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A comparative investigation of normal and irradiated Blastomyces dermatitidis

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A COMPARATIVE INVESTIGATION OF NORMAL AND IRRADIATED

BLASTOMYCES DERMATITIDIS

by

DONALD LOUIS LODMELL
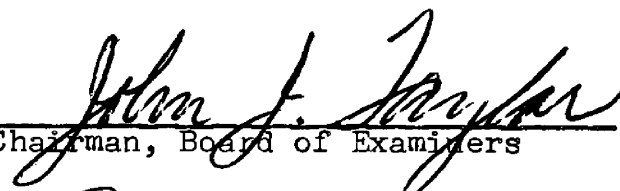
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CHAPTER I

INTRODUCTION

Blastomyces dermatitidis is a fungus which displays dimorphic cultural characteristics. Besides the normal mycelial growth form, a yeast-like form of growth may appear. In this parasitic growth phase, the fungus causes systemic infections in man and animals and occurs in the host tissue as simple yeast-like cells.

According to Levine and Ordal (1946), temperature was the principal controlling factor in phase determination. Salvin (1949) stated that 37 C was conducive for the yeast phase and 25 C for the mycelial phase and that no amino acid, carbohydrate, or "growth substance" was essential for the growth of the yeast phase. However, the extent of growth of the yeast phase did vary greatly according to the constituents of the medium.

Edwards and Edwards (1960) discovered that the Blastomyces yeast-phase cell was a highly complex, unicellular organism possessing varied nuclear and cytoplasmic components and was characterized by profuse, interconnecting membrane systems.

The cell wall is relatively thick averaging 190 m mu in cross section and is composed of fine filaments in layers, the outermost of which is denser and more compact than the others. Edwards and Edwards (1960) and Cochrane (1958) found that chitin, a nitrogen-containing polysaccharide, was present in the cell wall and that cellulose was

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absent. This observation was also made by Blank (1954) who stated that nitrogen-containing keratin was used for the synthesis of chitin, which in turn served as skeletal material of the cell wall. Later, Dalldorf (1962) identified galacturonic acid, mannose, glucose and glucosamine in the cell wall.

The cell membrane proper (plasma membrane) is dense, irregular, and averages 9 m mu in thickness. It shows invagination and vesiculation and frequently connects directly with the endoplasmic reticulum and peripheral mitochondria, or with the nuclear envelope (Edwards and Edwards, 1960).

Delamater (1948) and Edwards and Edwards (1960) agreed that the cell was multinucleate with the latter identifying 2 to 5 nuclei in the various cells examined. These nuclei were smooth to rugose in outline, spherical to ovoid in form and approximately 1.7 by 1.1 m mu in the long and short axes, respectively. Each nucleus examined possessed a matrix of fine filaments, granules of various sizes and densities, and but little identifiable chromatin. The nucleus was bounded by an envelope of the nuclear membrane proper, perinuclear cisterna, and outer nuclear membrane. Especially significant was the fact that the outer nuclear membrane was continuous from one nucleus to another and thus several nuclei could share a common perinuclear cisterna. Mitochondria were numerous and variable.

Edwards and Edwards (1960) stated that the continuity and possible interconvertibility of the cellular endomembrane systems should have the effect of producing great mobility in the cell (suggested by Delamater in 1948), on the basis of rapid communication between cyto-

plasmic and nuclear material, as well as free access to the periphery of the cell of products of reaction occurring in the membranes.

Therefore, of the pathogenic fungi examined with the electron microscope, the yeast cell of B. dermatitidis appears to be the most completely studied. It possessed all the definite constant components of a derivative cell, as in more advanced plant and animal cells, and these components were fully interconnected (Edwards and Edwards, 1960).

The lipids of B. dermatitidis have been investigated by several workers. Al-Doory (1962) stated that there was 6.96 per cent phospholipid, 33.63 per cent acetone-soluble fraction and 40.5 per cent total lipid by weight of dry organisms. Peck and Hauser (1938) stated that lipids made up 8 to 10 per cent of the weight of the whole dried cell and these were approximately one-third phosphatide and two-thirds acetone-soluble fat. The phosphatide on hydrolysis yielded glycerophosphoric acid, choline, ethanolamine and fatty acids which were probably palmitic, stearic, oleic and linoleic acids. The acetone-soluble fat upon saponification gave glycerol, ergosterol, and palmitic, oleic and linoleic acids.

Taylor (1961) stated that RNA of the yeast phase cells increased per mg dry weight after 7 to 10 days with a gradual decline to initial levels after 15 to 30 days. The DNA was relatively constant without significant variation during a 30-day period.

Halliday and McCoy (1955) proved that biotin was necessary for growth especially of laboratory strains of the organism, and that the degree of growth was proportional to the concentration of biotin. Other investigations with the nutritional requirements of Blastomyces

have been conducted by Gilardi and Laffer (1962). They concluded that

B. dermatitidis synthesized its own vitamins and utilized 21 carbon and

25 nitrogen compounds as the sole source of the respective elements.

Sulfur-containing compounds, reducing agents, "Tween 80", and p-amino-

benzoic acid inhibited growth. No additional amino acids or vitamins

were essential for growth in a liquid medium supplemented with casamino

acids and biotin. Furthermore, amino acids were not required for growth,

and the individual vitamins singly or in combination did not stimulate

growth. The oleic acid of "Tween 80" indicated that fatty acids might

have had an inhibitory role on growth. The inability to utilize all

intermediates of the Kreb's cycle did not imply that the organism lacked

the cycle. Methionine, thiourea and sodium thioglycolate each inhibited

growth at 500 mg/100 ml. Salvin (1949) stated that optimum growth ap-

peared in a medium with amino acids. Glycine, alanine, serine, valine,

glutamic acid, aspartic acid, proline and hydroxyproline produced the

best growth. Gilardi and Laffer (1962) discovered that the best growth

of their strains was in shake cultures in the presence of either ammon-

ium salts or organic nitrogen and that the optimum pH was between 5

and 9.

With regard to the actual systemic infection produced by the

organism, Dexter and Herndon (1960) found that B. dermatitidis was not

characteristically a parasite simply of phagocytic cells, nor were the

destructive effects of the fungus in tissue culture directly related

to depletion of essential cell nutrients by the growth and metabolism

of the organism. Instead the destructive effects on cultures of peri-

toneal exudates were said to be related directly to the rapid prolif-

eration of the fungus intracellularly and extracellularly. Ausherman et al. (1957) stated that in 55 canine cases of B. dermatitidis infection, 43 were of the chronic respiratory type, 4 had envolvment of the long bones terminating in generalized blastomycosis, and 8 has systemic infections, initially of which 2 had multiple subcutaneous abscesses.

Mackinnon (1959) said that experimental infection of the laboratory mouse gave a resultant metastasis comparable to conditions found in man suffering from similar infection. Such metastases occurred in animals inoculated by the intramuscular, intraperitoneal, intravenous, or pulmonary route and sometimes by subcutaneous infection or ingestion of infected material. Since Blastomyces infections in man produced similar pathology it was suggested that of the most probably route of infection, the pulmonary is the most logical, and that the infectious mechanisms are at least similar.

Although Denton et al. (1961) infected mice by intravenous inoculation into the tail vein of soil suspensions known to contain B. dermatitidis, Brandsberg et al. (1963) have recently demonstrated that the susceptibility of various experimental animals varied. Of the animals which they tested, including guinea pigs, golden hamsters, albino rabbits, white rats and white Swiss mice, they found that mice and hamsters were the most susceptible to infection. The hamsters proved to be the more susceptible of the two animals. The minimal number of viable particles necessary to produce a high percentage of infections was in excess of 100. It is also known that if the mycelial phase is inoculated into experimental animals these mycelial elements and conidia will convert to the yeast phase to produce infection (Demonbreun, 1935).

Although the literature contains references to studies of ultra-

violet effects on sexual spores of saprophytic fungi, no literature
could be found which reported the effects of ultraviolet irradiation
on B. dermatitidis or other human pathogenic fungi.

The effects of ultraviolet irradiation upon bacterial cells has
been under investigation for some time. Only a few pertinent recent
investigations have been summarized here. The relationship between the
recovery of cells after ultraviolet irradiation and the composition of
plating media has not been thoroughly investigated. Wainwright and
Nevill (1955) stated that the number of survivors which was recovered
was reduced when ultraviolet irradiated cells were incubated on a pep-
tone medium. Roberts and Aldous (1949) reported a greater recovery on
their chemically defined medium than on nutrient agar. Proof of this
point was accomplished when they added one per cent nutrient agar to
the chemically defined medium and observed a decreased number of sur-
vivors. Similarly, Alper and Gillies (1958) found that recovery of
irradiated cells on nutrient agar was greater than on "oxoid" blood
agar and that the addition of the latter to the former inhibited recov-
ery. They concluded that different plating media gave rise to widely
different viable counts after ultraviolet irradiation and that surviv-
ing fractions were smallest on media which were optimal for growth of
unirradiated organisms. Ramage (1961) also found that the medium which
was best for growth was poorest for recovery of ultraviolet irradiated
Shigella sonnei.

The investigations of the effects of ultraviolet irradiation on
respiration of bacteria have been rather extensive. In 1953, Kelner
investigated endogenous and exogenous respiration of Escherichia coli
after ultraviolet irradiation. He concluded that endogenous respiration

was not affected by irradiation even when the dosage was great enough to inactivate all the cells. Exogenous respiration was similar to the unirradiated cells for some time and then began steadily to decline. Billen et al. (1953) observed the same result. However, Heinmets and Kathan (1954) reported that oxygen uptake by irradiated cells in the presence of glucose was only very slightly affected. Billen and his co-workers also found that increased exposures to ultraviolet had no effect on the length of time during which irradiated endogenous equalled normal respiration. Giese and Swanson (1947) were in disagreement with Kelner (1953) in that endogenous respiration by yeast cells increased after ultraviolet irradiation in their experiments.

Sarachek (1958) and Pittman et al. (1959) found that an ultra-violet-induced respiratory deficiency was photoreversible and implicated the nucleus as the site of radiation damage.

The effect of ultraviolet irradiation on amino acids has not been investigated very thoroughly. One investigation (Nakanishi, 1958) dealt with the phenomenon of amino acid absorption of ultraviolet irradiation. The author stated that the lowest absorption was at or near the isoelec-tric points of the aliphatic amino acids, between pK_2 and pK_3 in the mono-amino di-carboxylic, and between pK_1 and pK_2 in the di-amino mono-carboxylic acids. On the other hand, -COOH and -OH groups showed an increase in absorption when they were charged. He concluded that the absorption was highest within a pH range in which the largest number of groups was charged.

The effects of ultraviolet irradiation on nucleic acids has re-ceived much investigation during the past few years. In 1954, Kanazer and Errera and Kelner pointed out that the synthesis of DNA stopped

immediately after irradiation. Kelner believed that the DNA inhibition

implied inhibition of gene reduplication. RNA syntheses were found to

continue in synchronization with cellular growth. Barner and Cohen (1956)

concluded that ultraviolet absorbed by DNA resulted in the breaking of a

critical bond and the formation of a toxic product. Kimball (1957) also

postulated that inhibition of DNA synthesis was the major factor in

irradiation damage to cells. He assumed that inactivation and mutation

were secondary consequences of the irradiation effects on DNA synthesis

and that inhibition of synthesis was a result of alteration in DNA and

interference with replication. Stuy (1959) attributed death of vigor-

ously growing cells not only to the radiation damage to the DNA, but

also to the length of the period necessary for repair. Drakulic (1959)

stated that inhibition of DNA synthesis was only temporary but could be

inhibited completely if irradiation of protein synthesis was prevented.

Hanawalt and Settow (1960) reported that the effects of ultra-

violet on RNA synthesis and on protein synthesis in E. coli were the

same. RNA synthesis was inhibited and much of the DNA synthesized after

recovery was non-functional. Although "particulate" RNA synthesis was

constant in the absence of DNA synthesis, both "soluble" RNA and protein

synthesis increased. Protein synthesis was more sensitive to ultravio-

let than RNA synthesis since apparently DNA specified "nonsense" RNA

which could not sustain normal protein synthesis. Damage to one strand

of DNA was sufficient for unit effect on protein synthesis, but both

strands must have been damaged to produce an effect on RNA synthesis.

