FURTHER STUDIES ON THE BEHAVIOR OF FUNGI IN THE PRESENCE OF RADIOACTIVE ISOTOPES.*

by

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A THESIS

Submitted to the Graduate School of Michigan State College of Agriculture and Applied Science in partial fulfilment of the requirements for the degree of

MASTER OF SCIENCE

Department of Bacteriology

1949

*Supported in part by a grant from the American Cancer Society, Committee on Growth, through the National Research Council

THESIS

ACKNOWLEDGMENT

I wish to express my gratitude to Iris A. Pearson, Henrietta S. Hayden, and Kenneth E. Corrigan who aided materially in this work and gave freely of their time, equipment, and special knowledge in their respective fields.

I wish to express my gratitude to Dr. W. L. Mallmann for his wise guidance and counsel, not only on this thesis, but over a period of many years.

Introduction

The widespread distribution of radioactive isotopes, shortly after World War II in 1946, for use by investigators interested in using them as tracers for various metabolic processes presented many new problems in technic and method. The isotopes themselves had to be handled with extreme care due to the hazards of the beta and gamma radiations. Although all laboratory personnel using isotopes were carefully instructed in their use, and only qualified personnel allowed to handle them, many of the technics used by present workers in biology were as yet undeveloped.

A very interesting experience revealed this lack of proper technic. Pearson et al (1 & 2) isolated an Alternaria and a Staphylococcus albus from contaminated solutions of I^{131} on two different occasions, early in 1946. The presence of these contaminants in the solutions interfered with the standardization of the isotope content by causing irregular and unpredictable Geiger counter recordings. Drops of these solutions examined under the darkfield microscope revealed the contaminants. The isotope solutions were filtered through Seitz filters to remove the organisms. When the filter pads were checked, they were found to be markedly radioactive. The pads were then washed repeatedly with sterile distilled water until the filtrates became radionegative. Upon rechecking one of the filter pads, the investigators found it to be still markedly radioactive. The material left upon the filter pad was examined microscopically and found to consist of mold spores. These were later identified as Alternaria. These spores were innoculated upon Sabaraund's medium and were found to be viable. The first cultures were radioactive, but subcultures from them were not. No alterations in growth or morphology occurred in the progeny

of the original radioactive spores, even though they were followed for several years through various subculturings. In an article recently released for publication by the U. S. Atomic Energy Commission, Kimball [3] reported the induction of mutations in paramecium aurelia by beta radiations.

Further studies demonstrated that the orgainsms were viable even after prolonged exposures to very large dosages** of beta and gamma rays.

This showed that solutions of these isotopes were not self sterilizing, and that it was necessary to autoclave them before parenteral administration to humans.

Pearson et al [1 & 2] in further studies exposed cultures of the Harper Hospital Stock Collection of Fungi to 20 microcuries of I^{131}

**The "Roentgen" (r) is the basic unit of radiation dose and is by definition the quantity of radiation which produces 1.62×10^{12} ion pairs per gram. Each ion pair (in air) represents 32 electron volts. The energy equivalent of this amount of air ionization, 83 ergs per gram, applies to air, water, and live tissue. The energy dose then is $\frac{32 \times 1.62 \times 10^{12}}{\beta eV} = r$. For I^{131} then $r = 8.9 \times 10^{7}$, or this number of beta disintegrations is required to give one roentgen unit. Each millicurie represents 3.7×10^{7} disintegrations per second. For the concentration in which the spores were originally discovered, 25 millicuries in 15 milliliters, or 1.6 millicuries per milliliter, or $\frac{1.6 \times 3.7 \times 10^{7}}{8.9 \times 10^{7}} =$ 0.67 roentgens per milliliter per second, or for short periods of time, as used in these studies, 58,000 roentgens per gram per day. For longer periods of time, this figure will decrease progressively by 10% per day.

and P^{32} for 4[illegible] hours, and also to an equivalent amount of high voltage X-ray to determine whether or not the different fungi, pathogenic and non-pathogenic could survive exposures to solutions containing radioactive isotopes. Table I illustrates the effect of the exposures on the growth of the stock fungus cultures.

