standard of 10,000 - 15,000 cells / ml of graded test media per standard 1 ml container was incubated approximately 5 days, then growth inhibitions were noted. In another early mass scale technique, cell agar plate screens collected positive results on a wide collection of chemicals and extracts. Agar streak assay methods produced similar

results. Becker (7) studied Paramecium caudatum cytotoxic activity of 583 strains of soil fungi extracts and found 61 percent of the extracts which were active against paramecia also had inhibitory effects against the dehydrogenase of Ehrlich mouse carcinoma cells.

Other protozoa such as Euglena, Ochromonas, Tetrahymena were used since they were considered closer to metazoa in kinship than bacteria (33). They also have exceptional ability to utilize high molecular and fat soluble materials. However, no results were correlated to findings of mouse screens.

Due to extensive knowledge of both genetic mapings (58) and the nutritional requirements of Drosophila melanogaster, abnormalities of body parts can be easily identified using antitumor test chemicals. Many purines produced the greatest abnormalities in eye, wing, leg, abdomen and melanotic tumors.

Viewing all diverse screening systems as a whole, no one assay system can identify all carcinostatic agents. However, as Gelhorn mentioned, there is no single tumor which could be expected to select all useful agents (26). A spectrum of assay systems are needed for the spectrum of cancers.

Action Mechanisms

Extracts, chemicals and derivatives viewed for possible chemotherapeutic value total in the thousands, and the chemical structure of each are in some way different. Due to this individual structural identification, the pathways of tumor inhibition of each chemical can be classified separately. Of the limitless media screened, only a fraction of the potential antitumor agents have been chemically studied and clinically followed in modes of action. A few writers have tried to compile and classify chemicals into areas of action, but their success has been very limited.

In view of the accepted fact that cancer is basically an increased mitotic process, all chemicals must act upon the immature cells. Adding to the difficulty of following the infinite number of action pathways, many biochemical effects can occur from each individual drug but some of these effects could have been indirectly caused by growth inhibition. This also increases the complexity of chemical classification.

Some authors have attempted to classify into general areas the antitumor chemicals (40, 41, 45, 55, 59). The findings were compiled from numerous laboratory reports and periodical articles. Not incorporated into their literature reviews are countless writings on carsinostatic chemicals,

however, those findings are basically similar to conclusions arrived at by the bibliographers. Much of the amorphous literature is based on only hypothesis which lead only to ambiguous conclusions that generally are disproven by other controversial statements.

Alkylating agents are mutagenic in that they damage the cytoplasm and nuclei of rapidly growing cells and cause injury to the chromosomal mechanism of rapidly dividing cells. Dealing only with those chemicals tested on the Achlya screening technique, the alkylating agents include several of the test compounds. The carsinostatic activity reaction of the alkylating agents has not been fully established but it is thought (55) the attack is on the chemical constituents of the gene (cellular polynucleotides) by electron exchange. Here the phosphate groups of the polynucleotides have a hydrogen ion replaced by a hydrocarbon radical or else the attack may be on the nitrogens of the polynucleotide. The alkylating agents of two nucleophilic reaction centers focus on deoxyribonucleic acid causing alterations of the chromosome. This results in cytotoxicity. The nitrogen mustards, benzimidazole, diethylstilbestrol, d-l sarcolysin and cyclophosphoramide all react under physiological conditions via the ethylamines (45). Nitrogen mustards are more susceptible to cells

of high proliferation activity and are thus sensitive to the neoplasms. They react irreversibly with nucleotide bases and nucleoproteins, thus causing chromosome malfunctions.

The ethylenimines, (41) TEM (triethylene malamine) and thio-TEPA (1-2, 4- dinitrophenyl-aziridine) are examples of monofunctional alkylating agents. The only conclusion concerning action modes is that in some ways these chemicals are associated with nucleoproteins.

Myleran as an alkylating agent has the ability to cross link with proteins while other biological action is by hydrolysis (41). Chlorambucil, and methyl carbomate are alkylating agents of undetermined action (40, 41). The rapid interaction of alkylating drugs with tissues removes them from the blood stream almost immediately upon intravenous administration, and undetectable tissue localization suggests only speculation as to the biological action (55, 41). Some authors (41, 44) suggested indirect action but this is unlikely due to swift effects of these agents. Perhaps the drug action is masked by other chemical processes in the tumor or the site of action could be located away from the tumor. Alkylating agents in general block the formation of RNA of highly metabolic cells by inhibiting the phosphorylation of mononucleotides to polynucleotides and interrupting protein synthesis.

The metabolite antagonists include purines and pyrimidines and their derivatives. Their action is against the synthesis and breakdown of nucleic acid molecules and of purine-pyrimidine containing co-factors incorporated in nucleic acids found in the abnormal cancer mitosis metabolism (59). Deoxypridoxine compiled with a vitamin B6 (pyridoxine)-deficient diet develops tumor inhibition (41) due to the formation of an inactive conjugate through their terminal amino acid groups with pyridoxal phosphate. DL-ethionine is also an amino acid (methionine) antagonist.

Included in the Achlya antitumor test chemicals are antifolic agents 2, 4-diamino-5-(3, 4-dichlorlphenyl)-6-ethyl-pyrimidine, 4-amino pyrazole pyrimidine, and daraprim (2, 4-diamo-5-chlorophenyl-6-ethylpyrimidine); chemical inhibitors of the B complex growth promoting vitamin. Growth is inhibited by the pyrimidines since antifolic agents inhibit the synthesis of purines by interferring with a carbon metabolism enzyme. Thymine synthesis is also inhibited and therefore DNA in mitotic cells. Daraprim is known to inhibit folic acid conversion to folinic acid.

The glutamine enzyme antagonist in the screening list is azaserine, an early antibiotic-anticancer agent produced by Streptomyces fragilis. Glutamine metabolism inhibition limits

nucleic acid and protein formation by deactivating pyridoxal phosphate, a coenzyme in glutamine transaminations (45). Azaserine is a competitive inhibitor of glutamine in place of a specific enzyme at the conversion of formylglycinamide ribotide to formylglycinamidine in rapidly growing cells. Azaserine has also caused the appearance of chromosomal fragmentations and agglutinations in some mitoses. Other Streptomyces species derivatives of unknown action modes include chloramphenical (chloramycetin), Mitomycin C from Streptomyces caespitosus (an antibiotic mutagen probably of DNA synthesis) and the nonmutagenic antibiotic actidione from Streptomyces griseus (41).

The inhibitor of pyrimidine synthesis and DNA pyrimidines is urethan, probably at the ureidosuccinic acid stage (40). Urethan also produces a thymine induced decrease of abnormal mitosis, producing chromosome damage.

The known purine antagonist listed is 2, 6-diaminopurine which affects the incorporation of purines into nucleic acid in the chromosome or nucleoportein molecule. It was also suggested that 2, 6-diaminopurine is a nucleic acid precursor which has a de novo inhibitory action of nucleic acid synthesis by fraudulent nucleotides. Protein synthesis is also affected probably by antagonizing the purine

nucleoside polyphosphates. Presumably closely related to 2, 6-diaminopurine in action is another purine, 5-methoxytoluguinone. Also 8-azaxanthine effects the incorporation of purines into nucleic acids by enzyme inhibition, and protein synthesis by affecting several enzyme systems. Potassium arsenite, colchicine, podophyllotoxin and hydrocortisone are all fairly recent antitumor chemicals inhibiting nucleic acid synthesis and reducing metabolic processes responsible for carbon dioxide production (41, 44, 45).

Syntheses of nucleic acids are generally inhibited through the antitumor chemicals by enzyme energy--coupling processes (45), precursor producing reactions or catalytic events. Little known action mechanisms exists for the majority of chemicals while those known are still of doubtful value. Unknown inhibitor actions include miracil, 1-methyl-3-nitro-1-nitro-1-nitroso-guanidine, testosterone proprionate (an intact hormone), 1-5-diaminobiuret, intact N-methyl formamide, 1-aminocyclopentane, nonmutagenic D-glucosamine and nonsoluble B-2-thienyl alanine. Chemical modes of action seem to center on genetic, enzyme and hormone effects. The environment, particularly pH, and chemical administrative methods also produce profound results to the antitumor action.

MATERIALS AND METHODS

Since Achlya sp. and other aquatic fungi are very sensitive to copper containing compounds and other toxins, it was considered desirable to try Achlya as an assay organism. In addition Olpidiopsis sp. which parasitizes Achlya sp. and forms a tumor-like growth in the hypha of the host was also used as an assay system. By using these systems, the toxic effect of the various chemicals could be determined.

Twenty-six representatives of the Saprolegniaceae from Brazil were tested by observing the morphological changes when placed in various concentrations of azaserine. These were: Achlya sp. (10v), Achlya sp. (2SP), Achlya sp. (C1), Achlya sp. (1Vic), Achlya sp. (BH9), Achlya sp. (2v), Achlya sp. (13SP), Achlya sp. (5v), Achlya sp. (3v), A. bisexualis (1SP), A. ambisexualis (1SP), A. americana (C2), A. klebsiana (5SP), A. radiosa (2SP), Achlya n.sp. (2SP), Dictyuchus sp. (3BH), D. monosporus (9SP), D. monosporus (4SP), Saprolegnia parasitica (2SP), Thraustotheca sp. (11SP), T. clavata (4v), Thraustotheca sp. (3SP), Thraustotheca (4SP), Thraustotheca sp. (3SP), and Thraustotheca sp. (6v). Two additional aquatic fungi from Illinois, Achlya flagellata and and A. flagellata parasitized by Olpidiopsis varians, were also checked with azaserine.

Three of the 28 organisms, Achlya flagellata, Olpidiopsis varians, and Achlya sp. (13SP), were selected as the assay organisms. Achlya flagellata has thick, dense, coenocytic, branched hyphae up to 1 cm in length and 150 microns in width (15). Septations occur only at the junction of reproductive structures. Subcylindrical sporangia are plentiful, generally one to a hyphal tip, with an apical pore. An inverted wall is evident at the base of the empty sporangia (Fig. 1). Primary spores are 11 - 11.5 microns in diameter after encysting inside or at the apical end of sporangia upon emergence after their first motile period (Fig. 2 & 3). The spherical spore mass remains against the sporangia or falls to the Petri dish bottom. A second motile period of secondary zoospores emerge from encysted spores, and encyst again. Spore germination follows the second encystment (Fig. 4). Up to five motility encystment cycles have been produced mainly from unfavorable germination temperatures and oxygen conditions, notably cool, redistilled, well aerated water (56). Environmental conditions seem to control zoospore emergence. Diplanetism, however, is most common. Primary zoospores are pear-shaped while secondary spores are reniform. Irregularly shaped sporangia or gemmae are abundant.

Fig. 1. Granular protoplasm of normal hyphae with an empty sporangium and an inverted wall at the base of the mature sporangium. (455x)

Fig. 2. Encysted spores around the apical end of a normal sporangium. (180x)

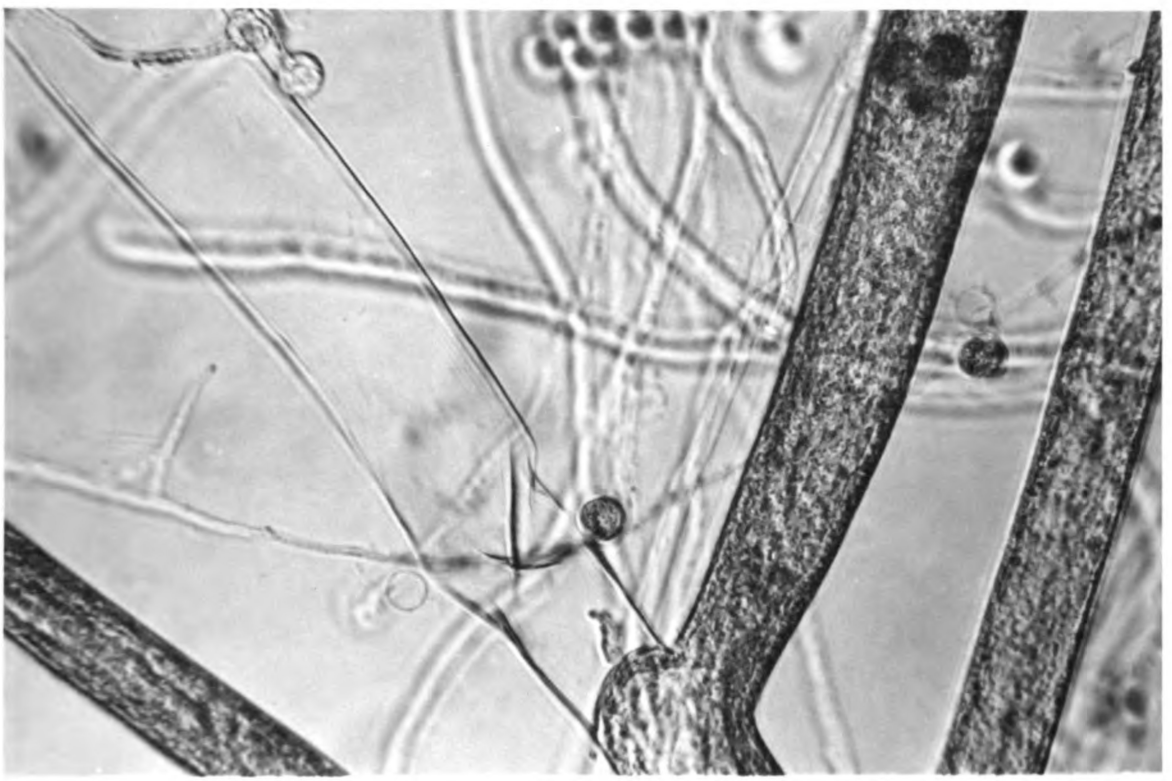

Fig. 1

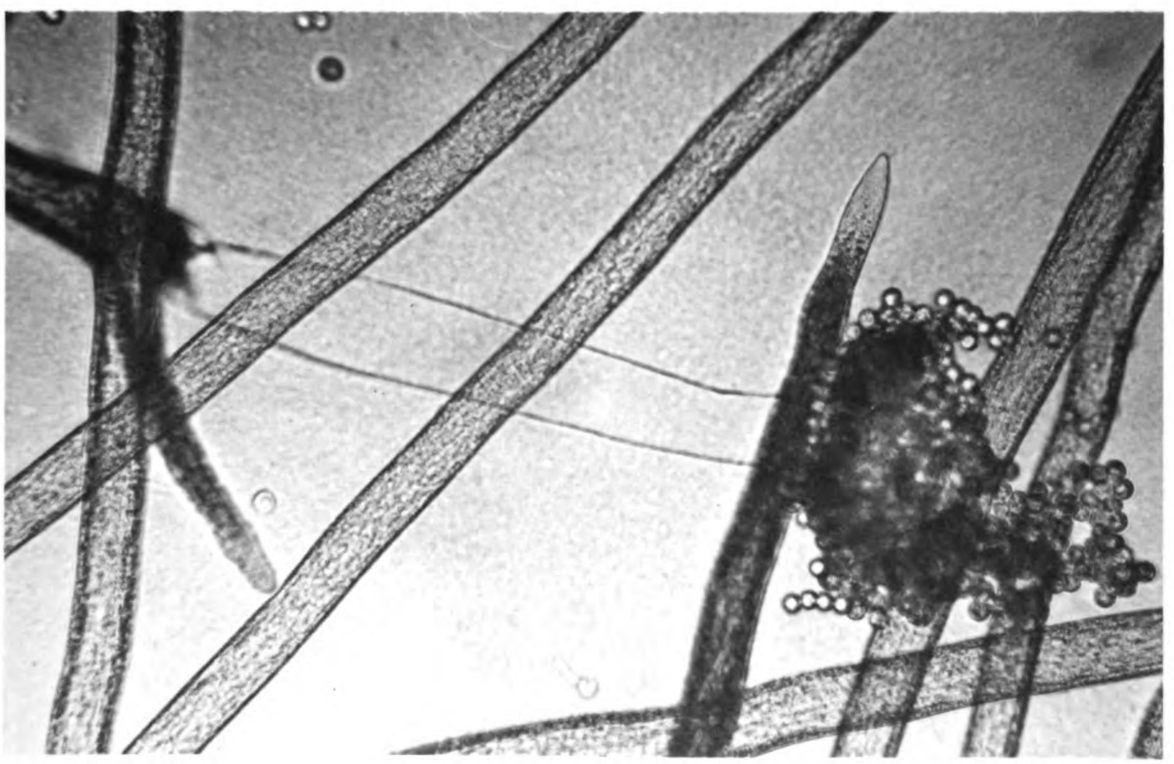

Fig. 2

Fig. 3. Normal hypha and sporangia, one empty sporangium and one with immature zoospores. (455x)

Fig. 4. Germinating spores after the last encystment showing normal developing hyphae. (455x)