Drakulic et al. (1961), while working with E. coli B said that

the ultraviolet effect on RNA was a disturbance in the RNA metabolism.

This disturbance was a decreased incorporation of adenine into the

"particulate" RNA and a greater incorporation into the "soluble" RNA. This additional "soluble" RNA in the irradiated cells was metabolically defective or unstable.

Marmur et al. (1961) said the initial action of ultraviolet irradiation was a chemical alteration of the pyrimidine residues. This was followed by a weakening of the DNA structure which resulted in interference in normal hydrogen bonding. Other noted effects were the cross-linkage of DNA strands resulting from dimerization of specific base pairs that were not normally opposite. Also noted were formation of dimers of thymine resulting in cross-linkage and impairment of DNA replication and proper base pairing.

Beukers and Berends (1961) stated that ultraviolet irradiation of thymine resulted in the conversion of thymine to mixed dimers and to I.P.T. (irradiation product of thymine), which was chiefly responsible for the mutagenic and lethal effects of ultraviolet.

Klouwen (1962) also discovered that ultraviolet was absorbed mainly by the purine and pyrimidine bases. The result was dimerization which prevented the uncoiling of the helix. A shift toward the keto form of the hydroxyl groups of the bases was also noted. This resulted in a strengthening of hydrogen bonds and the prevention of helix separation.

Photoreactivation is a very widespread phenomenon which effects changes of seemingly different nature in organisms damaged by ultraviolet irradiation. The fact that most of the known biological effects of ultraviolet irradiation have been photoreactivated seemed to indicate that they all include a common reaction.

Rupert (1961) summarized the effects of photoreactivation in the

following manner. Enzymes exist in photoreactivable cells which repair ultraviolet damage to transforming DNA in vitro. He also stated that photoreactivation was one of the mechanisms of cells to repair the damage produced by sunlight on their DNA. Because it can be demonstrated among many living things and because it could in principle occur with a low level of cellular organization, requiring only a suitable catalyst to favor the photoreversal of a photochemical change, photoreactivation could be considered a primitive mechanism evolving fairly early in the history of life on earth. Other mechanisms probably exist to perform the same function and may be responsible for some of the factors modifying the effects of ultraviolet on cells.

Hollaender (1955) believed that the effect of photoreactivation was almost completely specific for damages produced by ultraviolet irradiation. The site at which photoreactivable damage was thought to be produced was a nucleoprotein, and was probably nucleic acid. Mechanisms of reactivation require substances of "cytoplasmic" nature. The action spectra obtained seemed to indicate that different pigments were responsible for the absorption by the photoreactivating site in different organisms.

Kelner (1949) stated that with recovery of Streptomyces griseus from ultraviolet irradiation the ability of irradiated cells to grow and form a colony was restored. Recovery was proportional to the duration of illumination, within limits, and the rate of recovery increased with an increase in temperature up to approximately 50 C. Therefore, the killing effect of ultraviolet was due to a light labile alteration of some constituent in the cell. Exposure to visible light restored this altered constituent to its former state.

Hanawalt and Buehler (1960) worked with E. coli and found that when visible light was administered after ultraviolet irradiation the DNA synthesis behaved as though it had received an irradiation dose one-half the strength used previously. RNA and protein syntheses were capable of photoreactivation even in the absence of DNA synthesis. Therefore, it appeared that photoreactivation of bacterial RNA and protein syntheses basically involved a repair of DNA integrity rather than restoration of DNA synthesis.

Goodgal (1950) stated that the killing of microconidia of Neurospora crassa by ultraviolet was caused by lethal mutations. Light decreased killing action by destroying an ultraviolet-induced mutagenic agent, thus preventing the mutations.

Bellamy and Germain (1955) worked with various streptococci, E. coli and A. aerogenes. They found that E. coli and A. aerogenes were reactivated by blue and by white light while the streptococci were not. These results supported the theory that porphrins were involved in photoreactivation since streptococci contain fewer porphrins.

Rupert (1962) found that the photoenzyme from baker's yeast which repaired ultraviolet-inactivated transforming DNA was mechanically bound to ultraviolet irradiated bacteria in the dark, but not to non-ultraviolet irradiated DNA. In the bound condition it was stabilized against inactivation by heat and heavy metals. Both the mechanical bonding and stabilization were eliminated by illumination. These observations were consistent with the reaction scheme suggested by kinetics studies, in which the enzyme combined with ultraviolet lesions in DNA, after which the complex absorbed light and produced repair and subsequent liberation of the enzyme.

Goucher et al. (1955), while working with Azotobacter, stated that acetate oxidation by acetate-adapted cells was thought to be inhibited by ultraviolet light. This inhibition of a constitutive enzyme was observed to be diminished by exposing irradiated cells to visible light. This was also true for acetate cells adapted to growth in succinate.

Pittman et al. (1959), while working with yeasts, stated that the site of ultraviolet damage which affected respiratory activity was the nucleus. Although the photoreactivable damage of most ultraviolet-induced lesions appeared to be in the nucleus or associated with nucleoproteins, they thought photoreactibility of a given lesion did not imply necessarily that the reactivable damage was nuclear, since both nuclear and cytoplasmic damage have been shown to be photoreactivable.

Elevated temperatures have been found to enhance recovery of irradiated and photoreactivated cells to some extent. Charles and Zimmerman (1956) stated that E. coli B photoreactivated for 100 minutes after irradiation grew optimally at 55 C.

CHAPTER II

STATEMENT OF PROBLEM

This investigation was primarily undertaken to determine the nitrogenous constituents in normal B. dermatitidis, how these constituents may be altered by ultraviolet irradiation, and what role or roles they may have in infectivity.

CHAPTER III

METHODS AND MATERIALS

I. GENERAL METHODS AND MATERIALS

(1) Organism Employed.

B. dermatitidis, strain 6046, was employed throughout this investigation. It was obtained from the Rocky Mountain Laboratory, U. S. P. H. S., Hamilton, Montana, and was originally isolated by Dr. C. W. Emmons, U. S. P. H. S., Washington, D. C.

(2) Buffer and Saline Solutions.

The sterile phosphate-buffered saline used throughout this investigation was designated as SPBS. It consisted of 180 ml of 0.1M K_2HOP_4, 120 ml of 0.1M KH_2PO_4, 600 ml of distilled water and 10 g NaCl. After sterilization of the solution at 121 C for 15 minutes, the pH was 7.0.

The unbuffered saline solution was designated as SS and consisted of 8.5 g NaCl in one l of distilled water. It was also autoclaved.

(3) Maintenance of Cultures, Culture Media, and General Methods Employed.

B. dermatitidis 6046 was grown on Sabouraud Dextrose Agar pH 7.0, at 37 C. The medium contained 10 g Neopeptone, 40 g dextrose, and 15 g agar per liter of distilled water. The pH was adjusted to 7.0 with tribasic sodium phosphate and the medium was sterilized in the autoclave for 15 minutes at 121 C.

Cultures were checked periodically for contaminants by micro-
scopic examination.

The composition of the recovery media which were used in the
comparative study were as follows:

A. Sabouraud Dextrose Agar (SAB) as mentioned on page 14. (Balti-
   more Biological Laboratory).

B. Fluid Sabouraud Medium (FSAB). (Baltimore Biological Laboratory).

   1. Casitone - 5 g

   2. Peptamin - 5 g

   3. Dextrose - 5 g

   4. Distilled water - 1000 ml

The pH was adjusted to 7.0 by the use of tribasic sodium phosphate.
Fifteen g Bacto agar was then added and the solution autoclaved.

C. Sabouraud Dextrose Agar "80" (SAB 80).

   As SAB, with the exception that 0.1 ml "Tween 80" (Nutritional

   Biochemicals Corporation) was added per liter of medium.

D. Brain Heart Infusion Agar (BHI). (Baltimore Biological Laboratory).

   1. Calf brain, infusion from - 200 g

   2. Beef heart, infusion from - 250 g

   3. Peptone   - 10 g

   4. NaCl   - 5 g

   5. Disodium phosphate   - 2.5 g

   6. Dextrose   - 2.0 g

   7. Agar   - 15.0 g

   8. Distilled water   - 1000 ml

The pH was adjusted to 7.0 with 0.1 M HCl and the solution auto-claved.

E. Potato Dextrose Agar (PDA). (Baltimore Biological Laboratory).

1. Potato, infusion from - 200 g

2. Dextrose - 20 g

3. Agar - 15 g

4. Distilled water - 1000 ml

The pH was adjusted to 7.0 with tribasic sodium phosphate. After heating with frequent agitation and boiling for one minute the solution was autoclaved.

F. Eugon Broth (Difco).

1. Tryptose - 15 g

2. Soytone - 5 g

3. Dextrose - 5 g

4. 1-cystine - 0.2 g

5. NaCl - 4.0 g

6. Sodium sulfite - 0.2 g

7. Sodium citrate - 1.0 g

8. Distilled water - 1000 ml

Fifteen g Bacto agar was added and the solution autoclaved.

G. Mycological Broth (Difco).

1. Soytone - 10 g

2. Dextrose - 40 g

3. Distilled water - 1000 ml

After addition of 15 g agar it was sterilized in the autoclave.

H. Blood Agar (Difco).

    1. Veal heart, infusion from - 500 g

    2. Proteose peptone   - 10 g

    3. NaCl   - 5 g

    4. Agar   - 15 g

    5. Distilled water   - 1000 ml

After adjustment of the pH to 7.0 with 0.1 M HCl and steriliza-
tion in the autoclave, the medium was cooled to 55 C by placing it in
a water bath. Five per cent human blood was then added aseptically
and the poured plates were allowed to solidify.

(4) Preparation of Cells.

In the course of this investigation various concentrations of
cell suspensions were used depending upon the purpose of the experiment
being done at that time. However, for the most part, the experiments
were done with the following preparation. Five-day cells grown at 37 C
on SAB, pH 7.0, were removed from four slants with 10.0 ml of 0.85 per
cent SPBS. The cells were then washed twice with 10 ml portions of
0.85 per cent SPBS and centrifuged at 2,000 rpm in an International
Clinical Centrifuge. These washed cells were resuspended in enough
SPBS to equal the optical density of McFarland Nepthelometer #10 when
determined with a Coleman Junior Spectrophotometer at 500 m mu. A 1:50
dilution was prepared by adding one ml of the #10 concentration to 49.0
ml of 0.85 per cent SPBS. This 50 ml dilution was placed in a Waring
blender and agitated, at low speed, for 15 minutes at 8 C to assure
adequate separation of all clumps and cell aggregates. After blending,
10 ml aliquots were removed and treated in the manner designated in each

(5) Plate Counting Method.

The Quebec Colony Counter was used for those plates on which the number of visible colonies were too numerous to count with the unaided eye. The method employed for calculation of the total plate number involved the counting of the colonies in 5 squares, dividing the total by 5 and multiplying the result by 66.6. When possible, all colonies on a plate were counted to reduce the probability of error.

(6) Ultraviolet Light Source and Procedure for Irradiation.

The source of ultraviolet light was a Minerallight, Model SL 2537 short wave lamp. The maximum output of the lamp was at approximately 2,537 Å and was approximately 5 watts at the factory. The distance from the mercury tube to the petri dish bottom was 15.0 cm in all experiments. Cell suspensions which were to be irradiated were placed in 10 ml portions in sterile "Saran"-wrapped petri dishes. This volume covered the bottom of the dish to a depth of 3.0 ml. Before irradiation the lamp was turned on for 5 minutes to allow equilibration of output. During irradiation the suspensions were constantly rotated on a Yankee Rotator at 180 rmp to aid equal exposure of all cells. This work was done in a dark room with only a 10-watt General Electric blue bulb for illumination. Those cells which were irradiated and plated were wrapped in foil for the entire period of incubation. The irradiated cells used in the study of respiration were wrapped in foil during the entire process.

(7) Bright Light Source and Procedure for Photoreactivation.