TABLE I

Effect of radiations on fungi

	Organism	Growth After Exposure to		
		X-ray	P^{32}	I^{131}
1.	Actinomyces asteroides	++	++	++
2.	Alternaria	++++	+++	+++
3.	Aspergillus	++++	++++	++++
4.	Aspergillus niger	++++	++++	+++
5.	Blastomyces brasiliensis	0	+++	+++
6.	Blastomyces dermatitidis	+	+++	+++
7.	Candida albicans	+++	++++	++++
8.	Cephalosporium	+++	++++	++++
9.	Coccidioides immitis	++	++	++++
10.	Cryptococcus enoformans	++++	+++	++++
11.	Epidermophyton floccosum	0	++++	++
12.	Fusarium	++++	++++	++++
13.	Geotrichum	0	++++	+++
14.	Gliocladium	++++	++++	++++
15.	Helminthosporium	++++	++++	+++
16.	Histoplasma capsulatum	++	++++	+
17.	Hormodendrum	++++	++++	++++
18.	Hormodendrum compactum	++	+	++
19.	Hormodendrum pedrosoi	0	+++	++
20.	Microsporum audouini	0	++++	++
21.	Microsporum canis	+++	++++	+++
22.	Microsporum gypseum	0	++++	++++
23.	Microsporum lanosum	++++	+++	++
24.	Monosporium apiospermum	++++	+++	+++
25.	Mucor	++++	++++	++++
26.	Nigrospora	0	++++	++
27.	Ocspora	++++	++++	++++
28.	Paecilomyces	++++	++++	++++
29.	Penicillin	++++	++++	++++
30.	Phialophora verrucosa	0	++++	+
31.	Rhizopus	+	++++	+
32.	Rhodotorula	+++	+++	+++
33.	Scopulariopsis	+++	++++	+++
34.	Sporotrichum schenckii	0	++++	++++
35.	Streptomyces	+++	+++	++++

Table continued on next page.

Table I (continued)

Effect of radiations on fungi

Organism	Growth after exposure to X-ray	P^{32}	I^{131}
36. Syncephalastrum.....	++++	++++	++++
37. Trichoderma.....	+++	++++	++++
38. Trichophyton mentagrophytes.....	+++	+++	++
39. Trichophyton rubrum.....	0	+++	+++
40. Trichophyton schoenleini.....	++	++++	++
41. Trichophyton violaceum.....	0	++++	+++
42. Verticillium.....	+++	+++	++

The + signs indicate the amount of growth as compared with growth of control cultures. The amount of growth was graded from 0 (no growth) to ++++ (normal growth).

Examination of the table shows that the growth of most of the organisms was unaffected by the exposures to which they were subjected.

More recently it has become apparent that a considerable amount of bacteriological study was carried out under war time restriction, and the results are just how becoming available. Stapleton, Loftus and Armstrong (4) carried out some of the procedures of irradiating microorganisms with radioactive isotopes but their reports have only now become available and their results have not yet been published.

The general idea of metabolism of radioisotpoes by bacteria, fungi, and particularly yeast is not new. Ruben, Kamen and co-workers (5 & 6) reported the synthesis of propionic acid containing C^{14} by propionic acid bacteria which were fed $C^{14}O_2$ as early as 1940 and gave a complete report verbally

at a meeting in 1941. Since the bacteria absorbed the CO_2 and incorporated it into the propionic acid molecule, it is probable that it entered the metabolism of the living organism.

Under similar conditions Rothstein and Meier[7] studied the breakdown of ATP and similar organic phosphorus compounds by yeast. They did not, however, report incorporation of the P^{32} released by the breakdown of ATP into the spores or cell bodies of the yeast cells.