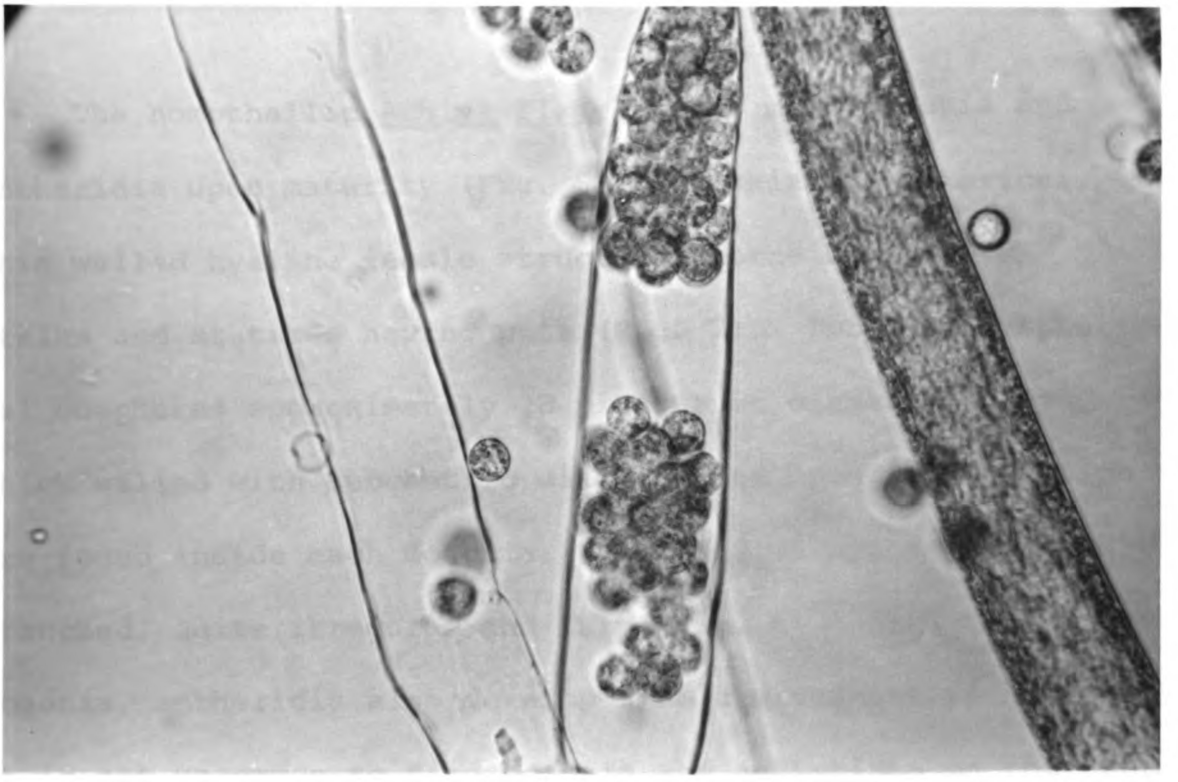

Fig. 3

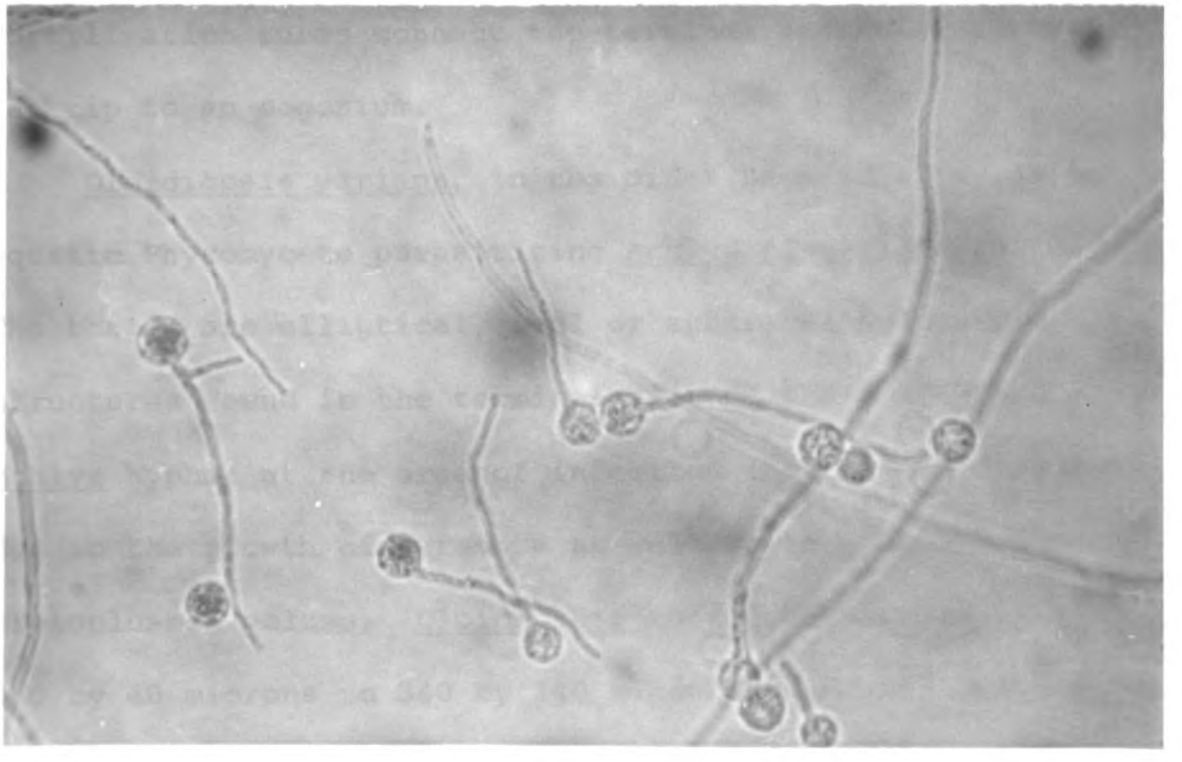

Fig. 4

The homothallic Achlya flagella has many oogonia and antheridia upon maturity (Fig. 5). Oogonia are spherical, thin walled hyaline female structures borne on slender stalks and at times having pits (Fig. 7). Two to six spherical oospheres approximately 28 microns in diameter become thick walled with subcentric oil droplets upon fertilization are found inside each oogonia. Antheridial hyphae are highly branched, quite irregular and thin (Fig. 6). Like the oogonia, antheridia also develop from the vegetative hyphae. It is not uncommon to find oogonia and antheridia on the same vegetative hyphae, but more often they occur separately. Fertilization tubes connect the terminal segmented antheridial tip to an oogonium.

Olpidiopsis varians, in the order Lagenidiales, is an aquatic Phycomycete parasitizing Achlya flagella (Fig. 8). The thalli are elliptical, oval or spherical holocarpic structures found in the terminal area of the host hypha. Achlya hyphae at the area of infection increase in diameter due to the growth of parasite as well as an increase in protoplasmic volume. Olpidiopsis zoosporangia vary in size (60 by 40 microns to 340 by 140 microns) and wall texture (smooth to extremely spiny) and upon maturity the biciliate oval zoospores (4.2 - 2.8 microns) escape through exit tubes.

Fig. 5. An oogonium of Achlya flagellata showing five oospores. Several antheridia are attached to the oogonial wall for oosphere fertilization. Dissolution of the oogonial wall at antheridial attachment points can be seen. (455x)

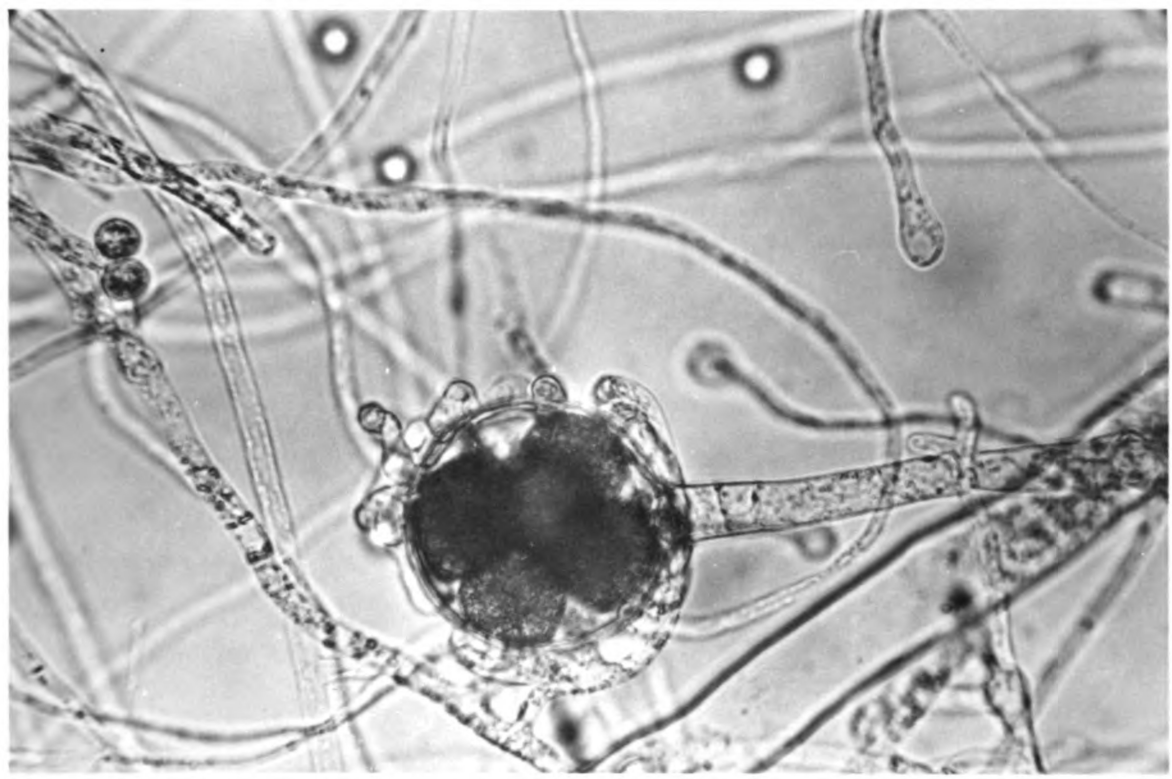

Fig. 5

Fig. 6. Large vegetative hyphae of Achlya sp. (13SP) with small antheridial hyphae of Achlya bisexualis male. Incompatibility is evident. (90x)

Fig. 7. Two immature oogonia with scattered encysted spores of Achlya flagellata. (455x)

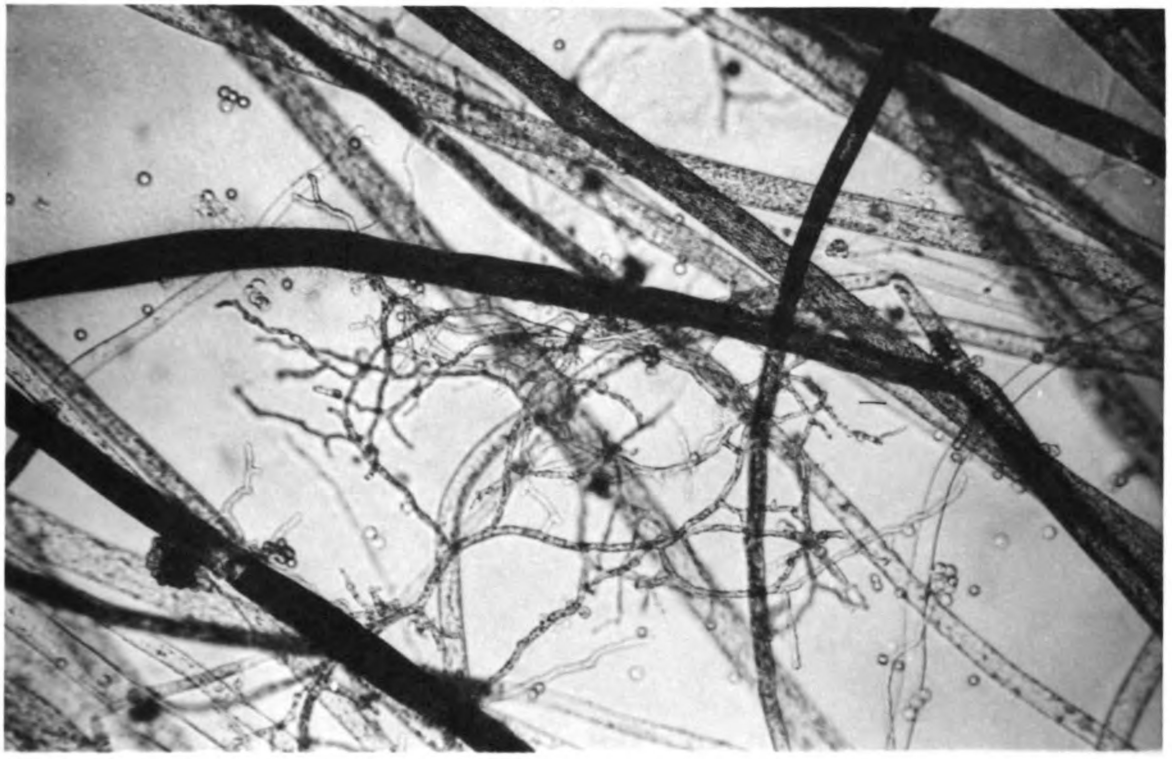

Fig. 6

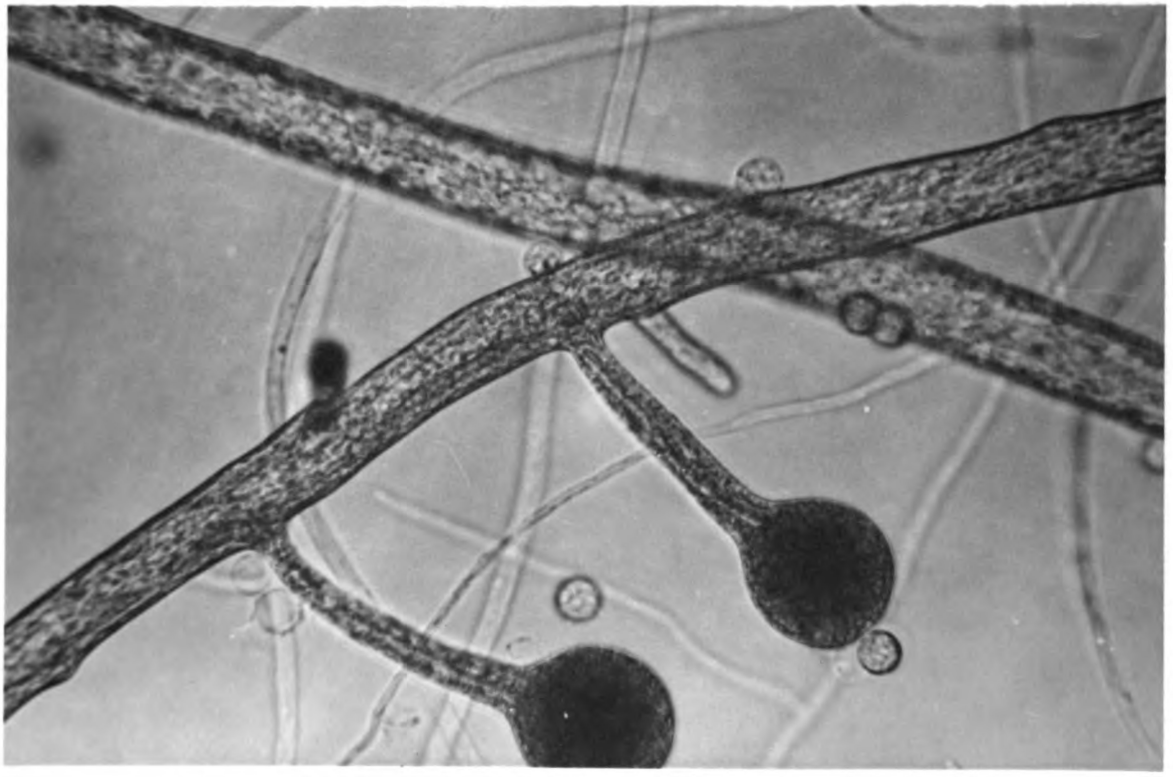

Fig. 7

Fig. 8. Spiny and smooth walled sporangia, and resting spores of Olpidiopsis varians in the Achlya flagellata hyphal tip. (930x)

Fig. 9. Five thick-walled resting spores and adjacent antheridia with one spherical and two oval shaped sporangia of Olpidiopsis varians. The mature oval shaped sporangia show many zoospores appearing like dots. (385x)