After the cells had been irradiated, photoreactivation was at-

tempted with a Sylvania 60-watt white light in a reflector. The bulb was 15 cm from the bottom of the petri dish and a glass plate one-half inch thick was placed 3 cm from the light source to absorb the heat emitted by the bulb. The duration of the illumination was equal to 4 times that of ultraviolet irradiation. As with the irradiated cells, the Yankee Rotator was again utilized to aid equal exposure to all cells.

(8) Mice.

All mice used in this investigation were of the Webster strain and were obtained from the Rocky Mountain Laboratory, U. S. P. H. S., Hamilton, Montana. The colony was maintained in the microbiology animal quarters. The average of those mice used for experimentation was 3 months.

II. EXPERIMENTAL METHODS AND MATERIALS

(1) Recovery of Visible Colonies on Various Media.

After 8 minutes of irradiation, triplicate plates of SAB, SAB 80, fluid SAB, BHI, PDA, eugon, mycological and blood agars were inoculated with 0.05 ml of irradiated cell suspension per plate. Control cells were also inoculated in triplicate with 0.05 ml per plate. All inocula were spread evenly over the plates with bent glass rods. The plates were sealed with rubber seals to prevent desication of the media and in- cubated at 37 C for 5 days. If the cells were to be illuminated another sample was irradiated 8 minutes and then exposed to the white light for 32 minutes. To note the effect of heat, if any, during the illumination process the cell suspensions were exposed to 2 different temperatures. One suspension was placed in a 8 C ice bath while being illuminated and the second was kept in water heated to 37 C. Inoculation and incubation procedures were the same as with irradiated and control cells.

(2) The Effect of Heat and Time on Illumination.

Subsequent to 8 minutes of irradiation 0.25 ml were removed in a Kahn pipette and 0.05 ml plated on each of 5 different SAB plates. The suspension was then illuminated (as previously described) for 0.5 minutes and another 0.25 ml removed and 0.05 ml plated as above. The illuminating and plating procedures were continued until the following total periods of illumination had resulted: 0.5, 1.0, 3.0, 5.0, 7.5, 10.0, and 30.0 minutes. Control cells were processed in a similar

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manner at each time interval.

(3) The Effect of Ultraviolet Irradiation on Respiration of B. dermatitidis.

The cell suspension was prepared as previously mentioned. The treatment of cells during irradiation and photoreactivation and the controls were also identical. The starved cells were stored in SPBS for 7 days at 8 C prior to use. In all other procedures they were treated as the freshly harvested cells. Standard manometric techniques were employed (Umbreit, Burris and Stauffer, 1959). Readings were taken at 10-minute intervals for a period of 2 hours. When the uptake of oxygen was great, readjustment to the 250 mm mark was necessary.

(4) Establishment of Rf Values.

In working with paper chromatography it was necessary that one establish Rf values for amino acids. Rf values found by using the methods of Smith (1958) and Block et al. (1958) for varying solvents were compared with the values established with my standard solutions. These standards were made by dissolving one mg of the amino acid in one ml of 10 per cent isopropanol. The standard solutions were kept frozen when not in use. The solutions were spotted $1\frac{1}{4}$ inch apart with the aid of a micropipette on Whatman #1 chromatographic paper $7\frac{1}{2}$ inches wide. Each spot was dried with an electric hair dryer. Two different amino acids with markedly different Rf values for the solvent being used were placed on one spot so there would be adequate separation for the determination. Descending chromatography was used for all experiments with an average elapsed time of 18-20 hours. The solvents used consisted of phenol (500 g + 125 ml water); pyridine-acetic acid-water

(50:35:15); butanol-ammonia (saturated 1-butanol with 2N NH_4OH); and pyridine-butanol-water (1:1:1). After the solvents had descended the length of the sheet, the sheets were removed from the chromatographic chambers and allowed to dry. Then they were dipped in 25 per cent ninhydrin in acetone (w/v), air dried, and placed in the 105 C incubator for 3 minutes. Rf values were established by the following formula (Block et al., 1958):

$$Rf = \frac{\text{distance traveled by substance}}{\text{distance traveled by solvent front}}$$

Since it was necessary to maintain rigorously controlled conditions, characteristics other than Rf values were also used in the identification of amino acids.

(5) Cell Extracts for Paper Chromatography and Their Development.

In the identification of amino acids by paper chromatography it was necessary that a large volume of cells was used, since many of the amino acids were present in such minute quantities they were almost undetectable. To obtain this needed amount, enough 5-day cells were removed from SAB slants to equal 6 ml of packed cells. These cells were washed 3 times with distilled water and resuspended in 15 ml distilled water. After the cells were evenly suspended, they were separated into 3 5-ml portions, each of which contained 2 ml of cells. One of the 5-ml portions was suspended in 35 ml of distilled water and placed in a large petri dish bottom. After placing the 40-ml suspension in the dish it was irradiated for 20 minutes. The second 5-ml portion was added to 35 ml of distilled water, placed in a petri dish, irradiated for 20 minutes and then illuminated for 80 minutes. The third

portion was added to 35 ml of distilled water and served as the control.
After their respective treatments the portions were collected, placed
in centrifuge tubes, spun down, and resuspended in 5 ml of distilled
water. These 5-ml suspensions were placed in separate vials with 4 ml
of #10 Ballantine beads which were washed 5 times previously with dis-
tilled water. The suspensions were agitated in the Mickel disintegrator
at maximum transit for one hour. After mickelizing, the liquid portions
within the vials were removed, the beads washed with 5 ml of distilled
water and the washings combined with their respective liquid portions.
The combined portions were then centrifuged until all sediment was re-
moved. One ml of each portion was then removed, evaporated to dryness
under vacuum (10 mm Hg at 50 C) and dissolved in $\frac{1}{4}$ ml of 10 per cent
isopropanol. If the cells being tested were to be starved the respect-
ive suspensions were placed in sterile flasks containing 35 ml of SPBS
and incubated at 37 C for the proper period of time. After incubation
the cells were washed 3 times with distilled water to remove all traces
of SPBS and the procedure continued as before by mickelizing 5 ml in
distilled water. Chromatographic development of the extracts was the
same as that for standard solutions.

(6) Two-Dimensional Paper Chromatographic Procedures.

For maximum resolution of the cell extracts, 2-dimensional paper
chromatography was recommended. The principle of this technique was the
successive development of the chromatogram with 2 different solvents,
whose advancing fronts were at right angles to each other. The extracts
for 2-dimensional paper chromatography were prepared as those described
earlier for one-dimensional analysis. One-half of the 0.25 ml of prepared

extract was applied one inch from the upper corner of a sheet 18 x 22 inches. After the first solvent had traveled almost to the bottom edge, the sheet was removed, dried, and rerun in a second solvent whose front was at right angles to that of the first solvent. After development with the second solvent, the chromatogram was ready for further processing and evaluation. Two different 2-dimensional experiments were used in the identification of the amino acids. The first consisted of the solvents butanol-pyridine-water (1:1:1) and phenol-ammonia (one ml NH_4OH:500 g phenol:125 ml water) (Smith, 1958). Identification of the amino acids was facilitated by the use of previously developed maps (Smith, 1958), Rf values and specific color reactions.

The second solvent system consisted of n-butanol-acetic acid-water (26:6:25) and n-butanol-methylethyl ketone-water (2:2:1). Cyclo-hxylamine was placed in a separate vessel in the chromatocab to permit resolution and diverse coloration of the amino acids without undesirable bluing of the paper (Mizell and Simpson, 1960). Identification of the amino acids was as above (Mizell and Simpson, 1960). The second system was selected for the major portion of the 2-dimensional chromatographic investigations.

(7) Location Reagents for the Identification of Specific Amino Acids.

The location of amino acids has usually been accomplished by means of ninhydrin alone. However, in order to confirm the location of particular amino acids it was often of value to use other more specific reagents. Many of these specific reagents were used in identification of the amino acids of B. dermatitidis.

A. The chromatogram was sprayed with 0.01 per cent alpha-napthol in ethanol containing 5 per cent urea. It was air dried a few minutes and sprayed lightly with 0.7 ml Br_2 in 100 ml of 5 per cent KOH. This was a demonstration of arginine which became a light pink (Acher, 1952).

B. The chromatogram was sprayed with a 0.1 per cent solution of alpha-napthol in NaOH. After it dried, the paper was sprayed with CaClO solution prepared from an equal mixture of ethanol and commercial Clorox. Arginine reacted and appeared as a red spot (Block and Bolling, 1951).

C. The chromatogram was dipped into a 0.02 per cent solution of o-phthalaldehyde in acetone containing also 0.2 per cent urea. After it was heated for 10 minutes at 50 C and then dipped in one per cent alcoholic KOH it was heated for 10 minutes at 50 C and for 10 minutes at 105 C. Taurine produced a red spot (Curzon and Giltrow, 1954).

D. After dipping the chromatogram in a 0.2 per cent solution of o-phthalaldehyde in acetone and air drying it, it was heated for 10 minutes at 50 C. Colored spots appeared with glycine, histidine and tryptophane (Curzon and Giltrow, 1954).

E. The chromatogram was dipped in a freshly prepared 2.0 per cent solution of vanillin in n-propanol and heated at 110 C for 10 minutes. It was then dipped in one per cent alcoholic KOH and heated again at 110 C for 10 minutes. Ornithine produced a salmon spot (Curzon and Giltrow, 1956).

F. After spraying the chromatogram with 0.2 per cent vanillin in acetone followed by one per cent alcoholic KOH, it was allowed to stand

at room temperature overnight. Ornithine, proline, and hydroxyproline produced red spots (Curzon and Giltrow, 1954).

G. A solution of one per cent p-dimethylamino-benzaldehyde in N HCl was sprayed on the chromatogram. Citrulline produced a yellow spot (Fowden, 1951).

H. The chromatogram was sprayed with a 0.2 per cent solution of isatin in acetone containing 4 per cent acetic acid and heated for 10 minutes at 100 C. After heating it was sprayed with Ehrlich's reagent (one g of p-dimethyl-aminobenzaldehyde, 90 ml of acetone, and 10 ml of concentrated HCl). The duck egg blue of hydroxyproline was replaced by an intense purplish-red color.

I. After the chromatogram was dipped in a 0.1 per cent solution of hydroxyquinoline in acetone and dried it was dipped in a solution of 0.2 ml Br_2 in 100 ml of 0.5 N NaOH. Arginine produced an orange-red spot (Jepson and Smith, 1953).

J. The chromatogram was sprayed with 0.25 per cent ninhydrin in acetone (w/v) and heated for 3 minutes at 105 C. It was then sprayed again with dilute $NaHCO_3$ (0.15 to 0.20 per cent). Phenylalanine produced a blue spot (Pasieka and Morgan, 1956).

K. A 0.2 per cent isatin in acetone solution with 4 per cent acetic acid was sprayed on the chromatogram and it was heated for 10 minutes at 100 C. A variety of colors for specific amino acids were obtained (Saifer, 1956).

L. After removal of the solvent in a cold air draught, the paper
was dipped in a 0.2 per cent solution of isatin in acetone. As soon
as the acetone had evaporated from the paper it was heated for 2 min-
utes at 105 C. There were again a variety of color reactions for
specific amino acids (Smith, 1953).

M. The chromatogram was dipped in a 0.2 per cent o-phthalaldehyde
solution and heated at 100 C for 2 minutes. It was then dipped in a
one per cent KOH in 95 per cent ethanol and reheated for another 10
minutes at 100 C. There were specific color reactions for glycine
and taurine (Smith, 1958).

N. After dipping the chromatogram in a solution of 1.5 g sodium
nitroprusside in 5 ml of 2N H_2SO_4 to which 95 ml methanol and 10 ml
20 per cent ammonia were added it was dried slightly. While still damp
it was dipped into a solution of 2 g NaCN dissolved in 5 ml H_2O and
diluted to 100 ml with methanol and allowed to air dry. This was for
the identification of cystine and cysteine (Toennies and Kolb, 1951).

(8) Analytical Methods for Nucleic Acids.