In further studies by Pearson et al [1-2-8] the iodine and phosphorus metabolism of these organisms was compared in an attempt to determine whether these isotopes were merely adherent to the outside surface of the fungi, or were actually metabolized. A non-pathogen, Fusarium, was used in this study. Separate cultures of this organism were exposed, one culture to 600 microcuries of I^{131}, and the other culture to 100 microcuries of P^{32}, for 48 hours. The 48 hour cultures were filtered through Seitz filters and washed until radio-negative filtrates were obtained. A filtrate that gave not more than 10 c/M/ml* was considered to be radionegative. Smears and cultures were made from the organisms on the washed filter pads. The smears were checked for radioactivity using the Geiger counter with the following results: counts on the slides of Fusarium exposed to 600 microcuries of I^{131} were 10,000 c/M/slide, showing that the P^{32} metabolism was about 30 times greater than that of the I^{131}. This marked difference in rate of metabolism of the isotopes by Fusarium was believed to be evidence that these elements were actually metabolized, and not merely adherent to the surfaces of the organisms.

*c/M/ml = counts per minute per millileter.

Experimental

Section A: A Simple Method of Transfer of Fungi.

Because it has been demonstrated that the spores of fungi, both pathogenic and non-pathogenic, can be made radioactive, it is essential that they be handled as rapidly as possible in order to protect the worker from undue exposure to harmful radiations. Further, it is important that the spores be retained in the culture and do not escape into the surrounding air of the laboratory, and thus create a hazardous condition for all personnel in the immediate vicinity.

To determine whether or not the spores escaped into the air during transfer, a series of experiments was made to demonstrate that the usual methods of transferring fungi using the conventional needles allowed spores to escape from the tubes.

Due to the fact that mold spores are formed on aerial hyphae, any undue disturbance in the tube causes the spores to disperse into the air, and they are carried out of the tube by the air currents set up by the movement of the transfer needle. To demonstrate this, a culture of the non-pathogenic Oospora was agitated with a needle in the usual manner. In order to photograph the cloud of spores, smoke was introduced into the culture tube before agitating it. (Fig. I A) The culture to which fluids were added were pipetted out after agitation showed no cloud. (Figs. 4 E & 4 F)

In radioactive cultures after manipulation with a needle, radioactive spores were detected in the air of the laboratory, on the furniture, and on personnel by means of a Geiger counter.

In several uncontrolled experiments fungus collections had been presented to a number of other institutions, and when these were sub-

cultured by competent technicians, the percentage of positive transfers was much lower than that obtained using the technique about to be described, and the incidence of cross-contamination considerably higher.

The method that was developed here consisted of adding fluid to cultures in order to wet the spores and disperse them into the solution rather than the surrounding air. This method has been used routinely with radioactive fungus cultures. It is adaptable to laboratory use for all fungi to protect laboratory personnel against infection by notorious air borne pathogens, such as Coccidioides, cross contamination of cultures by rapid growing non-pathogens such as Penicillium and Aspergillus, and laboratory contamination with numerous fungus spores.

Mallmann[10] suggested the use of an unionized wetting agent on fungus cultures such as Triton x-100 in a dilution of 1 - 5,000. This product is manufactured by Rohm and Haas. This agent when tested had no deleterious effect upon the spores, and insured wetting, which further reduced air contamination. This product has not been tested on cultures containing radioactive isotopes, therefore no conclusions can be drawn regarding the effect of this substance upon isotope metabolism in fungi.

The cultures to be transferred are placed upright in test tube racks, and enough sterile saline or water is added to each tube, using sterile technic, to bring the fluid level to the top of the agar slant. A Sterile 10 c.c. pipette or syringe is satisfactory for this purpose. The cultures are agitated with a sterile pipette and shaken gently until a suspension in which clumps of mycelia and spores are visible is obtained. A few drops of this suspension are transferred to the previously labeled

sub-culture tubes, using the same pipettes that were used to agitate the donor cultures. A suitable rubber bulb is attached to the end of the pipette to provide suction and to prevent oral contamination. A rubber bulb of 1 cc capacity was used for this purpose. The rubber bulb technic originated with the handling of isotope solutions, since it is a basic rule never to attempt the pipetting of radioactive materials by mouth.