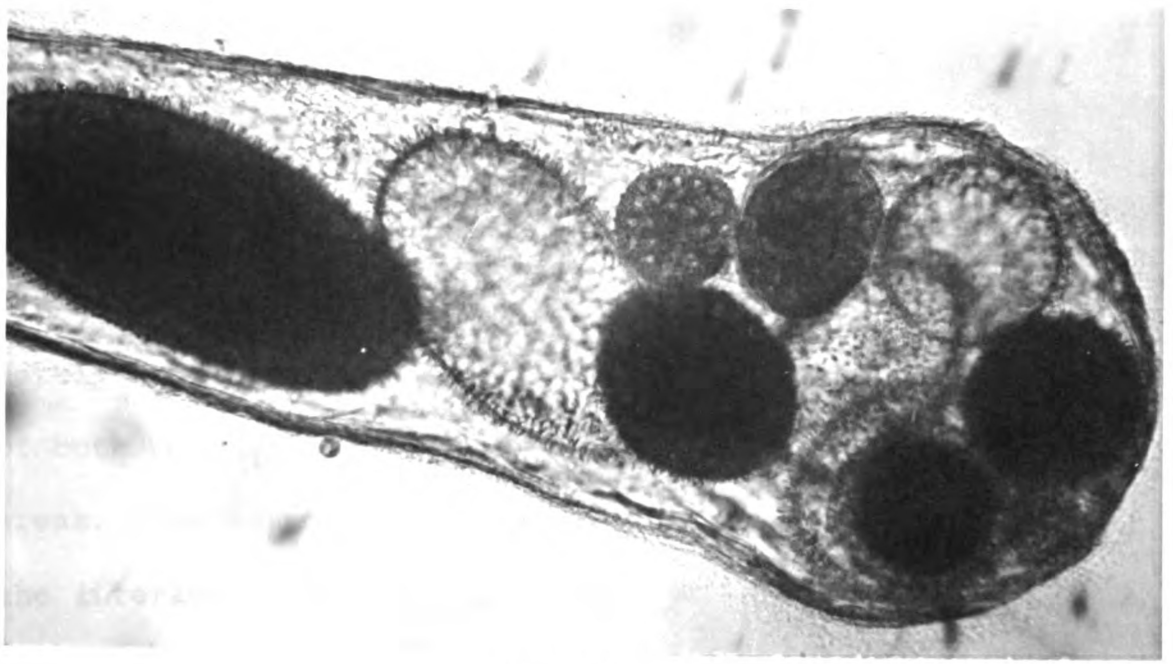

Fig. 8

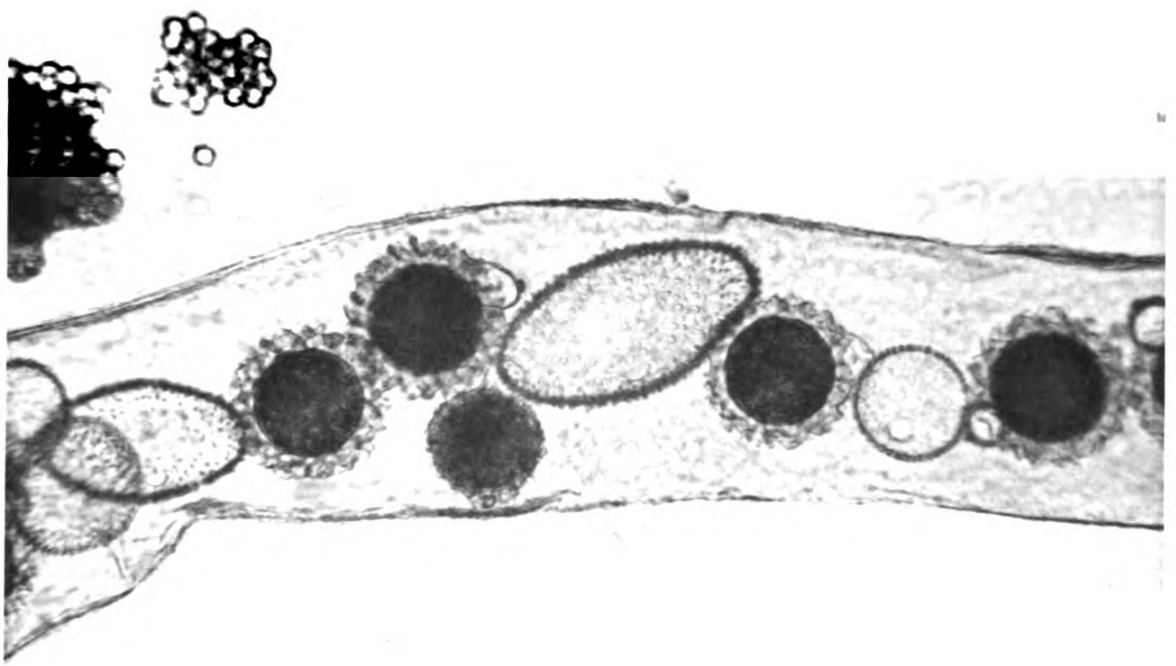

Fig. 9

The spherical oogonia (52-61 microns) have very thick (approximately 1.2 microns) yellow walls with coarsely tapered spines (up to 8.6 microns long). Adjacent to each oogonium is one or possibly two antheridia, also called companion cells (Fig. 9). Antheridia are spherical (approximately 26 microns) with scattered minute spines. The walls of both oogonia and antheridia have two distinguishable areas. The exterior wall with spines is colorless, while the interior is more yellow with high cellulose concentration. Thick walled resting spores remain upon completion of a fertilization tube. Germination emits biciliate zoospores from the resting spore. Zoospores penetrate immature host hyphae and develop new freely movable inter-host hyphal thalli.

Achlya sp. (13SP) was selected as the third test organism on the basis of preliminary sensitivity test to azaserine, a wide screen early antitumor chemical (4, 6, 8, 27, 34, 42, 46, 47). This thirteenth strain of sterile Achlya from the São Paulo, Brazil region was the most sensitive to azaserine of all Brazilian fungi tested. Azaserine was used as a preliminary chemical test for comparison with the amount required to inhibit tumors. Other Brazilian strains of

Achlya varied in degrees of inhibition in the presence of azaserine indicating possible differences in enzyme and hormone structures. Azaserine is known to affect enzyme actions.

Achlya sp. (13SP) is similar to Achlya flagellata in morphology except for its lack of sexual reproduction or "sterility." A series of crosses were attempted to determine the species of Achlya. Only two heterothallic Achlya species (A. bisexualis, A. ambisexualis) are known at present. The male and female strains of Achlya bisexualis and A. ambisexualis plus all the "sterile" Brazilian strains of Achlya collected were crossed with each other at room temperature (22-24°c). Actively growing mycelial masses of each were produced in Petri dishes so hyphal filaments of the "sterile" Achlya could be crossed and grown in contact with various heterothallic strains. Crosses were repeated at lower temperatures (12, 16, and 21°c) more inductive to interthallic sexual relations. Optimum crosses for known heterothallic species occurred at 16°c with fewer oogonia developing at the other two selected temperatures. Previous tests indicate heterothallic sexual organs initiate at 15-20°c. Above 20°c a reduction of oogonia and antheridia occurs until at 25°c most heterothallic strains lack sexual reproduction. At the lower temperatures, some pure male

heterothallic strains acted as females, while some pure females developed antheridia in the presence of the opposite heterothallic strain (5).

Sexuality is also related to hormonal activity. Individual homothallic and heterothallic strains exhibit degrees of maleness and femaleness according to the amount of hormone A complex (5). Weak maleness is negative for hormone A while hyphae of strong female tendencies possess large amounts of the hormone A complex, indicating a sexual gradient. Homothallics carry the complete hormone series (56). Many homothallics can cross with heterothallics as well as other homothallic species. Some strains of Achlya will remain "sterile" in the presence of a compatible strain or the absence de novo of hormone A complex. The bisexual relationship of two complementary hyphae remains unchanged but the antheridial member of one pair may be the oogonial initial in another bisexual union. The reverse is also true. Sexual reproductive sequence of two compatible strains undergoes a maturity development with each stage introducing another hormone segment of the diffusible A complex creating another step toward fertilization. Raper (50) states that the process is initiated with the hormone A complex secreted by the female initiating two A augments of the male for the production of the antheridia.

Antheridial hyphae secrete hormone B which induces female oogonia initials that in turn develop hormone C, the antheridial attraction and antheridial delimitation hormone. The mature antheridia secrete hormone D which is required for oogonia development and the differentiation of oospheres.

In crossing the Brazilian strains of "sterile" Achlya with the heterothallics, A. ambisexualis (A. ambisex.) and A. bisexualis (A. bisex.), the male A. ambisexualis did not cross with any of the "sterile" strains. Mature oogonial development occurred in other Achlya crosses as follows:

c-1/A. bisex. male	13 SP/BH 9	c-1/10 v
1 Vic/A. ambisex. female	13 SP/10v	BH 9/1 Vic
1 SP/A. bisex. female	3 v/BH 9	BH 9/2 v
	3 v/10 v	10 v/1 Vic
	c-1/BH 9	10v/2 v

In identifying Achlya sp. (13SP), used in the tumor assay screen, the following positive crosses were observed. Formation of oospores using c-1/A. bisex. male cross; (A. bisex. female) c-1/A. bisex. male; c-1/BH 9 (A. bisex. male); BH 9/13SP (A. bisex. female) indicated strains 13SP acted as an Achlya bisexualis female. From the crosses of 1 Vic/A. ambisex. female; (A. ambisex. female) BH 9/1 Vic; and BH 9/13SP (A. ambisex. male) strain 13 SP could also act as an

Achlya ambisexualis male. This indicates the "sterile" Achlya (13SP) is of the intermediate hyphal hormone gradient where sexuality is easily interchanged. The close relationship of the two heterothallic Achlya species is also noted since crosses occurred with both species. The term species could be a misinterpretation for the heterothallics A. ambisexualis and A. bisexualis, a hormone relationship or gradient is more indicative of the heterothallics and possibly the homothallics.

As a sequel experiment, four steroids were added to water cultures of Achlya. The six fungal cultures used were: Achlya sp. (13SP), Achlya sp. (c-1), Achlya ambisexualis male and female, Achlya bisexualis male and female. The two "sterile" Achlyas were chosen since both crossed with a mutual third "sterile" Achlya. Two related animal sex hormone steroids (stigmasterol and stilbesterol) and two plant steroids (phytosterol and beta sitosterol) were used. Agar plugs containing hyphae with attached hemp seeds were transferred directly from the agar Petri dish to sterile Petri dishes containing steroid concentrations of 100, 50, 25 and 12.5 gammas / ml. Two plugs were placed in each dish. Achlya sp.(13SP) and (c-1) were grown beside each strain of

the two heterothallic species. All six fungi cultures were also grown separately in the four steroids at each of the gamma levels. Temperature was constant at 19 degree centigrade.

The steroids are only very slightly water soluble, however, solubility was noted as reduced zoospore motility with increased steroid concentrations. Periodic observations were made on the cultures for abnormal growths, development of oogonia, and antheridia. Normal growth development in the control was used for comparison. Singular cultures of both Achlya sp. (13SP) and (c-1) growing in a solution of stigmasterol at 100 gammas /ml produced several immature oogonia at nine days. Achlya c-1 growing in the presence of beta sitosterol at 100 gammas /ml also produced oogonia at six days. Antheridial hyphae were also seen with c-1 and beta-sitosterol, fertilization occurred in three oogonia producing oospores. It would seem from this evidence that the hormone A complex is related in some way with the sterol complex.

The medium used for the Achlya inoculation was MP5, a weak nutrient medium consisting of maltose, 4 gm, peptone, 1 gm, Bacto agar, 20 gm, and 1,000 ml of distilled water.

All Petri dishes were dry heat sterilized at 180°c for three hours. Media was steam sterilized at 16 lb pressure, 121°c for 20 minutes. Approximately 20 ml of MP5 was poured into each Petri dish. A few hyphal strands were then removed from stock cultures by forceps and place upon the agar surface. Growth readily occurred at room temperatures (about 24°c). Stock Achlya growing in water culture quite commonly will have bacteria present and will be transferred with hyphal strands. Mycelium grows at a greater rate than the bacterial colonies found at the inoculation site. Peripheral hyphae are bacteria free. At the hyphal colony circumference, where young hyphae have outgrown the bacteria after 24 or more hours, discs were cut by means of a sterile cork borer. By sterile techniques, nine discs per Petri dish were placed equidistant from each other. Enough sterile distilled water was added to the plates so the plugs were slightly submerged. A thin water film completely covered the upper surface of each plug. Hemp seeds were cut horizontally, autoclaved then heat dried. One half of this cut part of a hemp seed was placed by sterile forceps on top of each agar disc. The water was kept at a minimum layer over the agar bloc in order to keep the hemp seeds from floating off the block surface. Hemp seed

cotyledons are an excellent medium for the Achlya hyphae. Achlya growing only from MP5 discs in a flooded Petri dish have thinner and shorter hyphae than mycelium growing from a hemp seed. In a hemp seed analysis; Benedict's test indicated no sugar upon boiling, iodine showed negative starch, Sudan III showed an abundance of fats, and the nitric acid and ammonium hydroxide test showed a large amount of proteins existed in the hemp cotyledons.

After 10 hours, before sporangial formation, the agar plugs with attached hemp seeds and young growing hyphae were transported by flamed forceps to the graduated chemical solutions for assay analysis. Mature sporangia formation required approximately 16 to 18 hours at room temperature (24-25^{o}c) after the hemp seeds were placed on the newly cut agar discs.

A slight variation in procedure was made in preparation for the third assay organism, Olpidiopsis varians. This Achlya flagellata parasite requires an aquatic media for hyphal inoculation. Clean stock cultures of Achlya flagellata infected with Olpidiopsis were kept refrigerated at 12^{o}c and renewed about once a month by adding sterile hemp seeds. Young cultures of Olpidiopsis used for the assay screens were not more than a week old. The stock cultures

not bacterial free, and contaminated cultures from airborne organisms were removed from the stock bottle and placed in Petri dishes. With the temperature change from 12 to 24°c (optimum temperature for greatest infection), the encysted Achlya spores formed secondary zoospores, and swam to the floating hemp seed halves that were placed in the water cut side down, encysted and germinated.

For more rapid colony development, it is desirable to float the hemp seed halves on the surface of the water instead of the bottom of the Petri dish since zoospores tend to accumulate and swim close to the surface. Approximately 10 hours were required for Achlya to develop, and have the Olpidiopsis zoospores infect the new host mycelium. Olpidiopsis zoospores do not penetrate older Achlya hyphae or develop tumor type host hyphal growths if sporangia or oogonia are developed. Mature and thin Achlya hyphae due to inadequate nutrients also are not parasitized by Olpidiopsis. Upon infection, new Achlya hyphae develop tumorous like growth of Olpidiopsis that differentiate into sporangia, oogonia and antheridia. The immature Olpidiopsis cultures were ready for the antitumor screening technique 24 hours after hyphal infection.

Airborne bacterial contamination was a greater problem with Olpidiopsis varians - Achlya flagellata cultures than with Achlya flagellata and Achlya sp. (13SP). Ciliated protozoa also infected the Olpidiopsis cultures at one time. Varied techniques were tried to clean the host--parasite cultures of contaminants. Achlya - Olpidiopsis cultures on hemp seeds were washed under a fast flow of tap water for one hour. Hemp seeds, placed in a beaker, were anchored by attaching a syringe needle to prevent loss of the cultures by the water flow. The fungi were not harmed, however; not all protozoa were eliminated and populations soon increased to their former numbers. Other suggested methods for protozoa removal from the cultures such as lowering the pH, making use of semipermeable membranes or developing anaerobic conditions also would inhibit or eliminate the fungi.

Sodium azide is a common eradicator of protozoa, which were inhibited at 0.01 percent but not fungi for 24 hours. The lethal dose was 0.1 percent for Achlya flagellata, Achlya sp. (13SP), and Olpidiopsis varians. However, sodium azide was not used for the chemical might in some way inhibit the screening mechanisms of the fungi.

The technique finally employed for the protozoa and bacterial problem consisted only of media already used for

the fungal cultures. Olpidiopsis is not easily transferable with Achlya on agar medium but it is successful if the Achlya host is only allowed a short period of growth, only long enough to grow one or two mm from the sight of inoculation. Achlya hyphae with the parasite were placed on the agar. Olpidiopsis is transportable in the coenocytic Achlya hyphae if the parasite is still in the very young immature stage. Greater movement can occur if the Achlya hyphae are also young for the flow of protoplasm is more active. The immature Olpidiopsis will migrate from the inoculation sight to the hyphal apex of some of the immature Achlya. The active hyphal growth will transport the parasite out of the bacteria and protozoa contaminated area in the site of the original inoculum. As soon as growth occurs, it is necessary to make agar discs of the new Achlya and Olpidiopsis infected hyphae in order to keep the Olpidiopsis alive as it will survive long in the agar medium. As soon as the agar discs containing the Achlya hypha and parasite are placed in Petri plates with sterile distilled water, the infected hyphae will continue growth. In the new uncontaminated culture, sterile hemp seed halves can be added to develop new cultures when Achlya zoospores develop followed by Olpidiopsis zoospores.

An airborne contamination of a Streptomyces species was eliminated from a culture of Achlya sp. (13 SP) by a streak plate method. Another problem arose when cultures became too old for adequate transfers. Because of the old mycelium and high contaminations of bacteria, porcelain rings were used to prevent the bacterial growth from spreading over the surface of the agar. The hyphae radiate from the inoculation sight and grow under the imbedded ring. Hyphae in the agar area outside the ring are free of bacteria and sterile plugs can be made. In old mature Achlya cultures, usually encysted spores or oospores germinate if present. Hyphal protoplasm of old cultures was usually highly infected with bacteria.

Other factors that might influence the screening results were investigated. Photoperiod affects were studied using a thick black cloth for a dark chamber on the laboratory bench. Achlya sporangia, encysted spores, zoospore motility, hyphal growth and maturity rate as well as Olpidiopsis infection and growth were equally good in continuous light, complete darkness and intermittent light. Temperature is influential. Room temperatures of 22-24^{o}c gave maximum Achlya growth and Olpidiopsis infection. Decreases resulted above and below these levels. Similar results were also noted by Slifkin with Olpidiopsis

incrassata (60). A greater number of Achlya oospores matured at temperatures below 20°c.