The procedures employed for analysis of both RNA and DNA were
similar to those used in "Methods of Biochemical Analysis," but because
sufficient specific information was lacking the method employed by Morse
and Carter (1949) was used as a supplement. The preparation of the
cell suspension and the treatment of irradiated, illuminated, and con-
trol cells were as before. Following these procedures, the volumes of
the cell suspensions were brought back to 10 ml. The cells were then
evenly suspended by shaking and one ml from each preparation was removed

and placed in separate tared crucibles for dry weight determinations. The crucibles were heated at 95 C for 12 hours. An additional 0.1 ml was removed from each suspension and plated on SAB to determine the number of viable (colony-forming) cells. After removal of these 2 samples, the suspensions were centrifuged for 3 minutes and the supernatant fluids were decanted. The volumes were then brought up to 9 ml with 1.8 ml of 50 per cent trichloroacetic acid and 7.2 ml of distilled water. The suspensions were centrifuged at 2,000 rpm for 20 minutes and the supernatant fluids were decanted. The sedimented cells were then hydrolyzed with 8 ml of 5 per cent trichloroacetic acid in a boiling water bath for 30 minutes. After hydrolysis, the preparations were centrifuged at 2000 rpm for 15 minutes and the supernatant fluids poured off for analysis.

A. Analysis of RNA.

The RNA reagent was prepared by dissolving 0.25 g of recrystalized orcinol and 0.169 g ferric ammonium sulfate in 6.25 ml of distilled water. To this solution was added 103.75 ml of concentrated HCl and 15 ml of distilled water. In the test for RNA, 3 ml of the test solution (containing 4-40 mu g pentose) was added to 9 ml of reagent. The mixture was heated in a boiling water bath for 20 minutes and then cooled to room temperature by placing the tubes in a bath of cold tap water. Optical densities of the mixtures were determined at 660 m mu with a Coleman Junior Spectrophotometer. A blank (zero optical density) was prepared with distilled water instead of the solution to be analyzed and treated as above. The readings obtained with the unknowns were compared with a reference curve prepared with solutions of RNA tested

at the same time. The reference solutions of RNA contained 20, 10, 5 and 2.5 mg per cent.

B. Analysis of DNA.

The DNA reagent was prepared by dissolving 0.25 g of diphenylala-mine in 24.5 ml of redistilled glacial acetic acid and 0.5 ml of con-centrated H_2SO_4. To test for DNA, one ml of the sample was added to 2.5 ml of reagent. The mixture was heated 5 minutes in a boiling water bath, then cooled to room temperature as above. A blank for setting zero was prepared as for RNA. The optical densities were determined at 540 m mu and the values were compared with a reference curve pre-pared with DNA solution tested at the same time. Concentrations of DNA in the reference solutions were 50, 25, 12.5, and 6.25 mg per cent.

(9) Mice Inoculations and the Effect of Irradiation on Infectivity.

Preparation of the cell suspension was the same as before. How-ever, after the suspension had been in the Waring blender for 15 minutes at low speed it was diluted to prepare the following cell concentrations: 1.5×10^5, 1.0×10^5 and 0.5×10^5. The number of cells in each of these was calculated by the use of a hemocytometer. The cells were counted which were present in 5 1/25 mm^2 squares. Of the cells which touched the boundary lines all those were counted which touched the upper and left lines. Those cells on the bottom and right lines were not counted. After the number of cells was determined it was multi-plied by 50, a constant, giving the number of cells per ml. Ten ml portions of each suspension were irradiated at 15 cm for 5, 8, 11, 14, 17, 20, 25, 30, 40, or 60 minutes. Upon completion of the irradiation

the suspensions were centrifuged for 5 minutes at 2000 rpm and 5 ml of
the supernatant were discarded. To the 5 ml remaining were added 5 ml
of 5 per cent aqueous sterile gastric mucin, pH 7.3. The suspensions
were mixed well by shaking and 0.5 ml were removed from each and plated
on each of 3 SAB plates. These were used to establish a relationship
between the minimal infective dose and the number of colonies recover-
able on SAB plates. After removal of the sample for plating, 0.5 ml
of the suspensions were inoculated intraperitoneally in each of 5 mice.
They were kept in glass mouse jars for a period of 10 weeks before
autopsying. Mice and SAB plates inoculated with unirradiated cells
served as controls and were included with the experiment. At the end
of 10 weeks the mice were etherized and autopsied. Before cutting into
the body cavity the abdomen of each mouse was swabbed with 10 per cent
lysol. Incisions were made with sterile forceps and scissors. If any
visible lesions were present on the liver or diaphragm they were removed
aseptically and placed on SAB slants of pH 5.6. If lesions were not
present, the liver, lungs, and heart were removed, placed in a sterile
petri dish and mashed with a glass vial. After maceration, 3 ml of
0.85 per cent SPBS were added to the dish and the mashed tissues were
mixed to uniform suspension. Approximately one ml of the suspension
was removed and placed on each of 2 tubes of SAB, pH 5.6. The tubes
were incubated at 25 C for one month and were then examined every 3
days for typical Blastomyces growth. Positive determinations of an
infected mouse were made by recovery of characteristic growth in these
cultures, microscopic examination of lesions stained with lactophenol
blue, and culturing of cysts on blood agar plates.

(10) <u>Determination of Minimal Infective Dose and Sex Susceptibility</u>.

A stock suspension containing 2 x 10^5 cells per ml was prepared by the methods used previously. From this stock suspension serial doubling dilutions were made to obtain 10 ml of each of following stock concentrations: 100,000, 50,000, 25,000, 12,500, 6,250, 3,125, 1,562, 781, 390, 195, and 97 cells per ml. Each of these suspensions was centrifuged for 10 minutes at 2,000 rpm and 5 ml of supernatant fluid was removed from each. To the remaining 5 ml was added 5 ml of sterile 5 per cent aqueous gastric mucin. Five-tenths ml of each suspension were plated on 3 separate SAB plates and 0.5 ml of each were injected intraperitoneally into each of 5 separate mice. Inoculation of both male and female mice was included to determine the effects of sex in susceptibility to infection. The mice were kept 10 weeks before being killed and examined in autopsy. The procedures were similar to those stated previously.

(11) <u>Mice Inoculations and the Effect of Illumination</u>.

The stock suspension containing 2 x 10^5 cells per ml was prepared as before. The irradiation procedures were also the same. Three cell suspensions were irradiated and illuminated. The first was irradiated for 17 minutes and illuminated for 60 minutes, the second suspension was irradiated 20 minutes and illuminated 80 minutes and the third suspension was irradiated 25 minutes and illuminated 100 minutes. The autopsy procedures and identification were the same as those mentioned previously.

CHAPTER IV

RESULTS

(1) <u>Recovery of Visible Colonies on Various Media</u> (Table 1, Figures 1-8).

Five per cent blood agar, with a veal infusion base, offered the
best growth of normal cells. BHI, SAB, and fluid SAB allowed somewhat
less growth, and because of the small differences in relative colony
numbers they were considered similar. SAB 80 produced fewer colonies
and mycological and eugon agars fewer yet. PDA produced the least
number of visible colonies. Many times these were almost too small to
count even after an incubation period of 5 days. The recovery of irra-
diated cells was best with blood agar (29.1 per cent). BHI agar was
next (19.8 per cent) and the group of SAB 80, SAB, and eugon media fol-
lowed it with respective recoveries of 17.8, 16.6, and 16.5 per cent.
Fluid SAB (14.2 per cent) came after this group of 3. PDA and mycolo-
gical agars were last with 10.4 and 9.8 per cent respectively.

The per cent recovery of those cells irradiated and then illum-
inated at 37 C was best with blood agar (31.6 per cent). SAB 80, eugon,
and SAB were less effective than the blood medium with recoveries of
19.6, 18.5 and 17.9 per cent. BHI and mycological agars showed 14.8
and 13.0 per cent, while fluid SAB and PDA were the poorest media with
only 11.3 and 11.1 per cent recoveries. For cells illuminated at 8 C
after irradiation blood agar was the best medium with a recovery of
32.2 per cent. Other effective media were eugon and BHI agars with
24.6 and 22.6, fluid SAB with 21.2, SAB 80 with 21, and SAB with 20 per

cent recoveries. Mycological agar and PDA with 16.0 and 15.5 per cent respectively were the poorest.

Only BHI and fluid SAB agars had a lower percentage of recovery with cells illuminated at 37 C when compared to the irradiated cells. All of the media produced a higher percentage of recovery with cells illuminated at 8 C when compared to either the irradiated cells or those illuminated at 37 C.

Blood agar was by far the best medium for growth of normal, irradiated, or illuminated B. dermatitidis cells. SAB 80, eugon, BHI, and SAB agars produced approximately equal numbers of colonies, but fewer than blood agar. Fluid SAB produced somewhat lower plate counts, but not so low as either mycological or PDA agars.

(2) The Effects of Heat and Light on Recovery.

The per cent survivors of those cells illuminated without the presence of the glass plate to absorb heat was fairly constant. There were only 1.3 per cent difference after 30 minutes of intense illumination.

The percentage of survivors of cells illuminated in the presence of the glass plate was variable. However, after an extended period of time, for example 30 minutes, the number of recoverable cells increased markedly to 4 per cent more than those illuminated in the absence of the plate (Table 2, Figure 9).

(3) The Effects of Ultraviolet Irradiation on Respiration.

A. Comparison of oxygen uptake by fresh and starved cells.

Freshly harvested and washed cells with glucose added as the

TABLE 1

RECOVERY OF VISIBLE COLONIES ON VARIOUS MEDIA

Media	Controls		Irradiated		Photoreactivated @ 37C		Photoreactivated @ 8 C	
	No. of Colonies	% Survivors	No. of Colonies	% Survivors	No. of Colonies	% Survivors	No. of Colonies	% Survivors
Blood	966	100	282	29.1	306	31.6	311	32.2
BHI	900	100	178	19.8	133	14.8	203	22.6
Eugon	767	100	127	16.5	142	18.5	190	24.6
F. SAB	883	100	126	14.2	101	11.3	187	21.2
Myco.	767	100	75	9.8	100	13.0	123	16.0
PDA	450	100	47	10.4	50	11.1	70	15.5
SAB	900	100	150	16.6	161	17.9	180	20.0
SAB "80"	833	100	149	17.8	163	19.6	175	21.0

TABLE 2

THE EFFECT OF HEAT ON PHOTOREACTIVATION

Time	No Glass Plate		Glass Plate	
	No. of Colonies	% Survivors	No. of Colonies	% Survivors
Control	900	100%	900	100%
IRR	111	12.3	101	12.1
30"	109	12.1	82.4	9.2
1'	93.4	10.4	38.6	4.3
3'	96.6	10.7	98.4	10.9
5'	90.0	10.0	70.6	7.8
7.5'	94.0	10.5	79.0	8.8
10.0'	93.6	10.4	90.6	10.0
30	97.6	10.8	131.6	14.6

substrate had an oxygen uptake 22.9 mu 1 greater than those with saline (endogenous) after 2 hours. Cells which were starved for 7 days had an uptake 19.0 mu 1 greater with glucose than endogenously.