It was found that the use of sterile pipettes instead of the routinely used wire loop was a much more efficient technic. It eliminated the need of the usual flame sterilization process between transfers, thereby reducing the amount of time consumed in making a transfer; time is an important safety factor when dealing with exposures to radioactive isotopes. Flaming volatile isotopes would contaminate the air of the laboratory, and breathing air and gases containing radioactive elements may produce pathological effects on the personnel so exposed. (Fig. I)

The employment of a different pipette for each transfer undoubtedly reduces the amount of cross contamination, which could occur when using the conventional loop needle. The ordinary 1 cc. pipette may be used for this purpose, but a less expensive, more convenient instrument can be made in the laboratory by drawing out 12-inch sections of glass tubing, one fourth inch in diameter, in a Bunsen Flame. These can be sterilized in the usual manner, are easier than the conventional pipettes to manipulate, and are less likely to become plugged with clumps of mycelia. After use, especially if contaminated with isotopes, they can be stored and destroyed when free of radioactivity, or resterilized and used again.

After the addition of the fluid, the donor culture often starts growing again and can be kept growing indefinitely by the addition of fluid to the dried agar slant.

Section B: A technique for making autoradiographs of microorganisms.

Autoradiographs were made to determine whether I^{131} and P^{32} were actually metabolized by microorganisms or were merely adherent to their surfaces. The autoradiograph was a photographic means of checking the amount of I^{131} and P^{32} metabolized, based upon time of exposure and size of colonies.

The culture from which an autoradiograph is to be obtained is covered with either sterile water or saline, and the calculated dose of the isotope is pipetted into this solution, using precautions against radioactivity. The culture containing the radioactive isotope is allowed to stand for a calculated length of time, usually 12 to 96 hours, after which the culture is agitated to suspend the organisms in the fluid. This suspension is filtered through a Seitz filter and washed repeatedly with sterile water until a radionegative filtrate is obtained. A filtrate that gave not more than 10 C/M/ML was considered radionegative, as before. Each filtrate was collected in a 15 cc. centrifuge tube placed within the vacuum flask (Fig. II). The presence of this tube prevents the contamination of the entire filtering system with radioactive material, and the use of a new tube after each washing permits accurate Geiger counts on the individual filtrates.

When a radionegative filtrate is obtained, the organisms remaining on the filter pad are tested for radioactivity. If they are radioactive, they are smeared on slides in the usual manner. The slides are checked with a Geiger

counter to determine the amount of radioactivity retained by the organisms in an effort to determine the length of exposure necessary to affect the photographic plates. The slides containing the radioactive organisms are taken to the darkroom where they are placed in direct contact with the photographic plates, the side containing the organisms being placed directly in contact with the emulsion. These plates are developed at previously calculated intervals of time, depending upon the amount of radioactivity present on the slides. The slides are then fixed bacteriologically and stained with an appropriate stain. The photographic plates are developed and fixed in the usual manner.

The stained slide and developed plate are matched grossly and corresponding areas are marked or encircled. These areas are examined under a microscope and photographed. The low power magnification is sufficient for colonies, but high power or oil immersion is necessary for individual fungi or bacteria.

Figures 3, 4E & 4F demonstrate corresponding areas under various magnifications.

Discussion

1. The method described for transferring fungi has been found to be satisfactory in our hands. It has the following advantages, especially for classroom study:

a. It keeps contamination of the laboratory with spores down to a minimum.

b. It prevents students from infecting themselves by means of air-borne spores while manipulating the cultures and attempting to transfer dry spores of pathogenic fungi, such as Coccidioides. (Fig. I)

c. It insures a high percentage of positive transfers for fungus cultures.

d. It decreases cross-contamination of stock cultures.

e. Growth is obtained in a shorter interval of time.

2. The technic of making autoradiographs of microorganisms was devised in an attempt to study quantitatively the physiology of various isotopes, and to determine what parts of the fungi utilized the radioactive isotopes, P^{32} and I^{131}. The technique of producing autoradiographs of tissues as originated by T. Evans (9) was a starting point for some of this work.