The screen procedure was started when sterile cultures of nonsporulating hyphae of Achlya flagellata, Achlya sp. (13SP) and immature Olpidiopsis varians infections in the Achlya flagellata were available. Using sterile forceps, one each of the three culture types were placed in 5 cm diameter glass Petri plates containing 10 ml of the sterile aqueous test solutions. The Petri dishes contained various concentrations in increasing proportions. The fungi proved highly sensitive, as measurements could be made in gamma units per ml. The control culture had 10 ml of sterile distilled water. Readings were taken at 6, 24, and 72 hour intervals. Observations on spore abundance as well as changes in the hyphal appearance were recorded at each of the readings. According to inhibition rates, chemical carcinostatic conclusions could be made after comparisons with other screening systems. Chemical screenings were repeated if results did not coincide with duplicated tests.

Observations on the development of asexual sporangia, occurrence of zoospores, presence of encysted spores and hyphal appearance were recorded at 6 hours. At 24 hours changes occurred mainly in the growth of hyphae, numbers of

zoospores and amount of motility. At 72 hours, sexual reproductive structures were clearly observed as well as morphological changes in the hyphae, amount of growth and lethal effects. Ten cm diameter Falcon Plastic Petri dishes were used for culture of the young Achlya and Olpidiopsis. The maturity rate and sporangial abundance of Achlya was reduced still further when grown in the 5 cm diameter plastic Petri dishes. Zoospore numbers and amount of motility also were reduced when compared with those in glass Petri dishes. Correspondence with Falcon Plastics Division of B-D Laboratories, Inc. of Los Angeles, California indicated the plastic used for the Petri dishes was known as polystyrene and contained no plasticisers. The trace elements were iron, 3 ppm, aluminum, 5 ppm, and copper, 0.5 ppm. Investigations have shown that plastics were considered devoid of toxicity for cell tissue cultures grown directly upon the tested materials (54). The plastic Petri dishes were sterilized with a combination of ethylene oxide and high frequency sound after the injection molding and packaging processes.

Ethylene oxide is highly germicidal and bactericidal by alkylation action while high frequency electron fields (ultrasonic) have a lethal action of physical destruction to cells by coagulation of cell proteins (63). In addition, DNA

and RNA in nucleoproteins were found to be modified (13). Dissolved gases are suddenly extracted from the cell during low pressure phases. Petri dishes of the larger size induced no abnormal Achlya or Olpidiopsis growth, while those grown in the 5 cm diameter dishes showed inhibitions. This may be due to the limited volume and tighter lid of the smaller Petri dishes, containing higher amounts of ethylene oxide. To eliminate this inhibition problem, 5 cm diameter glass Petri dishes were used in the assay.

Ten ml of dilution test material was used in each 5 cm Petri dish. To select the concentration levels used for chemical evaluations,previously published data on the optimum concentrations for inhibition, the tumors were used as a focal point. All test chemical doses were given in mg/kg weight of mouse and on the basis of 1/1,000 of a mg per ml of water in the Achlya test. Dilutions for each compound were selected both above and below the optimum concentration levels. A more accurate reading was made when repeated at concentrations closer to the optimum level of concentration. Generally concentrations increased or decreased from the selected inhibition level by 2, 5 or 10 gammas for the test chemicals. As an example of dilution methods, using chemical

9369, inhibition was noted at approximately 1 gamma / ml using Achlya flagellata. The maximum screening levels of 20 gammas / ml was then selected for the three screening organisms. A stock solution of 20 gammas / ml was made (by balance weight conversion .002 gm plus 100 ml equals 2,000 gammas, therefore 1 ml equals 20 gammas). Sericulture pipettes were used to measure the corresponding serial dilutions for the 5 cm Petri plates.

Concentration	Stock solution	Sterile H_2O
control	0 ml	10 ml
20 gammas/ml	10	0
18	9	1
16	8	2
14	7	3
12	6	4
10	5	5

About 150 ml of the stock solution was needed for this particular chemical, therefore, .003 gm were weighed out.

Since some chemicals screened were insoluble in water, carborymethylcellulose (Carbose D) was used to reduce surface tension and increase the wetting as well as penetrating action of insoluble chemicals. The viscosity of the material proved to be extremely viscous in water, creating conditions similar to agar, reducing the numbers of sporangia, zoospores and hyphal growth. Achlya sp. (13SP) was most

sensitive of the three test organisms. Inhibitory effects were observed at concentrations as low as 0.5 percent. Below this level, a normal increase of vegetative hyphae plus an increase in normal numbers of zoospore and sporangia were noted. No chemical suspensor was used for the insoluble chemicals. Inhibition levels were observed with several of the insoluble chemicals, thus indicating some degree of solubility. Benzimidazole, diethylstilbestrol and testosterone proprionate produced morphological inhibitions at 20 gammas / ml or lower, however, their solubility was very limited.

It was recently observed (unpublished experiments by Dr. Torres, University of Wisconsin-Milwaukee) that fruit coats of zinnia collected in an arid region in Mexico contain an unknown substance in inhibiting embryo growth. Achlya sp. (13SP) and (c-1) were grown in the fruit coat steep dilutions of 1:10, 1:100, 1:1,000 and 1:10,000. The steep was made by soaking 48 zinnia fruit coats in 10 ml of sterile distilled water. Readings were made at 24, 48, and 72 hours, 6 and 16 days. The controls had normal growth in distilled water. Spiral hyphal growth was seen in 24 hours at a 1:10 dilution for Achlya sp. (13SP). This spiral

growth could be observed in 48 hours at dilutions of 1:10, 24 hours at 1:100 and slight twisting in 24 hours at 1:1,000. Growth inhibitors present retarded spore germination period, lengthened the maturity rate such as time of zoospore production and encystment period.

RESULTS

Twenty-six representatives of the Saprolegnaceae from Brazil (3-28, Table I) were tested with azaserine, an early carcinostatic agent with a wide spectrum of inhibitory action in the animal tests. The 26 cultures were screened with azaserine to determine if any strains of Achlya, Thraustotheca or Dictyuchus from Brazil showed any greater chemical sensitivity than Achlya flagellata and the parasite of A. flagellata, Olpidiopsis varians which were from Illinois (1, 2, Table I). The majority of fungi were inhibited by azaserine at 8 gammas per ml or above, six fungi at 4 gammas per ml, and 5 below 4 gammas. Achlya sp. (13SP) from Brazil was selected as the third screening organism since it proved highly sensitive to azaserine, being inhibited at 1/2 gamma /ml.

Abundance of reproductive structures was also compared. The development or lack of development of sporangia and zoospores was one of the key identification techniques used for possible indication of antitumor chemicals. The three assay organisms selected develop quite normally under control conditions. Sporangia were abundant at 6 hours, zoospores at 24 hours, new mature sporangia and additional encysted zoospores at 72 hours (Table I).

TABLE I

Zoospore behavior for members of the Saprolegniaceae when tested with Azaserine.

Organism	Treated, no zoospores at gammas/ml	Untreated controls*		
	6 hours	6 hours	24 hours	72 hours
1. Achlya flagellata	4	s	sz	szzo
2. Olpidiopsis varians	2	s	szz	ssz
3. Achlya sp.(10 v)(sterile)	8	s	sz	sz
4. Achlya sp. (2 SP)	8	s	sz	sz
5. Achlya sp. (c 1)	8	sz	szz	sz
6. Achlya sp.(1 Vic)	8	sz	sz	sz
7. Achlya sp.(BH 9)	8	sz	sz	sz
8. Achlya sp.(2 v)	8	s	sz	sz
9. Achlya sp.(13 SP)	1/2	sz	szz	ssz
10. Achlya sp.(5 v)	8	s	szz	szz
11. Achlya sp.(3 v)	8	s	sz	szz
12. Achlya bisexualis(1 SP)	>8	s	sz	szz
13. Achlya ambisexualis(c 3)	8	sz	szo	sszzo
14. Achlya americana(c 2)	8	szz	sszzo	sszzo
15. Achlya klebsiana(5 SP)	>8	sz	szz	szz
16. Achlya radiosa(2 SP)	4	s	szo	o
17. Achlya n.sp.(2 SP)	1	sz	szo	szoo
18. Dictyuchus sp.(3 BH)	<8	s	sz	sz
19. D. monosporus(9 SP)	4	s	sz	sz
20. D. monosporus(4 SP)	4	sz	s	s
21. Saprolegnia parasitica (2 SP)	4	s	sz	z

	6 hours	6 hours	24 hours	72 hours
22. Thraustotheca sp.(2 SP)	<8	sz	szz	sz
23. Thraustotheca sp.(11 SP)	1	sz	sz	sz
24. Thraustotheca clavata (4 v)	<8	s	sz	sz
25. Thraustotheca sp.(3 SP)	8	s	sszo	ssz
26. Thraustotheca sp.(4 SP)	<8	szz	szz	szz
27. Thraustotheca sp.(3 SP)	2	sz	sz	sz
28. Thraustotheca sp.(6 v)	4	sz	sz	sz

*Code:

z = zoospres few
zz = zoospores abundant
s = sporangia few
ss = sporangia abundant
o = oogonia

Morphological changes observed when members of the Brazilian fungi were tested with azaserine are recorded in Table II. The results here indicate the morphological changes due to inhibition of hyphae as well as the changes in sexual and asexual reproductive structures. This gives a more complete assay reading indicating degrees of morphological inhibitions. All the Brazilian cultures were affected by azaserine. For example, Achlya flagellata mycelium was reduced in length and diameter but was alive during the first 6 and 24 hour periods. At 72 hours in concentrations of 4 gammas, protoplasmic movement of the original hyphae ceased. However, enlarged abnormal hyphae appeared. Achlya sp. (13SP) on the other hand was more sensitive as shown by short, slow-growing hyphae with few sporangia and zoospores at concentrations of 1/2 gamma. Above 1/2 gamma, zoospores were not present and hyphae remained unchanged. Other organisms not used for the assay produced varying degrees in inhibition of zoospores as indicated in Table II. The heterothallics were inhibited at low concentrations similar to Achlya sp.(13SP). All other organisms tested were either killed or inhibited at 8 gammas per ml with varying degrees and rates of hyphal toxicity below this level.

TABLE II

Morphological changes that were observed at 6, 24, and 72 hours in varied Azaserine dilutions.

1. *Achlya flagellata*:	Length and diameter of new growth retarded at 6, 24 hrs at 4 gammas; dead at 72 hrs, new, enlarged hyphae developed from base of colony.
2. *Olpidiopsis varians*:	Some zoosporangia showed the presence of denser protoplasm, irregular in shape at 6 hrs at 2 gammas.
3. *Achlya* sp.(10 v):	Normal vegetative growth only at 6, 24 hrs, sporangia appeared at 72 hrs at 8 gammas and lower.
4. *Achlya* sp.(2 SP):	New hyphae developed were of increased length and diameter at 24 hrs; zoospores at 24 hrs, and 72 hrs at 8 gammas and lower.
5. *Achlya* sp.(c-1):	At 6 hrs, growth inhibited, new hyphal development began at 72 hrs; few zoospores at 8-1/4 gammas, 24 hrs; many zoospores at 8-1/4 gammas at 72 hrs.
6. *Achlya* sp.(1 Vic):	Hyphae killed at 6, 24 hrs, few new hyphae at 72 hrs; zoospores at 24 and 72 hrs, 8 to 1/4 gammas.
7. *Achlya* sp.(HB 9):	Hyphae killed at 6, 24 hrs; new hyphae at 72 hrs, and at one gamma and below; zoospores at 72 hrs, 8 gammas and below.
8. *Achlya* sp.(2 v):	Hyphae retarded at 6 hrs, at one gamma and above; new hyphae at 24 hrs, 2 gammas and below; sporangia and zoospores, 24 and 72 hrs at 8 gammas and lower.

9. Achlya sp.(13 SP): Hyphae short, slow growth, few sporangia and zoospores even at 1/2 gamma, 6, 24, and 72 hrs.

10. Achlya sp.(5 v): Vegetative at 6 hrs, new normal hyphae at 24 hrs, 4 gammas and below; increased zoospores at 6, 24, to 72 hrs, 8 to 1/4 gamma.

11. Achlya sp.(3 v): Slow hyphal growth; sporangia and zoospores at 24 and 72 hrs at 1/4 to 8 gammas.

12. A. bisexualis(1 SP): Normal development of hyphae, new hyphae at 24 hrs; normal zoospores at 72 hrs and 6 hrs at 1/4 gamma, 24 hrs at 1/2, 1/4 gamma, reduced motility at 8 to 1 gammas.

13. A. ambisexualis (c 3) Hyphae retarded, few new hypha at 6 hrs, 1 gamma and below, 2 gammas and below at 24 hrs; zoospores, oogonia, antheridia at 72 hrs 8 gamma and below, 24 hrs at 1/2, 1/4 gamma.

14. A. americana(c 2): Killed at 8 gammas, inhibited at 4, few new hyphae at 2, vigorous vegetative growth, 1/2 gamma, 6 hrs; zoospores at 72 hrs, few at 4 - 2 gammas, normal at 2 and below; oogonia, antheridia, 1 gamma and below.

15. A. klebsiana(SP 5): Normal hyphal development; zoospores few at 6 hrs, 2 gamma and below; new hyphal growth and zoospores at 1/2, 1/4 gammas 24 hrs; at 72 hrs, 7 to 10 sporangia noted per hemp seed at 8 gammas, greatest numbers and zoospore activity at 2 gammas with gradual decrease to control.

16. A. radiosa(2 SP): Killed at 8 gammas; 4 gammas few new hyphae at 72 hrs; 2 gammas and below new hyphae developed at 24 hrs; sporangia immature on short hyphae at 2 gammas, few at 1, normal at 1/2, 1/4 gamma, 72 hrs; oogonia, antheridia, 1 gamma and below at 72 hrs.

17. Achlya sp.(2 SP): Hyphae inhibited at 8 gammas, new hyphae developed, 4-1 gammas, few sporangia, and zoospores at 1/2, 1/4 gamma, 6, 24, 72 hrs, few oogonia.

18. Dictyuchus sp. (3 HB): At 6 hrs, 8 gammas, hyphae inhibited; new hyphae developed at 6 hrs, 2 gammas, 24 hrs, 4 gammas; sporangia, spores few at 72 hrs, 8-2 gammas, 24 hrs, 1 gamma, 6 hrs 1/2, 1/4 gamma.

19. D. monosporus(9 SP): Few sporangial zoospores at 2 gammas and below, 6 hrs; new hyphae at 24, 72 hrs, 2 gammas and below; killed at 4 gammas by 72 hrs.

20. D. monosporus(4 SP): Killed at 4 gammas, few sporangia, 6 hrs; vegetative, 24, 72 hrs except for new sporangia and spores at 1/2, 1/4 - 72 hrs.

21. Saprolegnia parasitica (2 SP): Hyphae inhibited at 4 gammas, 6 hrs; new hyphae development at 24, 72 hrs; few sporangia, spores, many gemmae 72 hrs, at 2 and 6 gammas.

22. Thraustotheca sp. (2 SP): Hyphae inhibited, new hyphae developed at 24 hrs; sporangia, zoospores at 72 hrs above,8 gammas.

23. Thraustotheca sp. (11 SP): Hyphae inhibited to 1 gamma at 6 hrs, new large hyphae developed at 24 hrs; sporangia and spores at 1/2, 1/4 gamma, 72 hrs.

24. Thraustotheca clavata (4 v): Hyphae inhibited at 6, 24 hrs, 8 gammas; new hyphae developed at 6 hrs and 24 hrs, 4 gammas; vigorous new growth at 1/2, 1/4 gammas, 24 hrs, sporangia, spores, 72 hrs.

25. Thraustotheca sp. (3 SP): Killed at 8 gammas, 6 hrs; new hyphae developed at 6 hrs, 4 gammas and below, few gemmae, 1 gamma and below; sporangia, zoospores at 72 hrs, 4 gammas and below, 24 hrs, 1 gamma and below, gemmae also present.

26. Thraustotheca sp. (4 SP): Vegetative growth retarded, increasing at 6 hrs, few sporangia noted at 4, 1/2, 1/4 gammas; normal numbers of sporangia, spores at 72 hrs, 8, gammas and below.

27. Thraustotheca sp. (3 SP): Hyphae inhibited at 6, 24 hrs, 8-1 gammas, new hyphae developed at 72 hrs, 4, 2 gammas; few sporangia, spores at 6 hrs, 1 gamma and below, normal numbers at 1/4 gamma, new hyphae at 24 hrs, sporangia and zoospores at 72 hrs.

28. Thraustotheca sp. (6 v): Hyphae retarded up to 72 hrs at 8 gammas, 24 hrs at 4-2 gammas; new hyphae developed 4-2 gammas at 24 hrs; sporangia and zoospores at 72 hrs, 2 gammas and below, many at 1/4; few at 6 hrs, 1/2, 1/4 gamma, at 24 hrs all new hyphae were vegetative.