B. Comparison of oxygen uptake by normal cells, irradiated cells and irradiated and illuminated cells with glucose.

After 2 hours, freshly harvested unirradiated cells had an oxygen uptake 20.6 mu 1 greater than similar cells which were irradiated and wrapped with foil (not reactivated). The normal cells had only 1.2 mu 1 greater uptake than those irradiated and illuminated. Those irradiated and illuminated had an oxygen uptake 19.4 mu 1 greater than those irradiated and wrapped in foil.
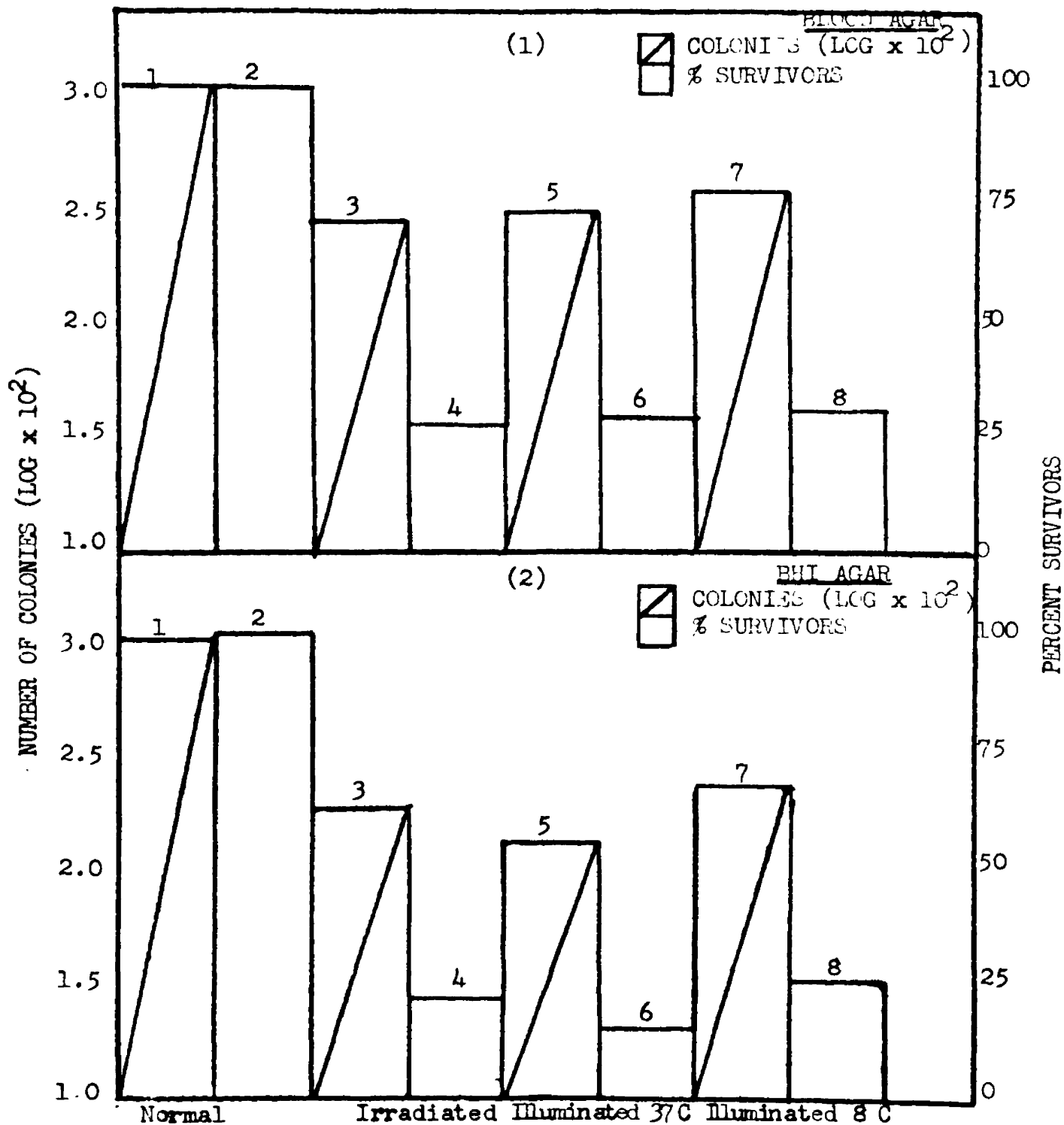
FIGURES I AND 2

The effects of different media on the growth of normal and treated cells: Columns 1&2-normal cells, columns 3&4-irradiated cells, columns 5&6-illuminated at 37 C, columns 7&8-illuminated at 8 C.
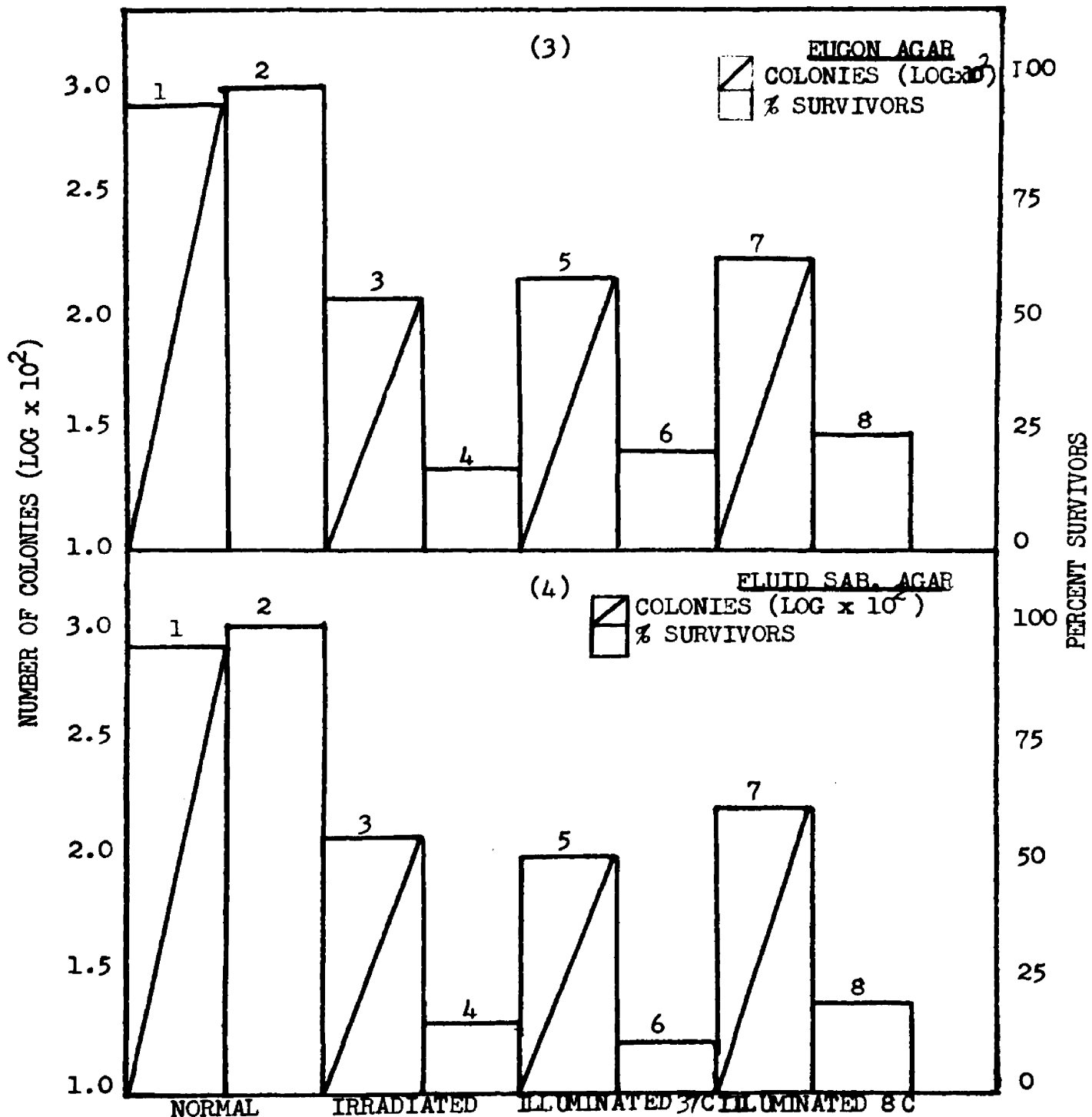
FIGURES 3 AND 4

The effects of different media on the growth of normal and treated cells: Columns 1&2-normal cells, columns 3&4-irradiated cells, columns 5&6-illuminated at 37 C, columns 7&8-illuminated at 8 C.
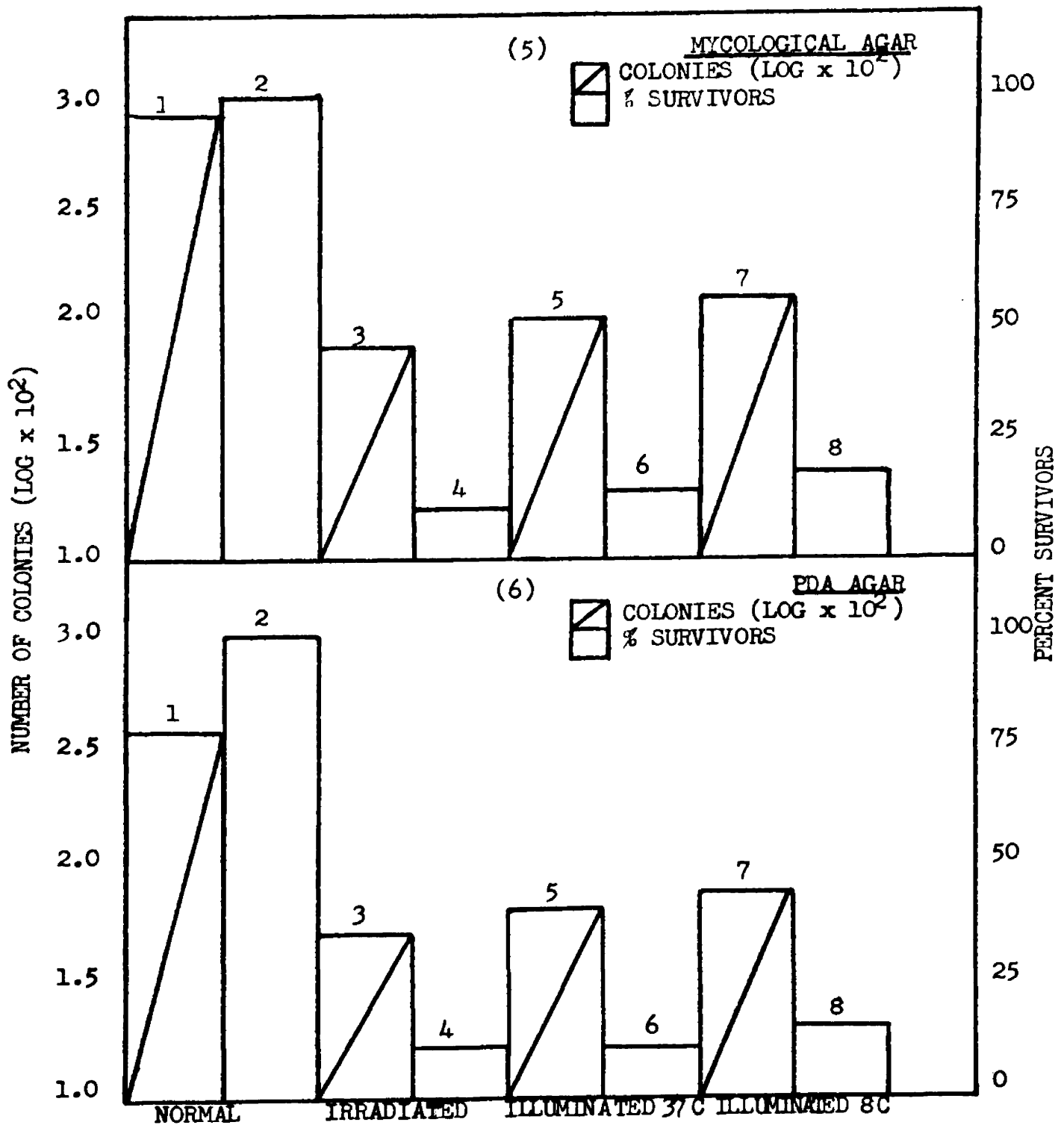
FIGURES 5 AND 6