The radioactive precautions mentioned in the experimental section of this paper cannot be overstressed and will be elaborated upon further

a. Protective rubber gloves and lead aprons should be worn when working with radioactive cultures to protect the individual. All work should be done behind a protective

lead or lucite shield, and all equipment should be placed on a metal tray covered with a thin coating of oil or petroleum jelly.

b. Competent assistants should be present in case of accident, and to aid in handling material.

c. All equipment and instruments contaminated with radioactive solutions should be stored in a safe place until they become radionegative. Waste products (urine, feces), and solutions (discarded cultures), and discarded specimens which are radioactive should be treated in a similar manner before being discarded.

d. The workers' hands, clothing, and the laboratory itself must also be checked with a Geiger counter at intervals to locate the radioactivity if present.

e. The properties of the isotope being used are of prime importance in determining the manner of handling, administering and disposal.

f. The personnel should be familiar with:

    1. The half life of the isotope.
    2. The type of radiations emitted.
    3. The radioactivity of the sample being used.
    4. The physiology, pharmacology and toxicology, if known.

g. Ability to operate and interpret Geiger counter readings.

h. Work should be carried out in a shower equipped laboratory in order to decontaminate personnel involved in case of accident.

It should be mentioned that the slides are not fixed before making the autoradiographs because heating might volatilize some of the isotopes, as for example I^{131}.

The fact that the slides are not fixed means that the slides containing virulent organisms, as Blastomyces, are dangerous and these slides, after being placed in contact with photographic plates, are placed in petri dishes during exposure to the plates. The equipment is then autoclaved after use. The slides with non-volatile isotopes can be flamed prior to exposure to the photographic plates and treated as any ordinary slide.

Any fine-grained photographic plates can be used for obtaining autoradiographs of colonies of organisms (Fig. 3), but special plates are necessary in order to obtain radiographs of individual organisms because the silver granules of ordinary plates are larger than the single organism. Kodak Nuclear Track plates, Type NTB (25 micron), which have a very fine grain, were used to obtain autoradiographs of M. tuberculosis. (Figs. 4A-B-C-D-E-F).

It should be mentioned that as the magnification is increased the depth of focus is decreased; and as a result, producing autoradiographs of individual bacteria, as in plates 4A-B-C-D-E-F requires patience and a certain degree of luck. It is like working out a jig-saw puzzle in that one can look at photographs for a long time without apparently accomplishing anything, and then suddenly be able to match up the corresponding areas.

The technic is a good one for developing microscopic technic and as a research tool, but at the present time no practical application has been devised.

Conclusions

1. A simple technic for transferring fungi is described.
2. A technic for obtaining autoradiographs of colonies and individual microorganisms is presented.

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I (A) SHOWS THE DISTRIBUTION OF FUNGUS SPORES UPON PROLONGED AGITATION WITH A LOOP NEEDLE WHILE ATTEMPTING TO MAKE SUBCULTURES.
NOTE THAT THE CLOUD PRODUCED IS INHALED BY THE TECHNICIAN, AND THAT THIS COULD BE DANGEROUS IN THE CASE OF PATHOGENS OR CONTINUED EXPOSURE TO VOLATILE ISOTOPES.
(NO RADIATION PRECAUTIONS ILLUSTRATED.)

I (B) NOTICE THE MARKED DECREASE IN THE SIZE OF THE CLOUD.