Thirty-five compounds from the National Service Center (NSC) were tested using the three selected fungi for screening. Table III lists the compounds with their corresponding NSC number. Inhibition levels in gammas per ml are shown for inhibition of the zoospores. Some of the chemicals nearly insoluble in water showed inhibitory effects. Two of the compounds showed miscible properties (1026, 3051). Olpidiopsis varians was inhibited at lower gamma concentration readings than Achlya flagellata. Achlya sp. (13SP) showed similar inhibition levels for zoospores as the homothallic species, A. flagellata, in some instances while with other chemicals, the results were closer to Olpidiopsis.

Table IV indicates morphological changes at various gamma readings for the three assay organisms with the tumor inhibiting compounds. Some of the chemicals caused the development of distinct differences in morphology other than only zoospore inhibition. These added morphological distinctions further characterized the chemical sensitivity in identifying carcinomstatic action. Such inhibitions of morphological structures as hyphae and sporangia aid in making the levels of zoospore inhibition distinct and clarifies the choice of such levels.

Table III

Levels of Zoospore Inhibition at Six Hours (gammas per ml)

NSC No.	Compound	Achyla sp. 13 SP	Achyla flagel-lata	Olpidi-opsis varians
185	actidione	4	2	1/2
741	hydrocortisone	-(160)	-	-
743	2,6-diaminopurine	50	60	40
746	urethane	40	30	10
747	n-methyl acetamide	-100**	100**	90
750	myleran*	-(50)	-	-
751	dl-ethionine	15	10	2
754	b-2-thienyl alanine	50	40	40
756	8-azaxanthine*	50**	50**	35
757	colchicine	40	30	30
758	d-glucosamine	-(40)	40	12
759	benzimidazole*	16	20	12
762	nitrogen mustard	2	2	1/2
1026	1-aminocyclopentane*	-(20)	14	8
1393	4-amino pyrazole pyrimidine	40**	20	20
3051	n-methyl formamide*	-(200)	-	-
3054	methyl carbamate*	-(80)	-	-
3060	potassium arsenite	16	20	10
3061	daraprim*	-(80)	-	-
3062	2,4-diamino-5(3',4'-dicloro-phenyl)-6-ethylpyrimidine*	50**	25	25
3063	desoxypyridoxine	35**	30**	25
3069	chloromycetin	80**	80**	40
3070	diethylstilbestrol*	2	1	1
3071	5-methoxytoluguinone	40	30	10
3088	chlorambucil*	50**	50**	40
3095	1,5-diaminobiuret	100	100	50
6396	thio TEPA	12	35	18
9166	testosterone proprionate*	10	40	4
9369	1-methyl-3-nitro-1-nitro-1-nitroso-guanidine	1	2	1
9706	TEM triethylene melamine	25	60	40
14210	d-l sarcolysin	50	75	50
14574	miracil	2	2	4
24818	podophyllotoxin	(20)**	-	-
26271	cycloposphoramide	50**	50**	40
26982	mitomycin-c	40	2	6

* insoluble in water

** reduced equals less 200 spores

TABLE IV

Fungal reactions to antitumor compounds (Morphological Changes).

Compound NSC No.	Achlya sp. (13 SP)	Achlya flagellata	Olpidiopsis varians
Key:	1.	2.	3.
185	1. Few sporangia and zoospores at 6 hrs, 1-2 gammas; inhibited at 24 hrs; only spiral vegetative growth at 72 hrs; vegetative at 6 hrs above 4 gamma inhibition level; killed at 24 hrs, protoplasm coagulated.		
		2. Vegetative above 2 gammas, 6 hrs inhibition level, few zoospores, 2-10 gammas, killed at 24 hrs, below had fewer sporangia and spores than control.	
			3. Zoospores less motile at 6 hrs, killed at 24 hrs; below 1/2 gammas inhibited at 6 hrs, not killed but no new growth.
741	1. Negative; sporangia and spores as control; however, at 6-24 hrs, many spores were nonmotile, 160 gammas or above.		
		2. Negative; sporangia and zoospores similar to control but at 72 hrs, fewer zoospores were present, 80 gammas and above.	
			3. Negative; new tumor formation at 24 hrs as control, zoosporangia at 6 hrs.
743	1. Sporangial maturation increased faster than control at a gradual rate to 50 gammas, but sporangial production decreased at 24 hrs, 50 gammas; motile spores at 72 hrs, above 70 gammas, zoospores encysted below 70 gammas at 72 hrs.		
		2. An increase in maturity was also noted at the various levels, oogonia and antheridia numerous, 40-60 gammas, decreased, 30 gammas and below.	

3. At 6 hrs, 40 gammas, tumor formation were inhibited, very few zoospores were seen at 24 and 72 hrs above 40 gammas; normal numbers of zoospores below 40 gammas, few new tumors.

746 1. At 40 gammas and above sporangia failed to mature, and no zoospores formed.

2. At 30 gammas and above, sporangia failed to mature but oogonia and antheridia were present; below inhibition level, zoospores developed at 24 hrs.

3. Zoospores inhibited at 24 hrs above 10 gammas; fungus killed above 40 gammas; at 6 hrs all were normal.

747 1. Reduced number of sporangia, zoospores, and hyphal growth at 100 gammas 6-24 hrs; like controls at 72 hrs and at 6-24 hrs 90 to 2.5 gammas.

2. Reduced sporangia and zoospores at 6-24 hrs, 100 gammas; like controls at 72 hrs and at 6-72 hrs at 100-2.5 gammas.

3. Retarded tumor formation at 6 hrs, 100-90 gammas, at 24 hrs, 100 gammas; new tumors at 72 hrs, 100 gammas at 24 hrs, 90-2.5 gammas; zoospores only at 6 hrs, 80-2.5 gammas.

750 1. No inhibition of sporangia and zoospores at 50 gammas.

2. No inhibition of sporangia and zoospores at 50 gammas.

3. No inhibition of tumor formation at 50 gammas.

751 1. Hyphae inhibited 50-20 gammas, 6-72 hrs; zoospores and sporangia few at 6-72 hrs, 10-1 gammas, normal at 1/2 gamma.

2. Killed at 10 gammas, protoplasm coagulated; few zoospores and sporangia, 5-1 gammas, 6-72 hrs, normal at 1/2 gamma; oogonia present at 72 hrs 3-1/2 gammas.

3. Killed at 2 gammas; few sporangia with zoospores at 1-1/2 gammas, 72 hrs.

754 1. Hyphae only at 6 hrs 100-50 gammas; few sporangia and zoospores at 24 hrs; sporangia and zoospores at 72 hrs, 100-50 gammas, at 24-72 hrs, 40 gammas, at 6-72 hrs, 30-2.5 gammas.

2. Loosely formed spiral hyphae only at 6-72 hrs, 100-40 gammas; spiral growth decreasing under 30 gammas; few zoospores at 24 hrs, 30-2 gammas, no inhibition at 72 hrs, 30-20 gammas, at 6-72 hrs, 10-2.5 gammas.

3. Gradient sporangial development only 100-40 gammas, 6-24 hrs; at 72 hrs normal zoospore numbers; zoospores numerous at 6-72 hrs, 30-2.5 gammas; new tumor formation at 72 hrs, 100-40 gammas, at 24 hrs, 30-2.5 gammas.

756 1. Reduced numbers of sporangia and zoospores at 6-72 hrs,50 gammas, no inhibition at 6-72 hrs 45-2.5 gammas.

2. Few sporangia and zoospores at 6-72 hrs, 50 gammas, increasing at 30 gammas to no inhibition from 30-2.5 gammas; oogonia present at 72 hrs, 50-2.5 gammas.

3. No tumor formation at 6 hrs, 35 gammas; at 24 hrs few zoospores present, inhibited at 72 hrs; control at 30-2.5 gammas, zoospores at 6-72 hrs; new tumor sporangia at 72 hrs 10-2.5 gammas.

757 1. Hyphae killed above 40 gammas at 6 hrs; at 24 and 72 hrs dense vegetative hyphae developed; from 30-14 gammas, zoospore numbers increased to normal levels at 6 hrs; 6, 24, and 72 hrs were normal in maturation below 14 gammas.

2. No zoospores at 30 gammas 6 hrs, but few oogonia appeared at 72 hrs; from 30-16 gammas, zoospore numbers increased at 6 and 24 hrs; few encysted spores, oogonia, and antheridia at 72 hrs.

3. Tumor formation inhibited from 6 hrs at 30 gammas and above; from 30-10 gammas, zoospores fewer than control, new tumor at 6-2 gammas, 72 hrs.

758 1. Negative; all levels like control in development.

2. Inhibited sporangia and zoospores at 6 and 24 hrs, 40 gammas; few zoospores appeared at 30 gammas, 72 hrs; zoospores numerous at 6 and 24 hrs, 30 gammas: encysted at 72 hrs with many oogonia and antheridia present.

3. Killed at 6 hrs, 30 gammas and above; inhibited zoospore production at 6 and 24 hrs, above 12 gammas, but present at 72 hrs; new tumor formation numerous below 10 gammas at 72 hrs.

759 1. Hyphae killed at 6 hrs, 16 gammas; few short hyphae developed at 72 hrs, 16-30 gammas; below 14, sporangia and zoospore production normal.

2. Hyphae inhibited at 6 hrs, 20 gammas, noted protoplasm coagulated by 72 hrs, killed; there was a gradual increase in zoospores from 18-1/4 gammas; few oogonia and antheridia.

3. At 6 hrs, the fungus was killed, 12 gammas and above; zoospores developed at 24 hrs, below 12 gammas.

762 1. Hyphae inhibited 20-40 gammas at 6 hrs, 20-12 gammas at 24 hrs; cytoplasm coagulated 20-14 gammas 72 hrs, killed at 14 gammas 72 hrs; short dense hyphae beginning at 72 hrs, 12 gammas increasing to 2 gammas; normal hyphae at 1 gamma 72 hrs; few sporangia at 72 hrs 6-2 gammas, at 6-24 hrs, 1 gamma,increased sporangia and zoospores were seen; many sporangia and zoospores at 72 hrs, 1 gamma, at 6-72 hrs, .5-.25 gamma.

2. Little if any hyphal growth, hyphae very short 6-72 hrs, 20-2 gammas, growth increased at 24 hrs, 2 gammas, at 6 hrs, 1 gamma; few zoospores at 1 gamma, 6-72 hrs, at 1/2 gamma, 6-24 hrs; zoospores

and sporangia at 1/2 gamma, 72 hrs, at 1/4 gamma, 6-72 hrs; oogonia present at 72 hrs, 2-1/4 gamma.

3. Killed at 1 gamma; few mature sporangia in tumors at 72 hrs 1/2-1/4 gamma but no new tumor development except controls at 24-72 hrs.

1026 1. Negative at 20 gammas, highest concentration tested; numerous sporangia and zoospores at 6-72 hrs.

2. Vegetative hyphae 6-72 hrs at 16 gammas and above, very long hyphae at 72 hrs; few zoospores 14-4 gammas, increasing in numbers 2-1/4 gammas at 6-24 hrs; decreased zoospore numbers at 72 hrs, 14-4 gammas, hyphae unusually long; hyphae control length 2-1/4 gammas, 6-72 hrs; oogonia present at 72 hrs, 14-1/4 gammas.

3. Tumor formation retarded at 6 hrs, 20-6 gammas, zoospores 6-1/4 gammas; zoospores and new tumors, 24-72 hrs at all concentrations.

1393 1. Sporangia, zoospores at reduced numbers, 50-40 gammas, 6-72 hrs, numerous at 30-1.5 gammas, 6-72 hrs; encysted spores many at 72 hrs.

2. Hyphae slightly spiraled at 50 gammas, 6-24 hrs, immature sporangia and oogonia at 72 hrs; very few zoospores at 45-20 gammas, 6-24 hrs, oogonia present at 72 hrs; sporangia normal at 6-24 hrs, 15-1/5 gammas, only oogonia present at 72 hrs.

3. Reduced zoospore numbers, 6-72 hrs, 50-20 gammas, many present in sporangia, 15-1.5 gammas; very few new tumors noted, 30-20 gammas, 24-72 hrs, many at 15-1.5 gammas.

3051 1. Negative; many sporangia, zoospores at 6-72 hrs, 200-10 gammas.

2. Negative; many sporangia and zoospores at 6-24 hrs, 200-10 gammas; many oogonia at 72 hrs.

3. Negative; many zoospores 6-72 hrs; new tumor formation 24 hrs and at 72 hrs, 200-10 gammas.

3054 1. Negative; numerous sporangia and zoospores at 6 hrs, 80 gammas.

2. Many sporangia and zoospores at 6 hrs, 80 gammas, oogonia at 72 hrs.

3. Negative at 80 gammas; all immature at 6 hrs, mature tumors at 24 hrs, zoospores and new tumors at 72 hrs.

3060 1. Slow inhibition; at 20 gammas, 6 hrs, many sporangia and zoospores, few at 24 hrs; hyphae killed at 72 hrs; no zoospores at 6-72 hrs at 16 gammas, normal at 14-1/4 gammas, 6, 24, and 72 hrs.

2. Negative at 20 gammas, sporangia and zoospores at 6-72 hrs, 20-1/4 gammas; many oogonia present at 72 hrs, 12-1/4 gammas.

3. Tumors retarded at 6 hrs, inhibited at 72 hrs, 10 gammas; zoospores and new tumors at 24-72 hrs, 8-1/4 gammas.

3061 1. Negative; growth appeared normal.

2. Negative; no difference noted between control and screening concentrations to levels tested.

3. Negative; no inhibition at 80 gammas, highest level tested.

3062 1. Decreased sporangia and zoospores at 6 hrs, 50-45 gammas, increased at 6 hrs, 40-15 gammas, slightly decreased at 6 hrs, 10-1.25 gammas; sporangia inhibited at 24 hrs, 50-15 gammas, few at 10-5 gammas, numerous sporangia and zoospores at 6-24 hrs, 5-1.25 gammas; only hyphae, some spiral at 50-5 gammas, 72 hrs.

2. Hyphae only at 6-72 hrs, 50-25 gammas; immature sporangia at 6 hrs, 20-2.5 gammas, few mature at 1.25 or less; few zoospores, spiral hyphae at 24 hrs 20-5 gammas, zoospore number normal 2.5 control; oogonia at 72 hrs, 35-1.25 gammas.

3. Zoospores in sporangia inhibited at 6 hrs, 50-25 gammas, at 24 hrs, 35 gammas, zoospores normal in numbers at 6 hrs, 20-1.5 gammas; new tumor sporangia at 72 hrs, 20-1.5 gammas.

3063 1. Sporangia, zoospores present at 6-72 hrs, 50-35 gammas, numbers gradually increased to 30-1.5 gammas, 6-72 hrs; many encysted spores at 72 hrs, 50-1.5 gammas.

2. Few zoospores and sporangia at 6, 24, 72 hrs, numbers increased at 30-15 gammas; normal, 15-1.5 gammas; at 72 hrs abnormal numbers of oogonia present from 50-1.5 gammas.

3. Zoospores inhibited at 25 gammas, 6 hrs, no new tumor formation; zoospores present at 24 hrs, 20-10 gammas, at 6 hrs, 5-1.5 gammas; new tumors at 72 hrs 4-1.5 gammas.

3069 1. Sporangia and zoospores reduced in numbers at 6, 24, 72 hrs, 80-30 gammas; control levels reached at 20-1 gammas at all three readings.

2. Reduced sporangia and zoospores at 6, 24, 72 hrs, 80-50 gammas, normal at 40-1 gammas; oogonia few at 70-50 gammas, 72 hrs, increasing to normal 40-30 gammas, normal oogonia 30-1 gammas.

3. Tumor sporangia inhibited 80-40 gammas, 6-72 hrs; zoospores present at 24 hrs, 30-1 gammas, at 6 hrs, 2.5-1 gamma; new tumors at 72 hrs, 30 gammas, 24-72 hrs at 20-1 gammas.

3070 1. Killed at 30 gammas; inhibited at 6 hrs, 30-8 gammas, at 24 hrs, 30-14 gammas; very short, dense, and thick hyphae at 6 hrs, 8-2 gammas, at 24 hrs, 12-2 gammas, at 72 hrs, 20-2 gammas; normal hyphae at 2 gammas or less; immature sporangia at 72 hrs, 4-2 gammas, zoospores few at 6, 24, 72 hrs, 1-1/4 gammas.

2. Killed at 20 gammas; very short dense hyphae at 6, 24, 72 hrs, 18-8 gammas, gradual increased

length 6-1/4 gammas, spiral hyphae at 72 hrs, 6-2 gammas, few sporangia at 72 hrs, 2-1/4 gammas.

3. Killed at 1 gamma, few zoospores at 24 hrs, 1-1/4 gamma, no new tumor formation even at 72 hrs, 1/4 gamma.

3071 1. Short inhibited hyphae 6-24 hrs, 100-40 gammas, at 72 hrs hyphal length gradually increased at 80-40 gammas; sporangia and zoospores were normal in numbers at 30-2.5 gammas, 6-72 hrs.

2. Short hyphae at 6-72 hrs, 100-30 gammas, sporangia and zoospores at 6-72 hrs, 30-2.5 gammas, oogonia at 72 hrs, 20-2.5 gammas.