The effects of different media on the growth of normal and
treated cells: Columns 1&2-normal cells, columns 3&4-irradiated
cells, columns 5&6-illuminated at 37 C, columns 7&8-illuminated
at 8 C.
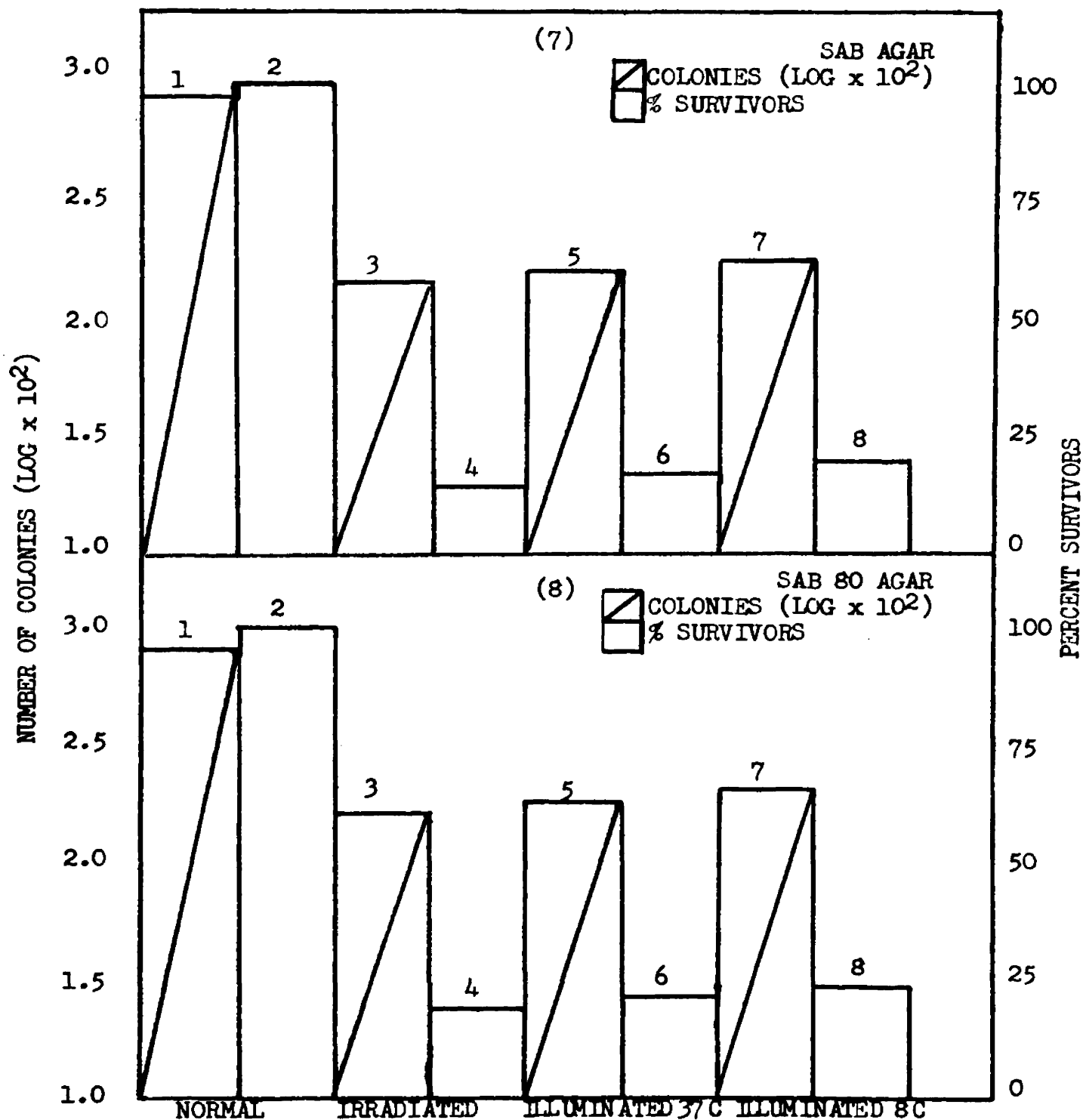
FIGURES 7 AND 8

The effects of different media on the growth of normal and treated cells: Columns 1&2-normal cells, columns 3&4-irradiated cells, columns 5&6-illuminated at 37 C, columns 7&8-illuminated at 8C.
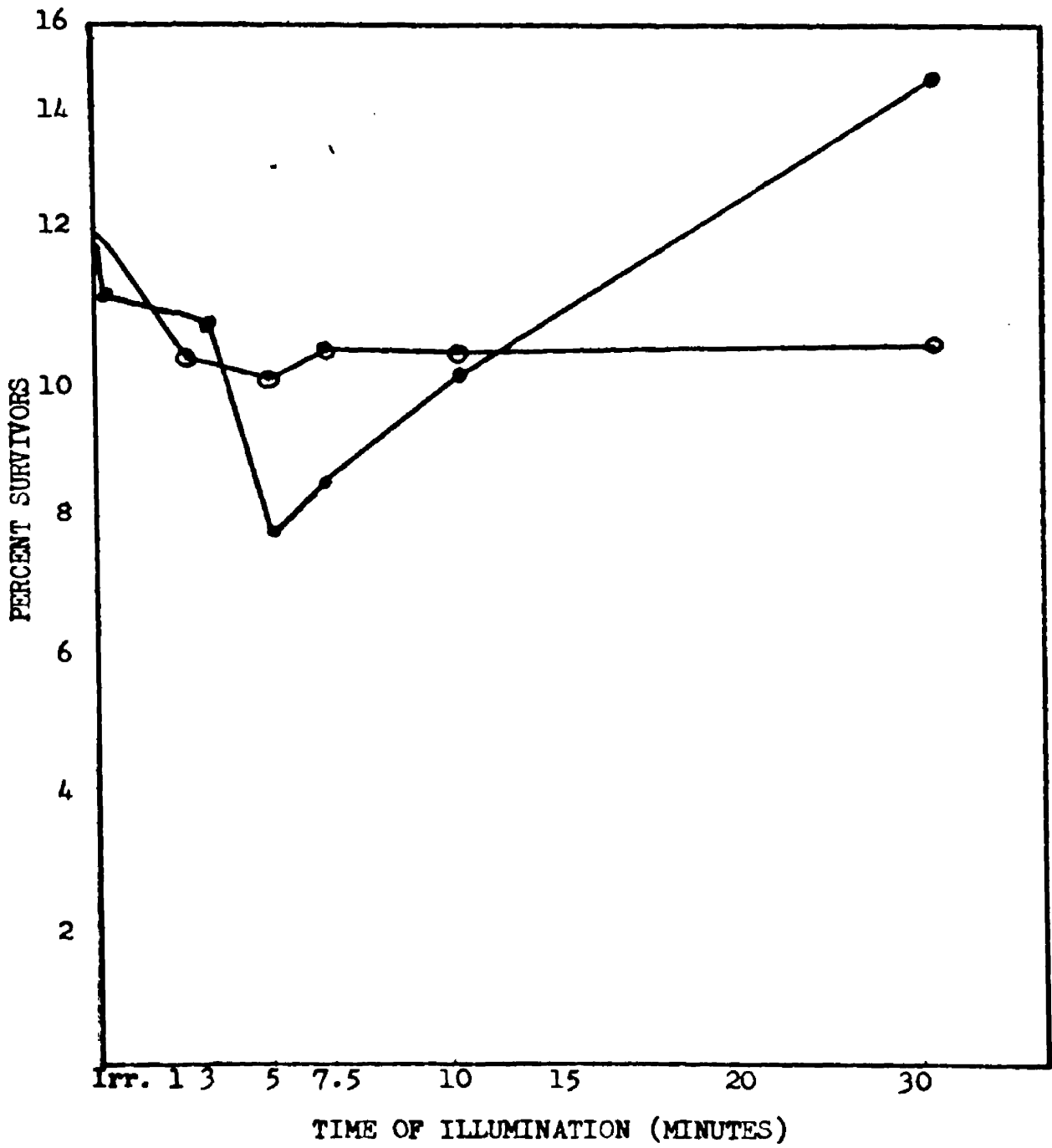
FIGURE 9

The effect of heat and light on recovery: ●–heat absorbed,
○–no absorbtion of heat

C. Comparison of oxygen uptake by normal starved cells, irradiated

   starved cells, and irradiated and illuminated starved cells with

   glucose.

   After 2 hours the starved unirradiated cells had an uptake 19.0

mu 1 greater than those irradiated and wrapped in foil and 19.1 mu 1

greater than those irradiated and illuminated. Illumination had no

apparent effect on the respiration of the irradiated starved cells

since it differed only 0.1 mu 1 from the non-illuminated cells.

D. Overall comparison between fresh and starved cells (Table 3,

   Figures 10-13).

   The respiration rates for fresh and starved cells were approxi-

mately equal with glucose as the substrate. The endogenous respiration

rates were also approximately equal. These endogenous rates were one-

half that of cells with glucose added as substrate. The oxygen uptake

was almost equal for both types of cells after they were irradiated,

and they demonstrated equal decreases in uptake from those cells which

were unirradiated. The fresh cells showed a marked increase in uptake

after illumination, but the starved cells remained unchanged from the

irradiated cells.

(4) Chromatography and the Identification of Amino Acids with Rf Values

A. A comparison between previously known and experimental Rf values

   with various solvents. (Reference solutions were Nutritional

   Biochemicals Corporation).

   1. Phenol (Table 4).

   For the most part, those Rf values determined in this experi-

TABLE 3

UL O_2 UPTAKE (An Average of 3 Experiments)

Time	Fresh Unirradiated Glucose	Fresh No. U.V. Endogenous	Fresh IRR Wrapped Glucose	Starved No. U.V. Glucose	Starved No. U.V. Endogenous	Fresh IRR Photo Glucose	Starved IRR Wrapped Glucose	Starved IRR Photo Glucose
10	1.52	0.47	0	1.0	0	1.50	0	0.5
20	5.52	2.6	3.6	3.0	2.6	4.9	2.9	2.8
30	8.32	4.8	7.7	5.4	4.1	7.4	5.0	4.9
40	9.82	6.2	8.4	8.4	5.1	9.4	7.1	8.3
50	14.85	6.7	10.2	12.6	6.6	12.3	7.6	8.9
60	20.15	7.7	12.6	18.3	10.3	16.8	11.4	11.3
1 Hr. Total	20.15	7.7	12.6	18.3	10.3	16.8	11.4	11.3
70	23.90	9.6	13.8	23.2	13.8	21.7	14.1	13.5
80	27.0	13.5	17.4	26.6	17.8	26.4	16.5	15.7
90	31.7	17.2	20.5	30.6	19.8	30.9	19.0	18.6
100	36.5	20.1	22.8	36.5	22.2	35.5	21.5	20.6
110	41.1	21.2	23.3	39.5	22.9	45.3	23.7	22.8
120	46.5	23.6	25.9	44.6	25.6	45.3	25.6	25.5
2 Hr. Total	46.5	23.6	25.9	44.6	25.6	45.3	25.6	25.5