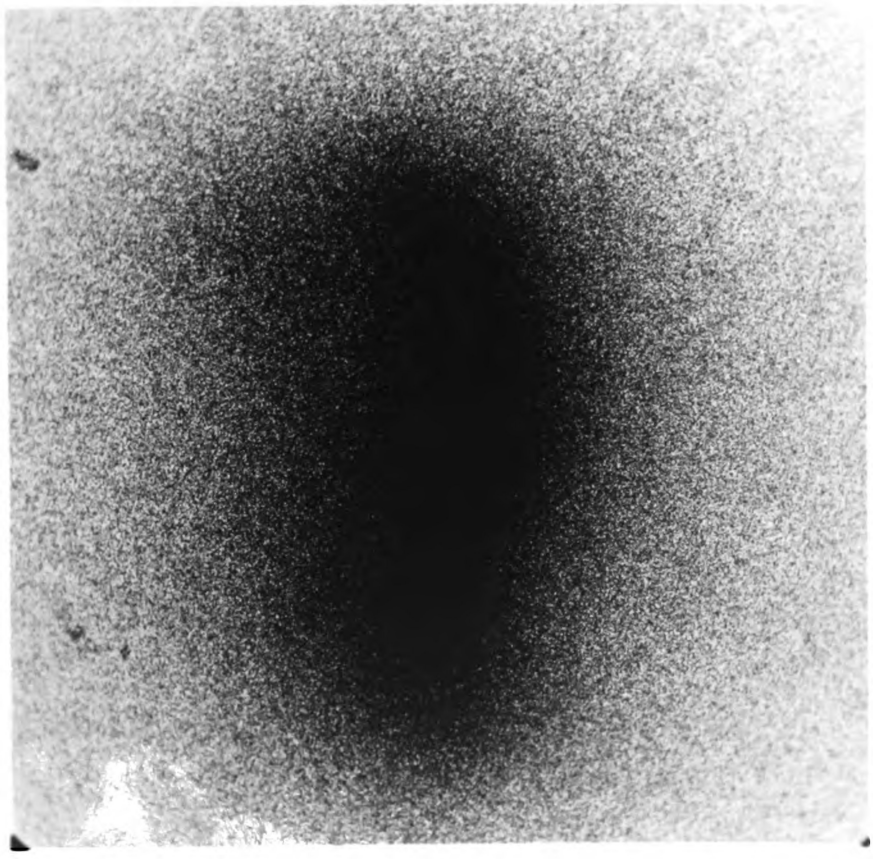
UPON
WHILE

D BY

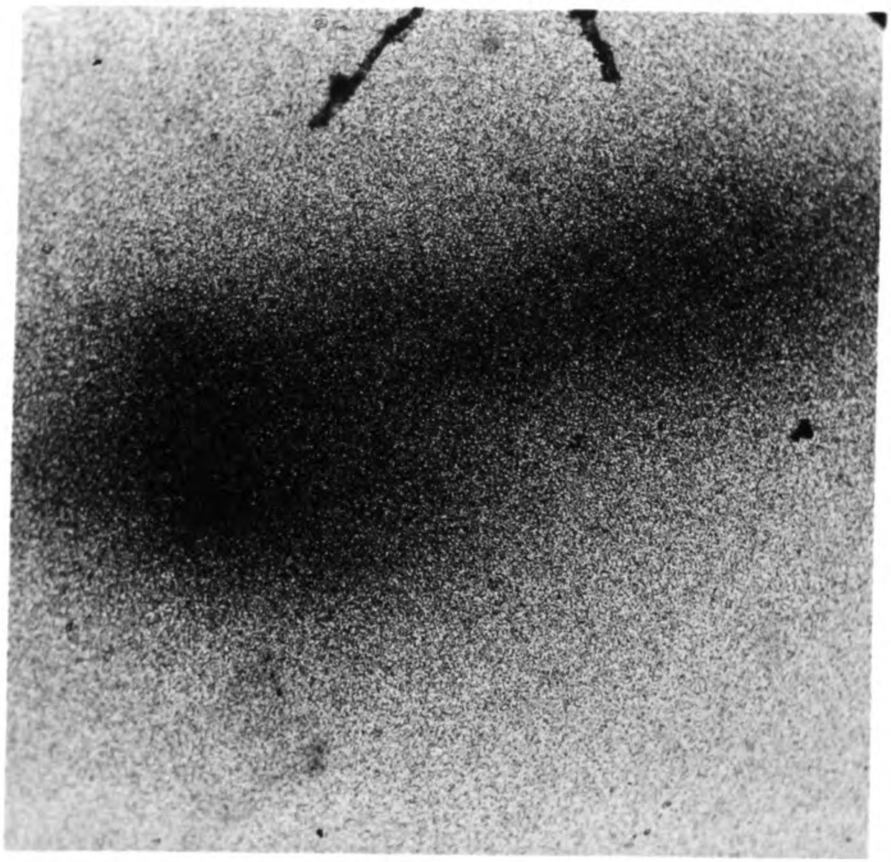
CON-

THE

II SEITZ FILTER SET UP.
NOTE THE TIP OF THE FILTER IN THE COLLECTING TEST TUBE WITHIN THE SUCTION FLASK TO COLLECT FILTRATE. THIS TUBE PREVENTS CONTAMINATION OF THE FILTERING SYSTEM WITH RADIOACTIVITY.
(TRAY AND SHIELD NOT SHOWN.)