3. Killed at 10 gammas, sporangial protoplasm coagulated; zoospores 6-72 hrs; and new tumors 72 hrs at 5-2.5 gammas.

3088 1. Few sporangia and zoospores at 6-72 hrs, 50 gammas, normal numbers 45-1.25 gammas; dense spiral hyphal colonies at 72 hrs, 50-15 gammas.

2. Few sporangia and zoospores at 6 hrs, 50 gammas, control numbers at 6 hrs, 45-1.25 gammas; an increased hyphal growth, more dense colonies than control was noted at 72 hrs, 50-10 gammas, normal growth, 10-1.25 gammas; oogonia appeared at 72 hrs, 35 gammas, increasing to normal levels at 15 gammas.

3. Zoospores inhibited at 40 gammas, 6-72 hrs, present at 6 hrs, 35-1.25 gammas, inhibited at 24 hrs, 35-25 gammas, present at 6-24 hrs, 25-1.25 gammas; new tumor sporangia at 24-72 hrs, 20-1.25 gammas.

3095 1. Killed at 250 gammas, 72 hrs, spiral growth 250-150 gammas, few gemmae 24-72 hrs; immature sporangia present at 72 hrs, 150-100 gammas, zoospores and sporangia at 6-72 hrs, 100-1 gamma; some immature sporangia developed hyphae.

2. Killed at 200 gammas 72 hrs, branched gemmae and immature sporangia developed hyphae at 6-24 hrs; spiral hyphae at 6-24 hrs, 150-100 gammas; sporangia zoospores 100-1 gammas, occasionally spiral hypha and gemmae at 72 hrs.

3. Killed at 6 hrs, 100 gammas, zoospores and few new tumors under 100 gammas at 24-72 hrs.

6396 1. At 12 gammas and above, zoospores and sporangia were inhibited, but developed at 24 and 72 hrs in decreasing numbers to 40 gammas; below 12 gammas, normal development.

2. Above 35 gammas, hyphae were vegetative at 6 hrs, germinating sporangia and oogonia developed at 24, 72 hrs; increased sporangia with zoospore production to normal numbers at 16 gammas; oogonia numerous at 24, 72 hrs, below 35 gammas.

3. Inhibition above 18 gammas at 6 hrs, few zoospores at 24 and 72 hrs; below 18 gammas, zoospores numerous at 6 hrs; new tumor sporangia at 24 hrs.

9166 1. Hyphae only at 50-10 gammas, 6-24-72 hrs, growth shorter at greater concentrations; some spiral hyphae at 25-10 gammas, 72 hrs; sporangia, zoospores few, 8-6 gammas, 6-72 hrs, normal sporangia and zoospore numbers 4-1/4 gamma, 6 to 72 hrs.

2. Vegetative hyphae to 40 gammas, 6-72 hrs; hyphal mat increased in denseness and diameter 35-1 gammas, 24-72 hrs; some spiral hyphae 35-1 gammas at 72 hrs plus few immature sporangia; no zoospores above 10 gammas.

3. Killed at 6 gammas, few zoospores at 24-72 hrs, 4-1 gammas, no new tumors.

9369 1. Killed at 20 gammas, 6 hrs, protoplasm coagulated; maturity slowed down, no zoospores developed until 72 hrs 18-1 gammas; zoospores and sporangia were present at .5-.25 gammas at 6-72 hrs.

2. Killed at 20 gammas, 6 hrs; hyphae inhibited at 6-72 hrs, 18-2 gammas, reduced growth, many gemmae; few zoospores at 6-72 hrs 1-1/4 gammas; few oogonia at 72 hrs 1-1/4 gamma.

3. Killed at 2 gammas 6 hrs, few zoospores in tumor sporangia at 72 hrs 1-1/4 gamma.

9706 1. No sporangia developed above 25 gammas, large vegetative hyphae developed at 24 and 72 hrs; zoospores few below 25 gammas to 10 gammas where numbers were normal at 24-72 hrs.

2. Above 60 gammas only vegetative hyphae; below 60 gammas few sporangia and zoospores at 6, 24 hrs to normal numbers at 20 gammas; few germinating spores within sporangia; oogonia at 20 gammas and below at 72 hrs.

3. Tumor sporangia killed at 40 gammas and above; zoospores normal below 35 gammas, developed witches broom affect below 35 gammas.

14210 1. Vegetative hyphae 25-200 gammas with few sporangia 75-50 gammas at 72 hrs; sporangia and zoospores developed normally below 25 gammas.

2. Above zoospore inhibition level of 75 gammas, hyphae long and narrow, spiral hyphae began at 24 hrs; below 75 gammas, normal development of sporangia; many oogonia at 6 hrs.

3. Killed at 50 gammas; zoospores normal in numbers and motility at 25 gammas and below at 6, 24, 72 hrs; new tumors at 24, 72 hrs below 25 gammas.

14574 1. Hyphae inhibited at 6 hrs, 30 gammas, hyphae very short and dense 30-12 gammas, 72 hrs; occasional spiral growth under 12 gammas after 6 hrs; sporangia developed normally at 1 gamma, 6 hrs, few seen at 72 hrs, 2 gammas.

2. Killed at 40 gammas, hyphae short down to 4 gammas, oogonia developed at 72 hrs between 18-10

gammas, seen at 24 hrs 8-1/4 gammas; sporangia and spores seen at 1 gamma and below at 6 hrs.

3. Killed at 20 gammas, little growth from 20 to 4 gammas; few zoospores at 24 hrs 4 gammas; normal sporangia and zoospores at 2 gammas 6, 24, 72 hrs.

24818 1. Sporangia and zoospore numbers reduced at 20 gammas 6 hrs but normal at 24 hrs; zoospores normal at 6 hrs, 18 gammas and below; few mild spiral hyphae at 16, 12 and 6 gammas, 6 hrs, normal growth after 6 hrs.

2. No inhibition at concentrations tested, up to 20 gammas; normal sporangia and zoospores at 6 hrs, oogonia and antheridia at 72 hrs.

3. Negative; immature sporangia tumors at 6 hrs, mature and new tumors developed at 24 hrs 20 gammas (highest concentration).

26271 1. Negative at 50 gammas; hyphae, sporangia zoospores like control.

2. Negative; like control results.

3. Negative at 40 gammas; no zoospores at 50-40 gammas at 6 hrs; normal zoospore number at 24 hrs 50-1 gammas, at 6 hrs 35-1 gammas; new tumors at 72 hrs 50 gammas, 24 hrs 40-1 gammas.

26982 1. Vegetative hyphae at 40 gammas 6, 24, 72 hrs; many sporangia and zoospores at 6-72 hrs from 30-8 gammas, fewer from 8-2 gammas, normal numbers 1-1/4 gammas.

2. Vegetative hyphae 40-2 gammas at 6-72 hrs, few sporangia at 1 gamma 6 hrs, normal at 1/2 and 1/4 gamma; oogonia 4-1/4 gamma at 72 hrs.

3. Zoospores inhibited at 6 hrs 40-8 gammas, present from 6 to 1/4 gamma, at 24 hrs, few zoospores to 8 gammas; many zoospores and tumor formation 6-1/4 gammas, both structures present at 72 hrs in all gamma levels, 40-1/4 gammas.

Substances other than NSC antitumor chemicals tested with the Achlya assay system included two polymers and their derivatives. Itaconic styrine with derivatives IS-lN-2, IS-lN-5, IS-lHlN-3, IS-4HlN-4, IS-lN-ll, and maleic styrine plus derivatives MS-lN-l, MS-lNal-lSh were screened with the three fungi. Only one, IS-lN-5, proved negative with mouse Sarcoma 180 test. It was also negative with the Achlya assay. The other styrines were inhibitory to mice tumors between 50 and 200 mg / kg. Achlya inhibitions were equally varied, being retarded between 80 to 800 gammas /ml. Wide variations were recorded with individual polymers on Achlya flagellata, Achlya sp. (13 SP) and Olpidiopsis varians. Styrine value in tumor treatment is still in the preliminary laboratory experimental stages.

DISCUSSION

The assay chemicals tested were shown to have, in most cases, inhibitory effects with Achlya sp. (13 SP), Achlya flagellata and Olpidiopsis varians. The chemicals were grouped into three levels of inhibition, based on concentrations in gammas. Highly carcinostatic agents included chemicals inhibiting the Achlya screen between 1-20 gammas /ml, higher levels of concentrations were chemicals within 20-50 gammas /ml, and the highest level of concentration required for inhibition were above 50 gammas / ml. Response to chemicals were considered negative (ex. 750, 758, 3051, 3054) if control and treated organisms continued to grow normally at all concentrations tested.

Alkylating agents causing damage to cytoplasm and nuclei by blocking formation of RNA and DNA were the most effective and sensitive to Achlya in the screen. Nitrogen mustard, benzimidazole, diethylstilbestrol and thio TEPA were all highly inhibitory to Achlya. Toxicity was at 20 gammas /ml or lower. The moderately inhibiting alkylating agents were d-l sarcolysin, TEM, cyclopposphoramide and chlorambucil. Myleran was slightly inhibitory while methyl carbomate was the only alkylating chemical showing no effect on Achlya

screening tests. The latter two chemicals were insoluble thus decreasing their effectiveness. Other than the insoluble methyl carbamate, all of alkylating agents were tested with the Achlya assay.

Amino acid antagonists such as d-l ethionine and desoxypyridoxine showed inhibitory effect on Achlya zoospore formation, also, either at high or moderate concentration.

The antifolic agents directly inhibitory to purine synthesis such as 2, 4-diamino-5-(3', 4'-dichlorophenyl)-6-pyrimidine and 4-amino pyrazole pyrimidine were both moderately inhibitory to Achlya zoospore development. The potential antitumor chemical daraprim was not effective with the Achlya assay. It is, however, insoluble in water.

Azaserine, the glutamine enzyme antagonist, inhibiting nucleic acid formation was highly toxic to the three assay fungi. All other nucleic acid inhibitors with varied modes of action were also moderately or highly effective at low concentrations. Purine antagonist effecting the incorporation of purines into nucleic acids in nucleoproteins such as 2, 6 diaminopurine, 5-methoxytoluguinone, and 8-azanthine showed only moderate Achlya zoospore inhibitions and morphological changes. Eight azaxanthine is another chemical

insoluble in water, therefore little if any material went into solution from the chemical crystals, thus ineffective on the Achlya hyphae.

Potassium arsenite, colchicine, podophyllotoxin and hydrocortisone have been listed as nucleic acid synthesis inhibitors with reduced CO_2 production. These four unrelated compounds are diverse in requiring a wide range of concentrations to show toxicity on Achlya. Potassium arsenite gave highly positive reactions while hydrocortisone was negative in response.

Chemicals having unknown modes of action undoubtedly have widely diverse effects on cell metabolism to show inhibitions of zoospores in the Achlya assay method studied. Several of these chemicals were highly inhibitory or mildly inhibitory to Achlya. One compound with unknown mode of action, 1, 5-diaminobiuret, was not inhibitory to Achlya until 150 gammas per ml, was added, however, Olpidiopsis varians showed inhibition at 50 gammas /ml.

All carcinostatic chemicals effecting the nuclear material of malignant cells were either highly or moderately inhibitory to Achlya zoospore development and Olpidiopsis tumor development. Nucleic acid synthesis was apparently directly or indirectly affected. Those chemicals showing

little or no inhibition when screened with the Achlya method were insoluble or nearly insoluble in water. Slightly insoluble chemicals such as diethylstilbestrol, still were highly inhibitory at low concentrations.

Morphological changes other than zoospore emergence and motility were also similar with the nucleoprotein inhibitors. The Achlya mycelium showed varying degrees of reaction to toxicity, usually at higher concentrations than required for zoospore inhibition. Either spores were nonmotile or absent at the chemotoxic levels. The absence of spores occurred if sporangia did not develop in the test solutions or sporangia maturity was halted just before zoospore emergence. At times spores did not produce the first motile stage of passing through the sporangial pore and encysting at the sporangial apex. The first motile period was eliminated and spores germinated within the sporangium (Fig. 10). The new spore germinated and hyphae penetrated the sporangia walls and produced a witches broom appearance on the sporangia. Eventually the sporangial wall disintegrated leaving a small dense mat of thin hyphae. Disintegration also occurred at times with the Olpidiopsis. If inhibition levels were moderate, between 20-50 gammas /ml, in a few cases, Olpidiopsis tumors were retarded and the Achlya hyphae continued to produce a witches broom like growth of hyphal branches.

Fig. 10. Achlya flagellata with germinating spores within the sporangium produced with TEM (NSC No. 9706). (135x)

Fig. 11. A germinating immature sporangium or gemmae type growth from 1-methyl-3-nitro-1-nitro-1-nitroso-guanidine (NSC No. 9369). (165x)

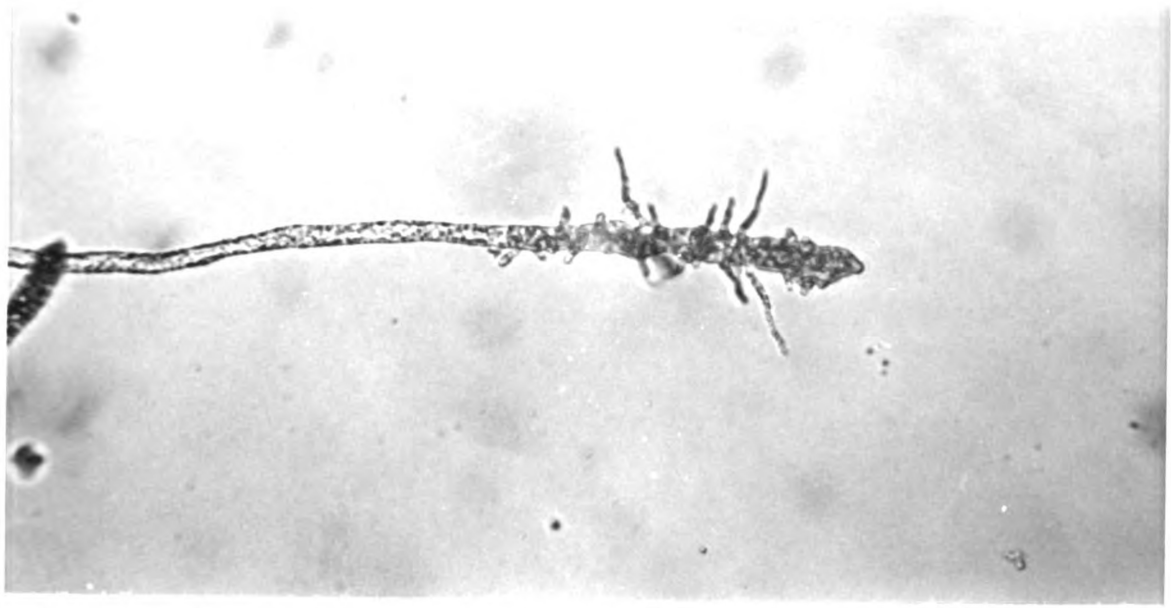

Fig. 10

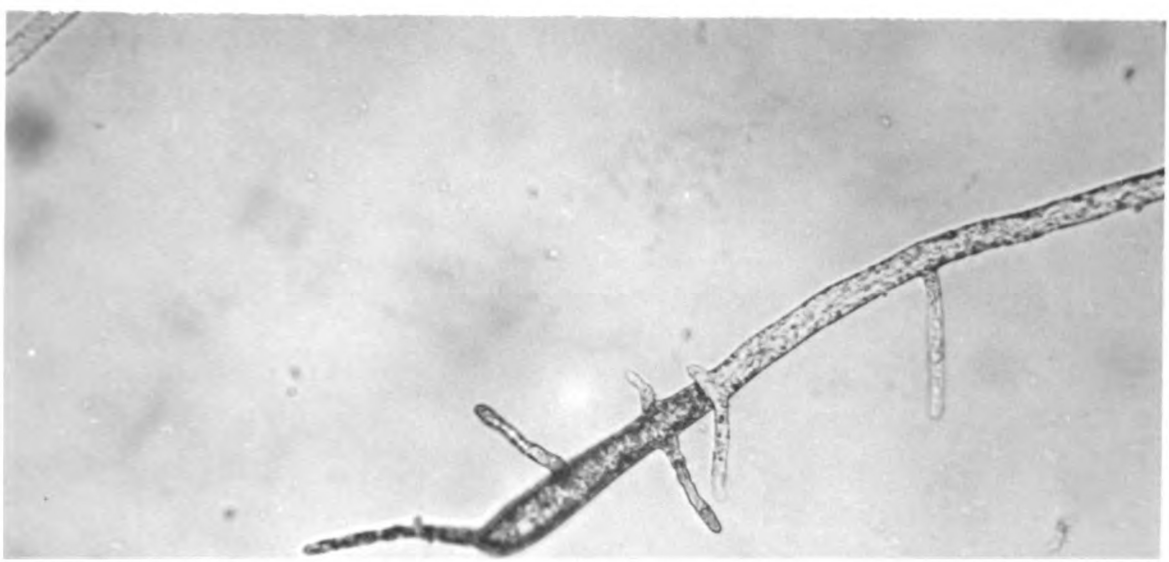

Fig. 11

Fig. 12. Achlya sp. (13 SP) with a spiral hypha produced with miracil d (NSC No. 14574). (135x)

Fig. 13. Severe protoplasmic coagulation of Achlya flagellata hypha with actidione (NSC No. 185). (250x)