52
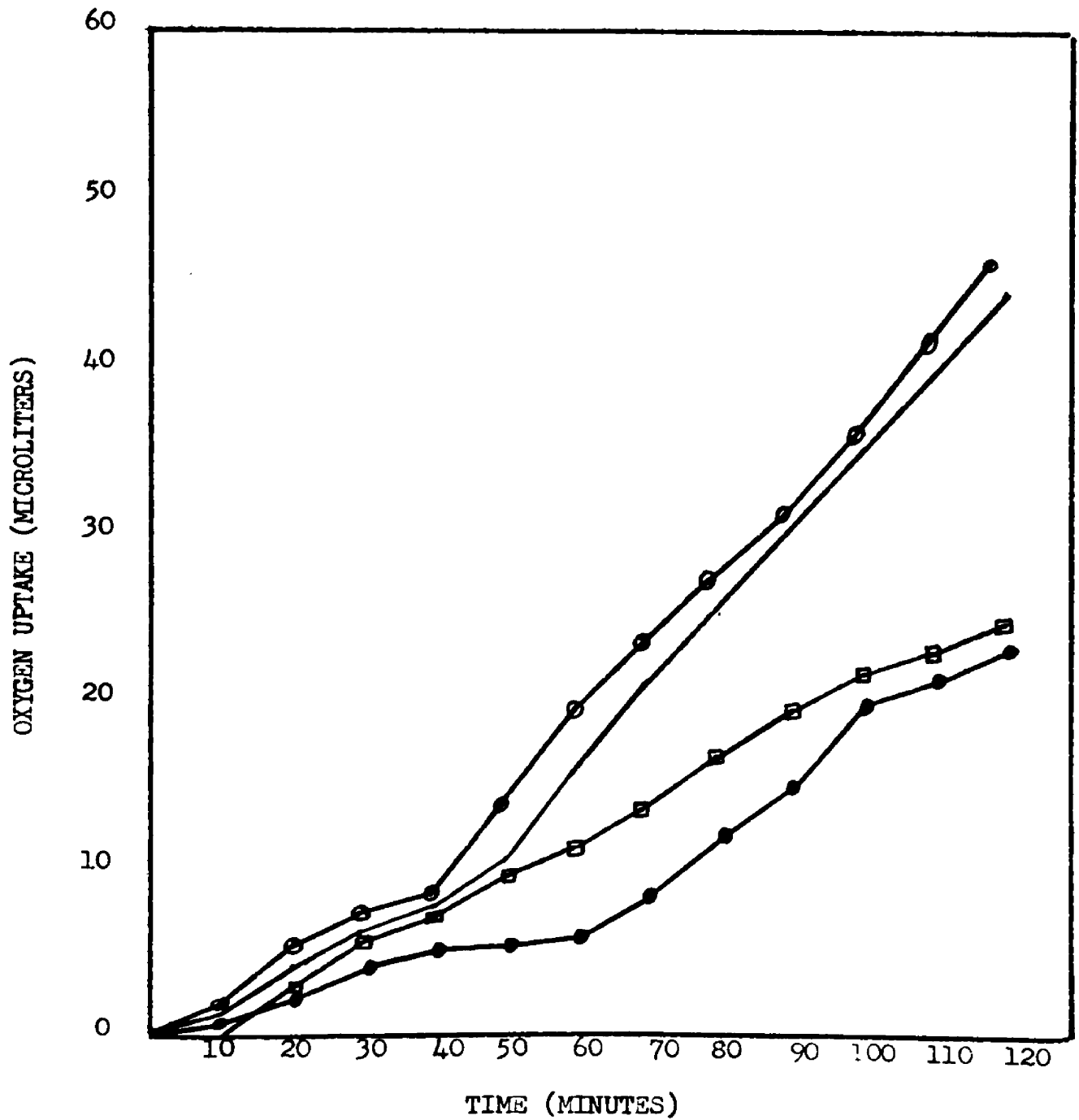
FIGURE 10

Respiration of fresh Blastomyces dermatitidis: O -normal with glucose, ●-normal endogenous, ▫-irradiated with glucose, ▬-irradiated, illuminated with glucose.
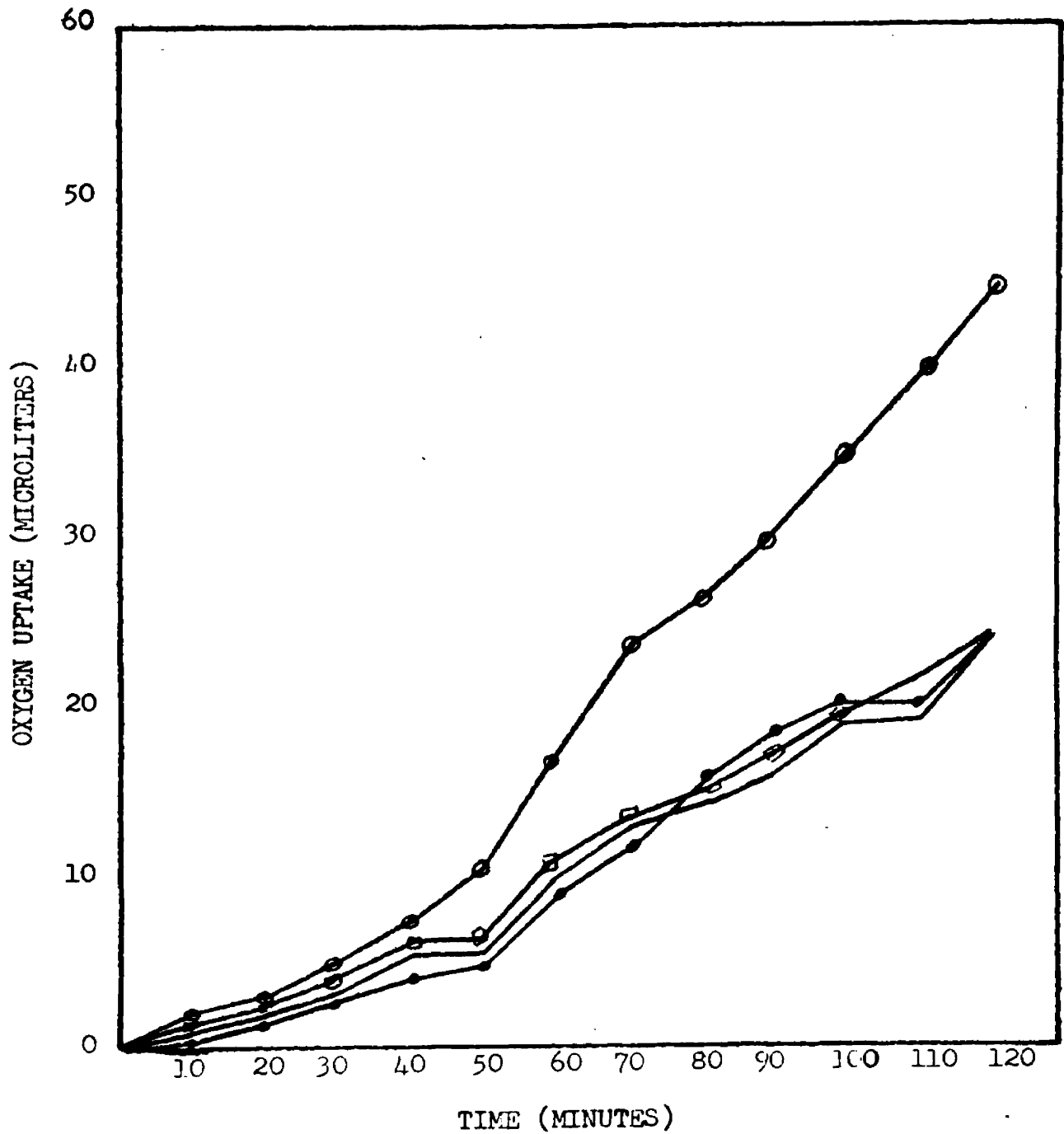
FIGURE II

Respiration of starved Blastomyces dermatitidis: o -normal
with glucose, ● -normal endogenous, �‫ -irradiated with glucose,
━ irradiated, illuminated with glucose
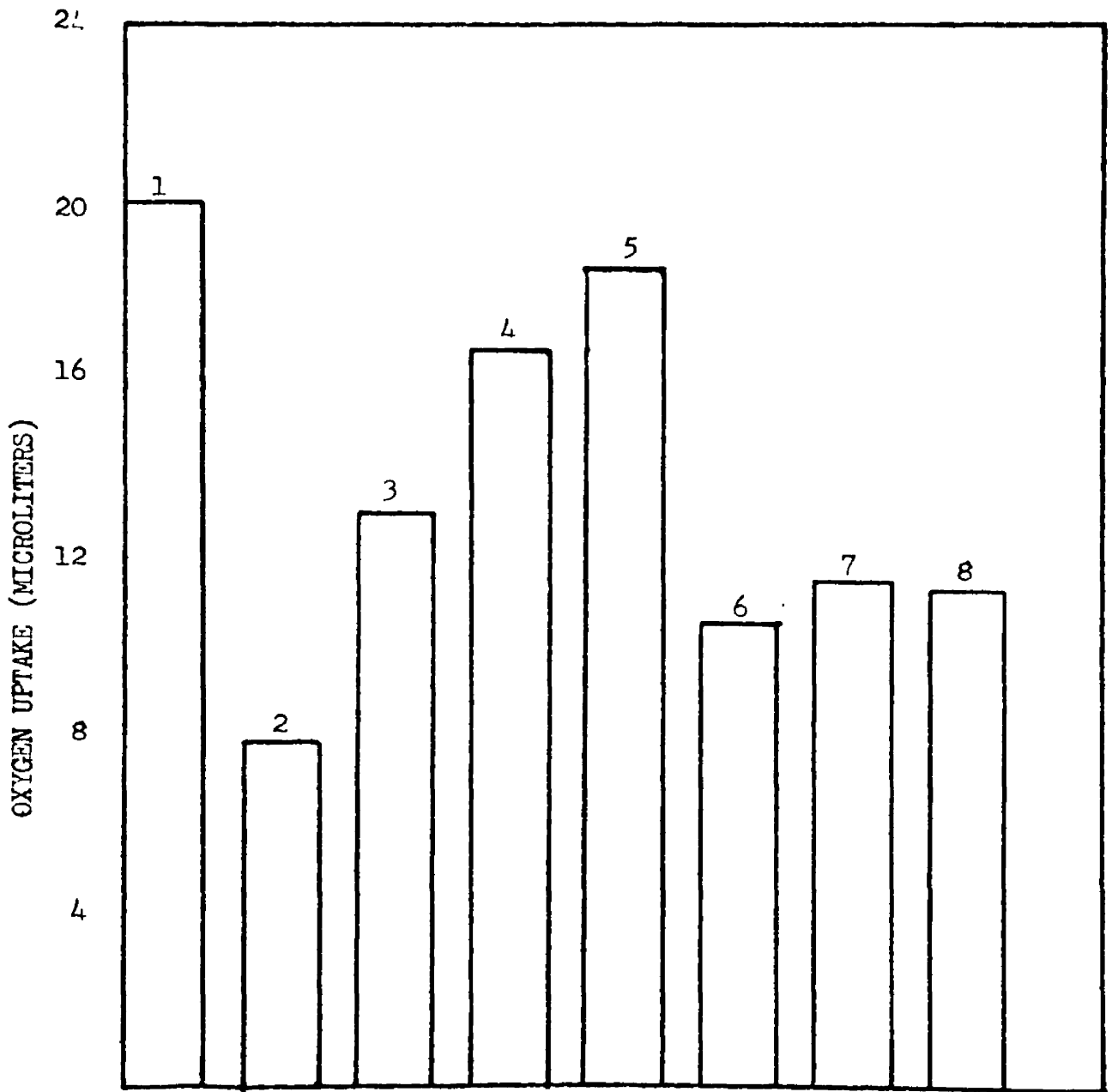
FIGURE 12

Oxygen uptake (microliters) in one hour: 1-fresh normal
with glucose, 2-fresh endogenous, 3-fresh ultraviolet with
glucose, 4-fresh ultraviolet illuminated with glucose,
5-starved normal with glucose, 6-starved endogenous,
7-starved ultraviolet with glucose, 8-starved ultraviolet
illuminated with glucose.
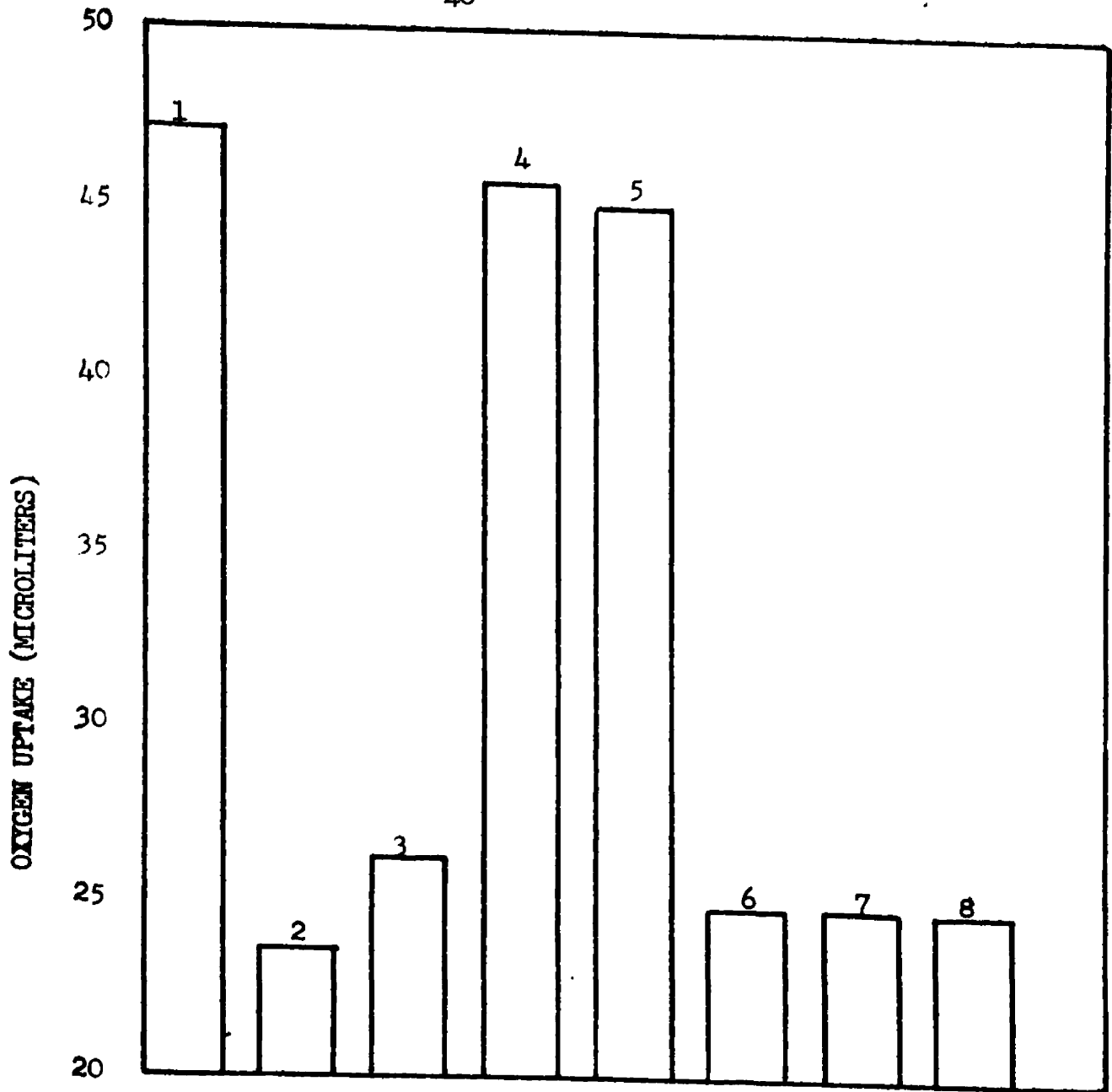
FIGURE 13