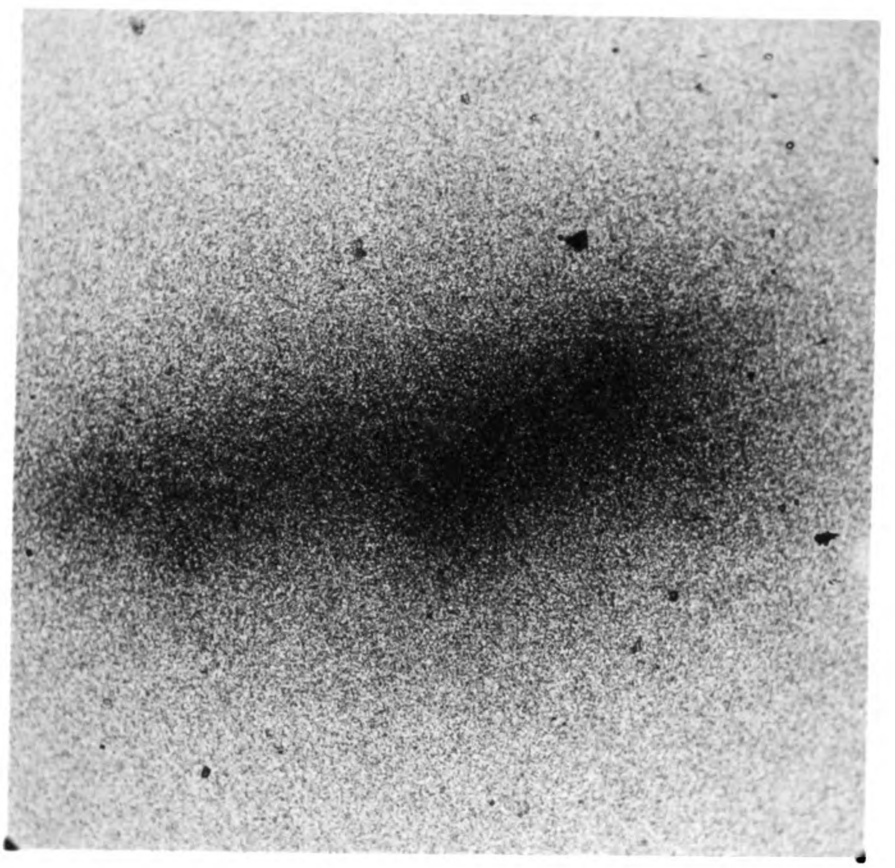


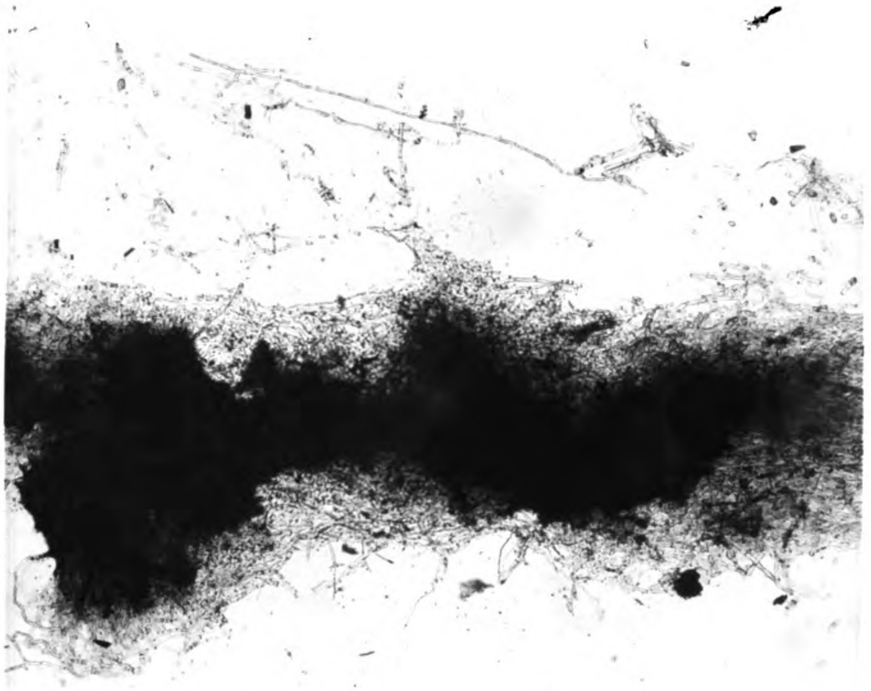
Same As Fig. III

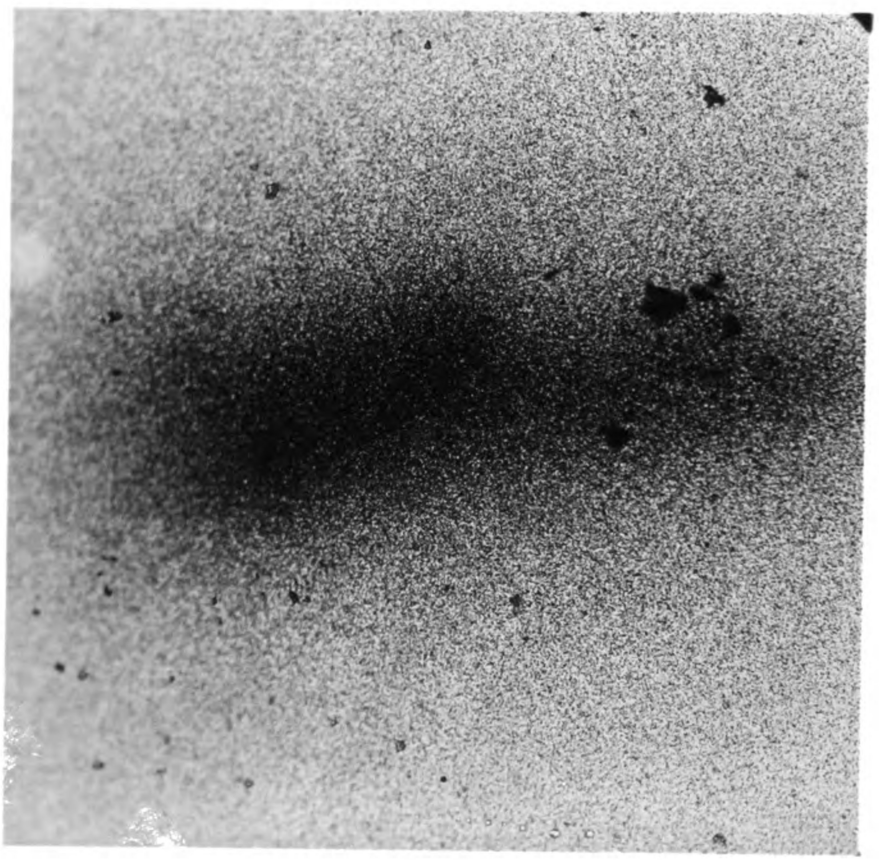


III (A) A FUSARIUM COLONY AFTER A 48-HOUR EXPOSURE TO 100 MICROCURIES OF P-32. X 125
DILUTE METHYLENE BLUE.

III (B) AUTORADIOGRAPH OF (A) X125







Same As Fig. IIC

IV (A) TUBERCLE BACILLI (HUMAN) FROM CULTURE AFTER A 96-HOUR EXPOSURE TO 300 MICROCURIES OF I^{131}. X 1250 ZIEHL-NIELSON STAIN.

(B) AUTORADIOGRAPH OF (A) X 1250

(C) TUBERCLE BACILLI (HUMAN) FROM CULTURE AFTER A 96-HOUR EXPOSURE TO 300 MICROCURIES OF I^{131}. X 1250 ZIEHL-NIELSON STAIN.

(D) AUTORADIOGRAPH OF (C) X 1250

(E) TUBERCLE BACILLI (HUNAN) FROM CULTURE AFTER A 96-HOUR EXPOSURE TO 300 MICROCURIES OF I^{131}. X 1500 ZIEHL-NIELSON STAIN.

(F) AUTORADIOGRAPH OF (E) X 1500

ROOM USE ONLY