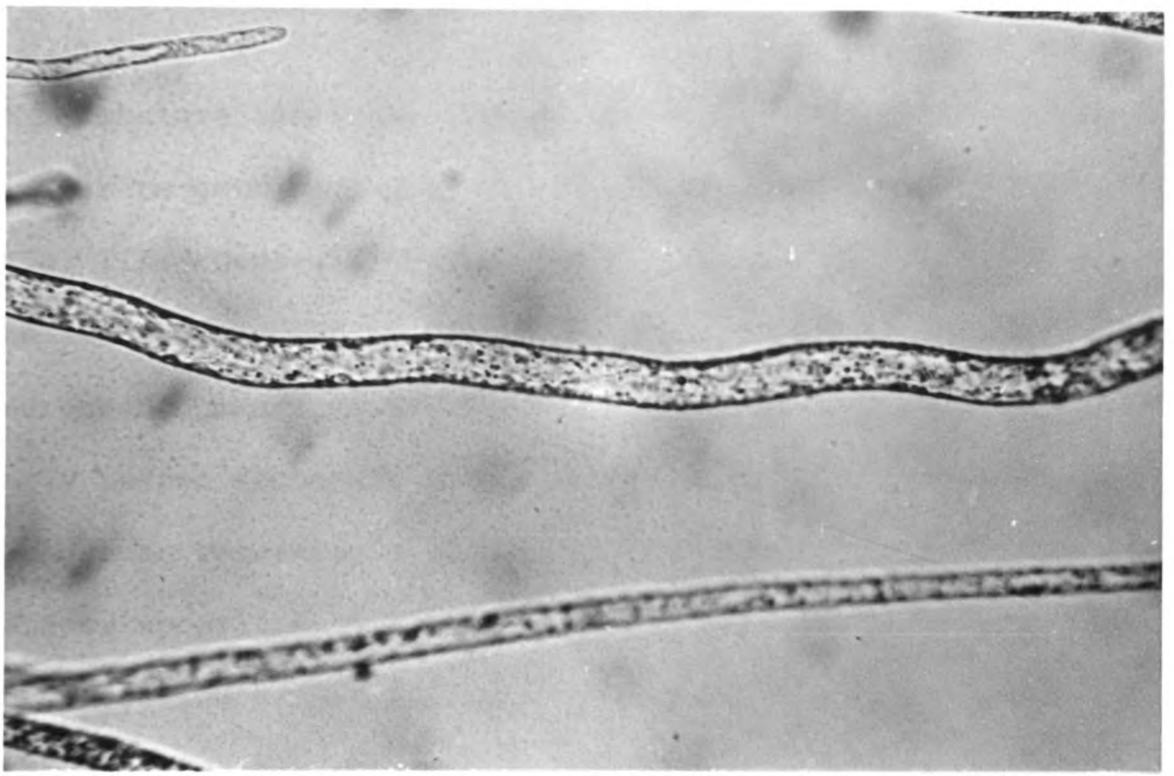

Fig. 12

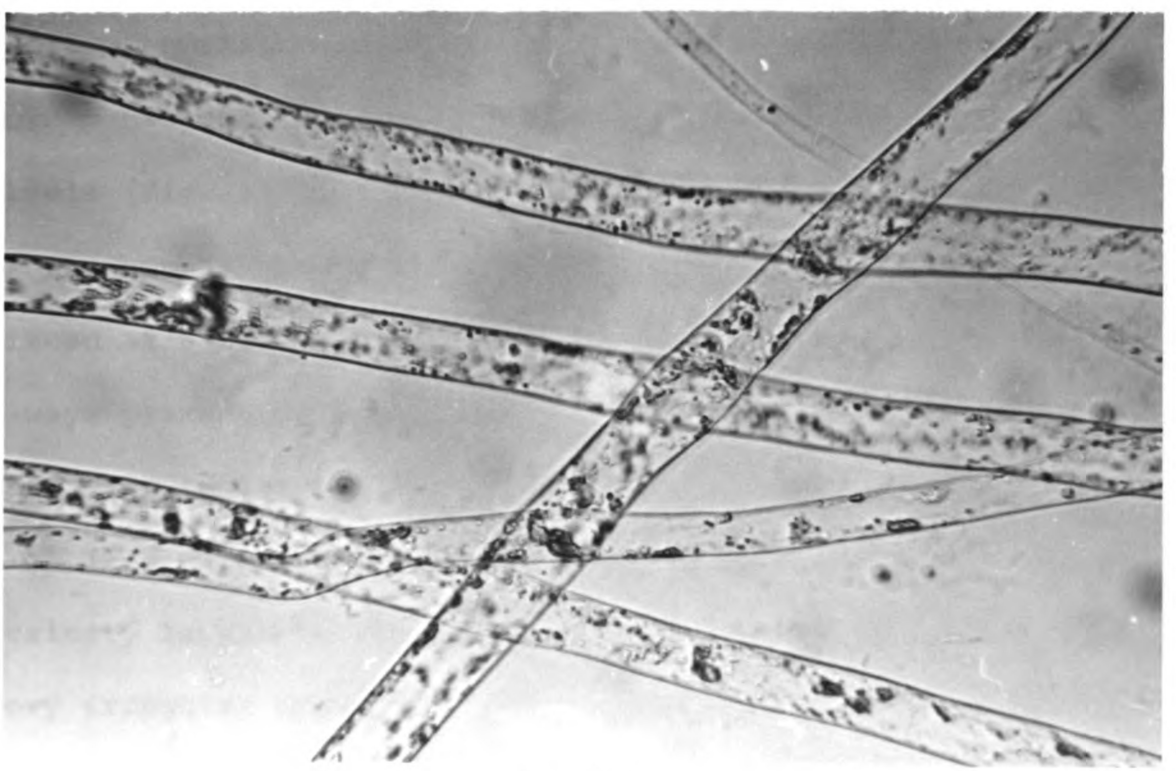

Fig. 13

Immature sporangia also produced several branch hyphae similar to germinating spores within the sporangial walls (Fig. 11). Generally branch hyphae were fewer in number in the immature sporangia than those with germinating spores. Increased numbers of gemmae (thick walled cells) or irregularly shaped sporangia also appeared with some test chemicals. The gemmae remained in an immature state and did not germinate or sporulate.

Mycelium produced varying morphological changes according to the degree of toxicity. Within the highly toxic chemical range of 1/4 - 20 gammas /ml, hyphae were quite short with slight irregularities in the cylindrical growth. More protoplasmic coagulation was also recorded at these levels (Fig. 13). Coagulation appeared prior to the death of the hyphae, however, at times hyphal growth still continued at a reduced rate. Severe protoplasmic clumping was always present in hyphae killed by the antitumor chemicals. Normal protoplasm in the hyphae appear finely granular.

If disarrangement of protoplasm did not occur at higher toxicity levels in the Achlya assay, hyphae would produce very irregular branches. Normal growth discontinued at and above the toxic level as soon as the mycelium was placed in the test solutions. In many instances in the highly toxic

chemicals, growth continued at the normal rates as the irregular branches continued to elongate (Fig. 14, 15). The branches were very irregularly shaped with many raised protrusions in the wall instead of the usual cylindrical appearance.

Sometimes hyphae appeared inhibited yet sporangia developed at 6 hours. This occurred only at inhibition levels of 2 - 1/4 gammas. When the plug was placed in the test solution, inhibition of hyphae might not be sudden. Instead inhibition could be a slow process, a gradual gradient build up of inhibition at low concentrations. This allowed sporangia to develop but when concentration apparently reached the temporary inhibitory level, sporangia were mature. Inhibition at low concentrations (2-1/4 gammas) generally were temporary. Original hyphae were killed but new hyphae developed from the transferred plug in the test solutions after apparent changes in effectiveness of the concentration occurred.

Moderately inhibited hyphae generally have more spiral growths (Fig. 12). As the hyphae elongate in the test solutions, growth continues in a coiled or spiral pattern instead of the normal linear fashion. The spiral growth was produced

from mildly inhibiting chemicals where growth continued at or near its normal rate. More severe spiral effects and coiling developed when the chemicals caused a reduction in the lengthening rate of the hyphae (Fig. 16, 17). In many cases where a moderate inhibition in zoospore numbers occurred or motility decreased, increased vegetative growth also developed. If hypha elongated at a much greater rate over the controls, the filaments were very narrow and few sporangia were present. Other moderate inhibitions by chemicals produced only normal vegetative hyphae without any sporangia and in the case of Achlya flagellata, few if any oogonia or antheridia appeared.

The gametangia of Achlya showed no irregular morphological growths with the carcinostatic chemicals, only maturation rates were reduced. Oogonia formed at 72 hours instead of 24 hours. The abundance of antheridia was also lessened in highly effective chemicals. The reverse was true also. Oogonia were inhibited at lesser concentrations than zoospores (ex. 3071). Oogonia remained immature but usually in normal numbers with these tumor toxic chemicals. At times oogonia formed when sporangia were inhibited (ex. 3062).

Fig. 14. Abnormal hyphal branch development after normal hyphae were placed in diethylstilbestrol (NSC No. 3070). Growth continued under these conditions. (250x)

Fig. 15. Abnormal branching and some protoplasmic coagulation of Achlya flagellata with benzimidazole (NSC No. 759). (250x)

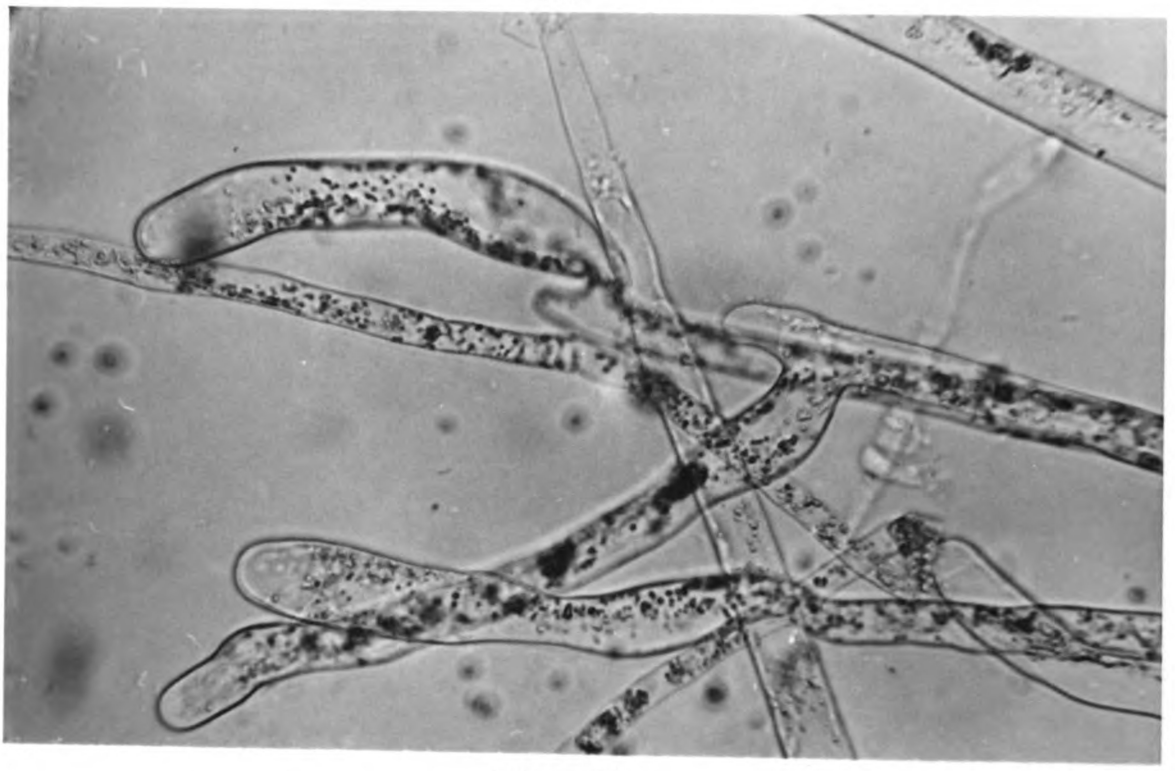

Fig. 14

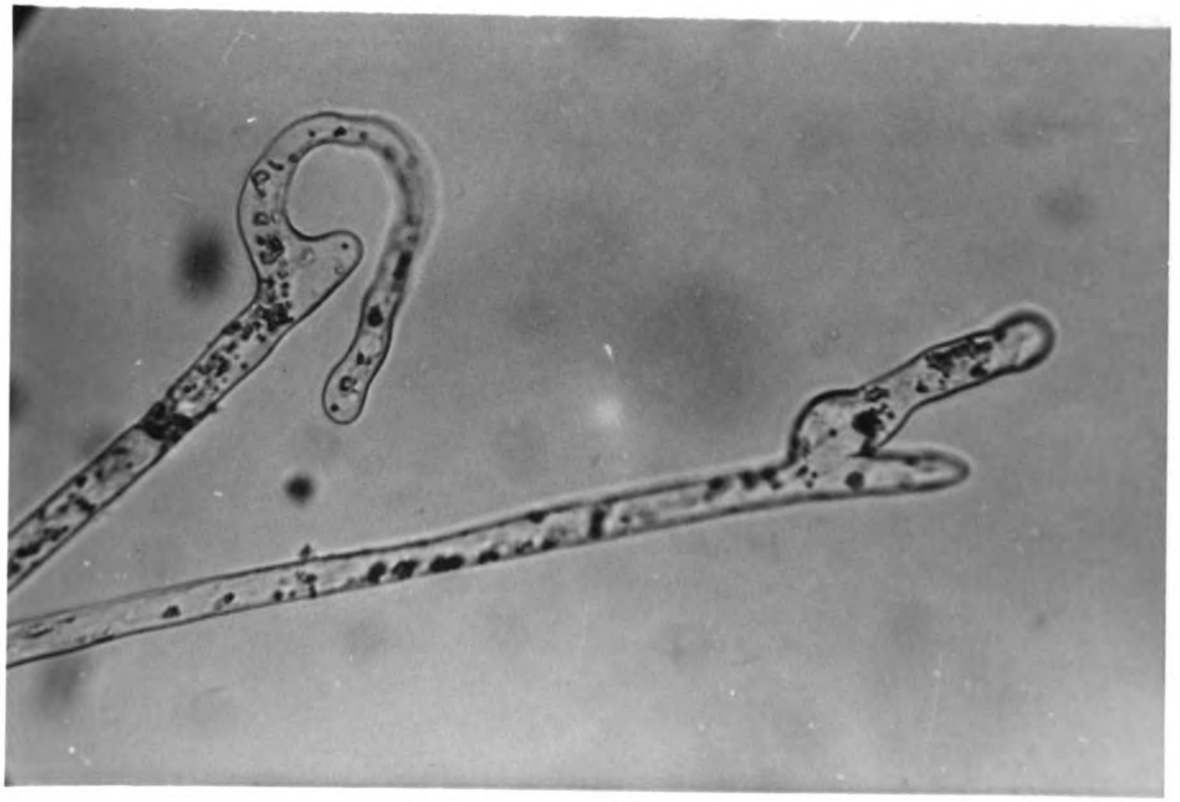

Fig. 15

Fig. 16. Marked spiral hyphal development with scattered encysted spores produced by testosterone proprionate (NSC No. 9166). (185x)

Fig. 17. A spiral mature sporangium with encysted spores. (180x)

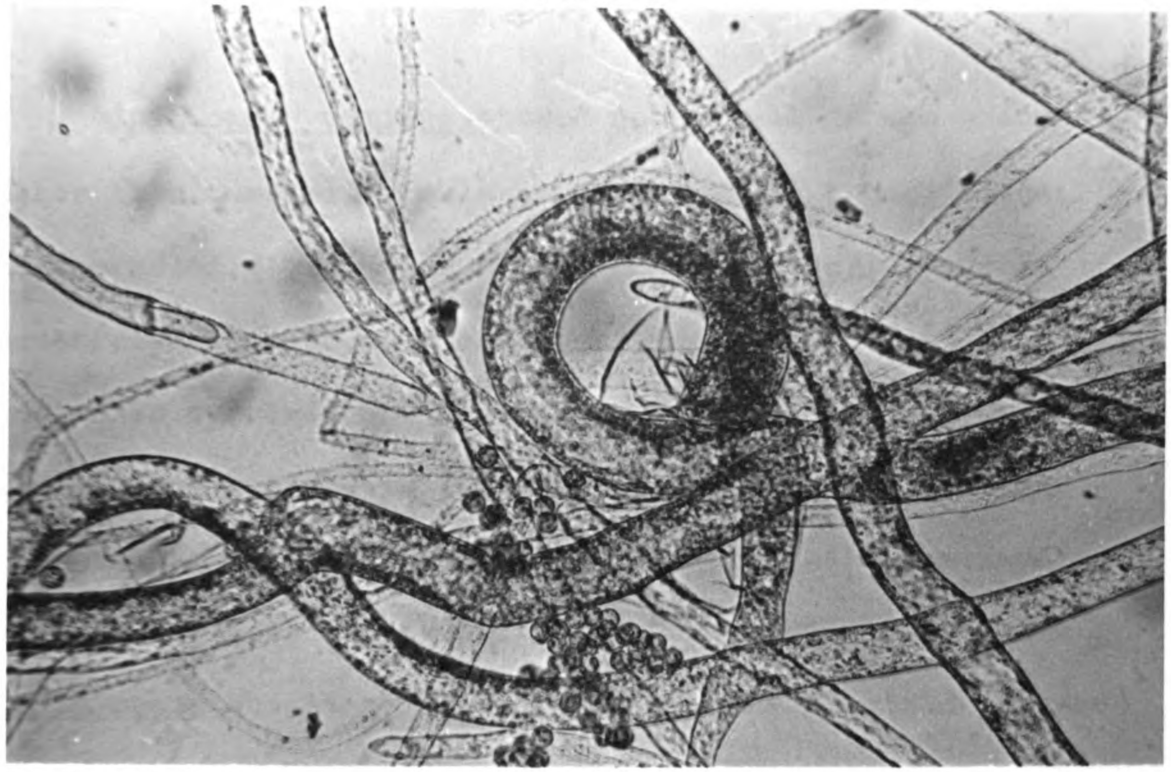

Fig. 16

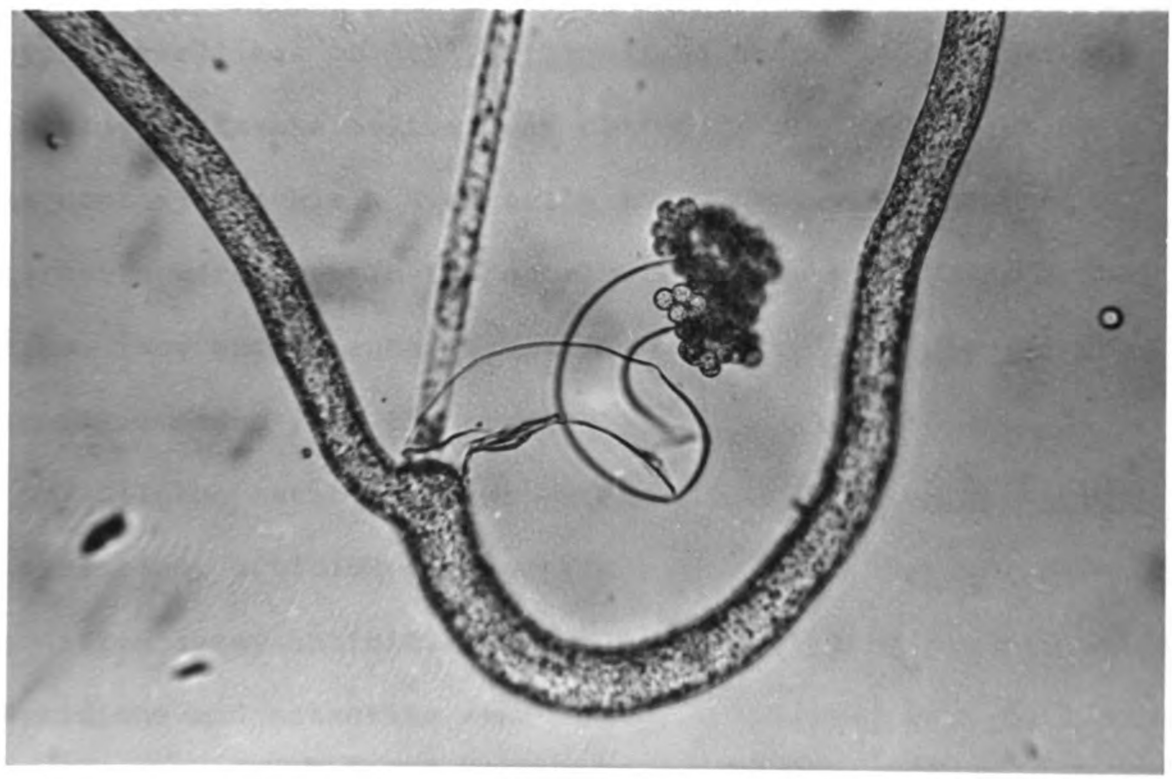

Fig. 17

Olpidiopsis varians showed no unusual growth changes other than tumor-like enlarged host hyphal tips. Either zoospores of Olpidiopsis on the Achlya flagellata hypha penetrated and produced new parasitic growth or else no zoospores survived and the hyphae were not parasitized. Olpidiopsis was always inhibited at lower concentrations than the Achlya. At times the chemical inhibition levels between the host and parasite were quite different. New Olpidiopsis thalli solutions at toxic levels would not develop even thought Achlya flagellata continued to grow at its normal maturity rate. Olpidiopsis was not inhibited when hyphal swellings of Achlya flagellata continued to increase in size after the culture was placed in the test solution at nontoxic levels. Eventually motile zoospores of the parasite were seen in the sporangia in the tumor-like hyphal tips. New encystments were seen soon after Olpidiopsis zoospores emerged from the sporangia.

All the antibiotics produced by species of Streptomyces (azaserine, actidione, mitomycin c and chloromycetin) gave positive assay inhibitions, but not at the same concentrations. Actidione and azaserine were highly inhibitory at 4 to 1/4 gamma / ml range for the Achlya species and Olpidiopsis.

Mitomycin C and chloromycetin were moderately inhibitory to the Achlya-Olpidiopsis assay at 40-50 gammas, however, Achlya flagellata zoospores and Olpidiopsis zoospores were eliminated at 2 and 1 gamma levels respectively.

Comparing the Achlya-Olpidiopsis assay method with other screening techniques is difficult due to different test chemicals used and the variations in reactions in different systems. The three fungi used in the screen produced positive inhibition levels with all water soluble alkylating agents, folic acid and nucleic acid derivatives. Lactobacillus casei, L. arabinosus, and Streptococcus faecalis also produced similar results with these chemical groups, but a few variations were noted. Escherica coli was screened with agents mainly involving amino acid synthesis. Clostridium feseri was positively inhibited by carsinostatic alkylating agents, antitumor antibiotics and steroids.

Other assay organisms including Kloeckera brevis, Saccharomyces cerevisae, Clostridium tetani, Neurospora crassa and Rhodotorula sp. were to some degree affective in antitumor screening programs, but may show negative results in higher animal test systems.

A more comprehensive comparison for the Achlya system can be made with the various mouse assay techniques, as shown in Tables V and VI. These tables are composed of screening

information on antitumor compounds from Cancer Chemotherapy, National Service Center, National Institutes of Health. Five tumor tissues transplantable in mice are routinely used for testing. All test chemical doses were given in mg/kg weight of mouse and on the basis of 1/1,000 of a mg per ml of water in the Achlya test.

Achlya flagellata zoospore inhibition levels are compared with the tissue ED-50 culture test, and the S-37 tumor tested with one dose administered subcutaneously (see Table V). In many instances, chemicals were not screened with a particular system, therefore, a clear picture comparing all screening systems is difficult. Table VI lists three other screens systems, namely S 180, Ca 755, and L1210. The denominator under "Other screening data" gives the number of screening programs studied, mainly microbiological. The numerator shows the number of positive screening programs for each individual chemical. Blanks indicate little or no information is known for the newer chemicals. Few, if any, screening techniques have incorporated these new chemicals into other programs due to their relative newness in chemotherapy.

The Achlya assay seemed to be the most sensitive to alkylating agents. The highly toxic alkylating agents to

Table V

NSC No.	Compound	Achyla flagellata sporangia* inhibited at µg/ml: (initial test)	Tissue** culture ED-50	S-37** Single dose mg/kg sub q
185	actidione	1-20	<1	100+
741	hydrocortisone	-(50)	--	-(100)
743	2,6-diaminopurine	50	<1	--
746	urethane	1-20	10-90	--
747	n-methyl acetamide	40	<1000	--
750	myleran	-(50)	>100	-(500)
751	dl-ethionine	40	>100	--
754	b-2-thienyl alanine	50	10-99	-(1000)
756	8-azaxanthine	20	10-99	--
757	colchicine	20	<1	3++
758	d-glucosamine	20	10-99	-(1000)
759	benzimidazole	20	9-100	-(1000)
762	nitrogen mustard	0.5	1-9	-(5)
1026	1-aminocyclopentane	2	--	--
1393	4-amino pyrazole pyrimidine	20	<1	-(250)
3051	n-methyl formamide	-(50)	>100	--
3054	methyl carbamate	-(20)	>1000	-(1000)
3060	potassium arsenite	1	<1	-(6)
3061	daraprim	-(50)	1-9	-(400)
3062	2,4-diamino-5 (3',4'-dichlorophenyl)-6-ethyl-pyrimidine	20	10	-(400)
3063	desoxypyridoxine	-20 reduced	<100	--
3070	diethylstilbestrol	20	3.4	-(1000)

Table V. Continued

NSC No.	Compound	Achyla flagellata sporangia* inhibited at µg/ml: (initial test)	Tissue** culture ED-50	S-37** Single dose mg/kg sub q
3071	5-methoxytoluguinone	-(50)	13	50+
3088	chlorambucil	20 reduced	8-12	-(250)
3095	1,5-diaminobiuret	>50	370	-(500)
3069	chloromycetin	40	<10	-(1000)
6396	thio TEPA	20	1-9	--
9166	testosterone proprionate	20 reduced	6-36	-(1000)
9369	1-methyl-3-nitro-1-nitro-1-nitroso-guanidine	1	0.7-6	-(100)
9706	TEM triethylene melamine	50	<1	--
14210	d-l sarcolysin	--	1-27	-(100)
14574	miracil	20	2-15	-(1000)
24818	podophyllotoxin	--	0.00095	20(10++,5++)
26271	cycloposphoramide	-- (50)	>100	--
26980	mitomycin-c	20	0.006	100

*Preliminary investigations with Achyla flagellata by Dr. Yamuna Lingappa, research assistant, Dept. of Botany and Plant Pathology, Michigan State University, in 1960.

**Information furnished by Mrs. Dorothea B. Fitzgerald, and chemicals furnished by Mrs. Fay Funk, Cancer Chemotherapy National Service Center, National Institutes of Health, Bethesda, Maryland.

- = negative

-- = no test made

Table VI

Antitumor Tests on Selected Compounds Utilizing Different Systems

NSC No.	Compound	CCNSC screening results mg/kg*			Other screening data
		S 180	Ca 755	L 1210	
185	actidione	+32**	-16**	-32**	3/5
741	hydrocortisone	+35	+37.5	-37.5	8/14
743	2,6-diaminopurine	-90	-90	-90	5/28
746	urethane	+250	+250	-750	19/28
747	n-methyl acetamide	-1500	-650	-300	6/15
750	myleran	+30	+15	-30	7/21
751	ethionine	-100	-75	-75	5/22
754	b-2-thienyl alanine	-750	-750	-400	1/17
756	8-azaxanthine	-750	-750	-250	0/22
757	colchicine	-0.05	+0.2	-0.5	24/51
758	d-glucosamine	-1500	-1500	-750	1/18
759	benzimidazole	-250	-250	-200	1/21
762	nitrogen mustard	+1.5	+0.7	-1.5	27/40
1026	1-aminocyclopentane	-62.5	+30	-62.5	____
1393	4-amino pyrazole pyrimidine	-___	-___	-___	____
3051	n-methyl formamide	+175	+175	-200	12/12
3054	methyl carbamate	-750	-750	-300	0/2
3060	potassium arsenite	+15	+13	-8	7/23
3061	daraprim	+200	+100	-40	____
3062	2,4-diamino-5(3',4'-di-chloro phenyl)-6-ethyl-pyrimidine	-12	+6	-12	10/15
3063	desoxypyridoxine	+225	-225	-50	2/19
3069	chloromycetin	-250	-1000	-250	0/18

Table VI. Continued

NSC No.	Compound	CCNSC screening results mg/kg* S 180	Ca 755	L 1210	Other screening data
3070	diethylstilbestrol	+250	-250	-250	____
3071	5-methoxytoluguinone	-___	-___	-___	____
3088	chlorambucil	-8	1.25	+16	____
3095	1,5-diaminobiuret	+150	+75	-87.5	3/14
6396	thio TEPA	+___	+___	+___	____
9166	testosterone propionate	-720	-1000	-1000	____
9369	1-methyl-3-nitro-1-nitro-1-nitroso-guanidine	+___	+___	+___	____
9706	TEM triethylene melamine	-2	-2	-0.75	____
14210	d-l-sarcolysin	_10	_10	+10	____
14574	miracil	∓20	+80	+39	____
24818	podophyllotoxin	-1	-0.7	-0.7	____
26271	cycloposphoramide	+___	+___	+___	____
26980	mitomycin c	+2	+2	+___	____

* + = tumor inhibition; - = no tumor inhibition

** highest nontoxic dose tested

Information collected from the appendix of Ann. N. Y. Acad. Sci., vol. 73: 905-970; and unpublished information furnished by Mrs. D. B. Fitzgerald, Cancer Chemotherapy National Service Center, National Institute of Health, Bethesda, Maryland.

Achlya (nitrogen mustard, thio TEPA) also were highly inhibitory to S 180, Ca 755, L1210, and S 37. Diethylstilbestrol, positive for the Achlya test, was negative to Ca 755, L1210, and S 37. Similarly the moderately inhibitory alkylating agents in higher animals such as TEM, d-l sarcolysin, chlorambucal, and cycloposphoramide showed no inhibition in the mouse tumor tests. Fewer mice assay tests produced positive results with chemicals moderately inhibiting Achlya zoospore emergence and other morphological changes. Methyl carbamate was the only alkylating agent that failed to show inhibitory effect with Achlya, however, it was also negative with all mouse assay techniques.

Similar results were shown with the other chemicals. If the chemical had high Achlya-Olpidiopsis toxicity levels then in general most if not all mice assay systems were positive. Moderately good inhibitory chemicals assayed with Achlya had fewer positive results in mouse tumor tests. Chemicals that were negative with Achlya were usually negative in most of the mouse tests.

Various chemicals of unknown modes of action which were good carsinostatic agents in mouse tests, corresponded to similar higher concentrations required for inhibition in

the Achlya-Olpidiopsis assay. Difficulties may occur in identifying chemical modes of action due to an external physical cause. Inhibitions were registered by osmotic pressures thus producing a false positive action instead of a chemical reaction. Abnormal growth was a plasmolysis process. When Achlya was placed in screening solutions of varied gamma / ml concentrations, no observable abnormal Achlya-Olpidiopsis growth was evident, due to the osmotic pressure, since cells must be completely turgid for growth to occur. A reduction of turgidity would stop growth, however, growth continued in several instances when Achlya protoplasm appeared coagulated.

The Achlya flagellata, Olpidiopsis varians and Achlya sp. (13 SP) assay system for identifying carcinostatic chemicals appears to show some correlation with some of the mice tumor screens. The degree of inhibition based on gammas per ml in the Achlya chemical screening coincided in some cases at concentrations of 1,000 times the gammas used per ml. This would equal the mg per kilogram of mouse weight. Accuracy was relatively high with alkylating agents, amino acid antagonists and antifolic agents. Inhibitions were associated with nucleic acid production directly or indirectly by blocking purine and pyrimidine incorporation into nucleic acids.

SUMMARY

Twenty-eight cultures of aquatic fungi (26 from Brazil, 2 from Illinois), including species of Achlya, Dictyuchus, Thraustotheca, Saprolegnia and Olpidiopsis were used to find a very sensitive strain for inhibition of zoospores by anti-tumor agents. Azaserine was used in the preliminary tests. Three of the fungi, Achlya sp. (13 SP), Achlya flagellata, and Olpidiopsis varians were selected to further test diverse chemicals for fungal morphological inhibitions.

A single test system was developed with Achlya sp. for detecting levels of inhibition of various antitumor agents within a six hour period. This was based on the inhibition of zoospore development.

A double assay system with Achlya flagellata and Olpidiopsis varians, which forms tumor-like swellings in the hyphal tips of the host, was also tested with the tumor inhibiting chemicals. The single system proved to be more easily handled in the laboratory and nearly as accurate.

Achlya sp. (13 SP) from Brazil was the most sensitive of any of the aquatic fungi tested with the various antitumor agents. Olpidiopsis varians was more sensitive to the chemicals than the host fungus, Achlya flagellata.

Compounds identified as alkylating agents required high levels of concentration for zoospore and hyphal inhibition corresponding to similar levels for mouse tumor regression. Amino acid antagonists also produced inhibitory action while antifolic agents and purine antagonists showed some inhibitory effect.

Many other chemicals with unknown modes of action also inhibited fungal growth or caused abnormalities. In some cases severity of the abnormalities increased as chemical concentrations increased. Water insolubility reduced the effectiveness of some chemicals since a freely diffusible condition must exist in order to have the fungal hyphae contact and absorb the test material. A few of the compounds were slightly soluble as indicated by inhibitions of fungal growth.

Chemical inhibitory effects were observed by changes in Achlya zoospore numbers and rate of activity, hyphal abnormalities, oogonial and antheridial development, maturity rate, and Olpidiopsis tumor development.

Steroids and interthallic relationships were tested on heterothallic strains of Achlya. Variability was shown in the action of the Achlya hormone complex indicating varied concentrations of the steroid could control the maleness or femaleness of a strain.

A total of 36 antitumor agents were tested with the aquatic systems and found to be somewhat comparable in inhibitory effect to the results obtained in the various tumor assay systems in animals in a number of cases.

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