Oxygen uptake (microliters) in two hours: 1-fresh normal with glucose, 2-fresh endogenous, 3-fresh ultraviolet with glucose, 4-fresh ultraviolet illuminated with glucose, 5-starved normal with glucose, 6-starved endogenous, 7-starved ultraviolet with glucose, 8-starved ultraviolet illuminated with glucose.

TABLE 4

PHENOL AS A SOLVENT

Amino Acid	Reference Rf	Experimental Rf
1. L-Alanine	.58	.58
2. DL-Valine	.78	.79
3. DL-Isoleucine	.85	.83
4. L-Glutamic Acid	.33	.31
5. Glycine, Anhydrous	.42	.44
6. DL-Phenylalanine	.84	.87
7. L-Tyrosine	.60	.61
8. L-Proline	.90	.69
9. L-Cysteine (free base)	.22	.30
10. L-Histidine Monohydrochloride	.65s	.66

mental investigation were similar to those established by Smith (1958). However, phenol was not used as a solvent because of the heavy streaking it produced and its slowness of descent.

2. Pyridine - acetic acid - water (Table 5).

This solvent was very good for separation of the amino acids. The experimental Rf values were approximately 0.6 less than values previously reported (Block et al., 1958).

3. Butanol - ammonia (Table 6).

This solvent did not produce an adequate spread of the amino acids and proper determinations of Rf values were almost impossible (Smith, 1958).

TABLE 5

PYRIDINE - ACETIC ACID - WATER AS A SOLVENT

Amino Acid	Reference Rf	Experimental Rf
1. L-Alanine	.57	.46
2. DL-Valine	.70	.65
3. DL-Isoleucine	.65	.69
4. L-Glutamic Acid	.47	.42
5. Glycine, Anhydrous	.41	.29
6. DL-Phenylalanine	.73	.69
7. L-Tyrosine	.65	?
8. L-Proline	.55	.49
9. L-Cysteine (free base)	?	.15
10. L-Histidine Monohydrochloride	.37	.26

4. Butanol - pyridine - water (Table 7).

Of all the solvents used for one-dimensional descending chromatography, this was the best. The spread of the amino acids was excellent and the specific amino acids could be easily established since the experimental values were nearly the same as those reported by Smith (1958).

B. Amino acids identified with the 2-dimensional solvent system of butanol - pyridine - water and phenol - ammonia. (Reference solutions were Nutritional Biochemicals Corporation).

The 2-dimensional system with phenol was of some value in identification of 2 or 3 amino acids but the chromatograms were generally

TABLE 6

BUTANOL - AMMONIA AS A SOLVENT

Amino Acids	Reference Rf	Experimental Rf
1. L-Alanine	.09	.06
2. DL-Valine	.22	.17
3. DL-Isoleucine	?	.26
4. L-Glutamic Acid	?	.004
5. Glycine, Anhydrous	?	.03
6. DL-Phenylalanine	.46	.29
7. L-Tyrosine	.14	.12
8. L-Proline	?	.095
9. L-Cysteine (free base)	?	.007
10. L-Histidine Monohydrochloride	?	.04

very streaked and the descent never produced an even front. After development, the phenol left the paper dark brown which interferred with the identification of the spots which were very light.

C. Amino acids identified with the 2-dimensional solvent system of n - butanol - acetic acid - water and n-butanol - methyl ethyl ketone - water (Table 8).

This system for 2-dimensional paper chromatography was excellent since it alleviated the problems which arose with the phenol solvent system. The color reactions obtained were very exact and the spots, as compared to the map prepared by Mizell and Simpson (1961), were comparable in position (position implies Rf value).

TABLE 7

BUTANOL - PYRIDINE - WATER AS A SOLVENT

Amino Acid	Reference Rf	Experimental Rf
1. L-Alanine	.37	.31
2. DL-Valine	.48	.44
3. DL-Isoleucine	.56	.55
4. L-Glutamic Acid	.20	.15
5. Glycine, Anhydrous	.29	.23
6. DL-Phenylalanine	.63	.57
7. L-Tyrosine	.60	.55
8. L-Proline	.34	.33
9. L-Cysteine (free base)	.14	.11
10. L-Histidine, Monohydrochloride	.24	.21
11. L-Citrulline	.23	.19
12. L-Aspartic Acid	.20	.12
13. L-Cystine	.15s	.09
14. L-Arginine, Monohydrochloride	.15	.12
15. L-Asparagine	.20	.15
16. DL-Ornithine, Monohydrochloride	.11	.08

TABLE 8

TWO-DIMENSIONAL CHROMATOGRAPHY TO REPLACE PHENOL:
(n-butanol-acetic acid-water, and n-butanol-methyl ethyl ketone-water)

Amino Acid	Reference Rf[*]	Experimental Rf	Color
1. Glutamic Acid	.12	.17	Purple
2. Aspartic Acid	.11	.16	Blue
3. Arginine	.07	.12	Purple
4. Lysine	.09	.14	Purple
5. Asparagine	.15	.20	Beige
6. Glycine	.18	.24	Purple
7. Histidine	.25	.30	Grey-Green
8. Serine	.26	.31	Purple
9. Alanine	.22	.27	Purple
10. Proline	.24	.28	Yellow
11. Valine	.42	.47	Purple
12. Methionine	.46	.51	Purple
13. Tyrosine	.43	.48	Grey
14. Isoleucine	.54	.60	Purple
15. Leucine	.57	.62	Purple

[*] Mizell and Simpson (1961).

TABLE 9

RF VALUES ESTABLISHED FOR BLASTOMYCES DERMATITIDIS WITH
ONE AND TWO-DIMENSIONAL CHROMATOGRAPHY

Amino Acid	1-Dimensional		2-Dimensional	
	Solvent	Rf	Solvent	Rf
1. Proline	Bu-P-H$_2$O	.34	Mizell	.28
2. Asparagine	Bu-P-H$_2$O	.15	Mizell	.20
3. Aspartic Acid	Bu-P-H$_2$O	.22	Mizell	.16
4. Alanine	Bu-P-H$_2$O	.33	Mizell	.27
5. Valine	Bu-P-H$_2$O	.45	Mizell	.47
6. Glutamic Acid	Bu-P-H$_2$O	.17	Mizell	.17
7. Arginine	Bu-P-H$_2$O	.12	Mizell	.12
8. Histidine	Bu-P-H$_2$O	.21	Mizell	.30
9. Ornithine	Bu-P-H$_2$O	.08	Smith	.13
10. Citrulline	Bu-P-H$_2$O	.19	Smith	.25
11. Cystine	Bu-P-H$_2$O	.09	Smith	.15s
12. Cysteine	Bu-P-H$_2$O	.11	Smith	.14
13. Serine			Mizell	.31
14. Glycine	Bu-P-H$_2$O	.25	Mizell	.24
15. Threonine			Smith	.38
16. Taurine			Smith	.40
17. Tyrosine			Mizell	.48
18. Lysine			Mizell	.14
19. Isoleucine			Mizell	.60
20. Leucine			Mizell	.62
21. Methionine			Mizell	.51

D. Various concentrations of amino acids observed in the investigations with paper chromatography.

Different concentrations of various amino acids were qualitatively observed with the unaided eye throughout this investigation. Glutamic acid and alanine always provided the most dense spots. Following these in concentration were aspartic acid, valine and glycine. All the other amino acids occurred in lower concentration and produced spots of nearly equal density.

(5) Amino Acids Identified and Those Not Detectable.

A. Amino acids identified in B. dermatitidis and the methods utilized for their demonstration.

1. Proline

   a. yellow with ninhydrin
   b. light red with vanillin
   c. blue with isatin
   d. Rf value with 2-dimensional and one-dimensional chromatograms.

2. Asparagine

   a. light brown with ninhydrin
   b. Rf value with 2-dimensional and one-dimensional chromatograms
   c. light brown on 2-dimensional chromatogram

3. Aspartic acid

   a. blue with ninhydrin
   b. Rf value with 2-dimensional and one-dimensional chromatography
   c. blue on 2-dimensional chromatogram.

4. Alanine

   a. purple with ninhydrin
   b. Rf value with 2-dimensional and one-dimensional chromatography
   c. purple on 2-dimensional chromatogram

5. Valine

   a. purple with ninhydrin
   b. Rf value with 2-dimensional and one-dimensional chromatography
   c. purple on 2-dimensional chromatogram

6. Glutamic acid

   a. purple with ninhydrin
   b. Rf value with 2-dimensional and one-dimensional chromatography
   c. purple on 2-dimensional chromatogram

7. Arginine

   a. red on yellow background with sulfanilic reagent
   b. pink with 8-hydroxyproline
   c. pink with alpha-napthol
   d. Rf value with 2-dimensional and one-dimensional chromatography
   e. purple on 2-dimensional chromatogram

8. Histidine

   a. red on yellow background with sulfanilic reagent
   b. green with o-pthalaldehyde
   c. Rf value with 2-dimensional and one-dimensional chromatography
   d. grey-green on 2-dimensional chromatogram

9. Ornithine

   a. yellow brown with vanillin
   b. Rf value with 2-dimensional and one-dimensional chromatography

10. Citrulline

   a. yellow with Ehrlich's reagent
   b. Rf value with 2-dimensional and one-dimensional chromatography

11. Cystine

   a. purple with sodium nitroprusside
   b. immediate blue with isatin
   c. Rf value with 2-dimensional and one-dimensional chromatography

12. Cysteine

   a. (as cystine)

13. Serine

    a. yellow with $NaIO_4$ and Nessler's reagent
    b. Rf value on 2-dimensional chromatogram
    c. purple on 2-dimensional chromatogram

14. Glycine

    a. green with o-pthalaldehyde
    b. Rf value with 2-dimensional and one-dimensional chroma-
       tography
    c. purple on 2-dimensional chromatogram

15. Threonine

    a. yellow with $NaIO_4$ and Nessler's reagent
    b. Rf on 2-dimensional chromatogram

16. Taurine

    a. pale red with o-pthalaldehyde
    b. Rf on 2-dimensional chromatogram

17. Tyrosine

    a. blue with isatin
    b. Rf on 2-dimensional chromatogram
    c. gray on 2-dimensional chromatogram

18. B-alanine

    a. blue with isatin, faded with Ehrlich's reagent on ninhy-
       drin developed chromatogram

19. Lysine

    a. Rf on 2-dimensional chromatogram
    b. purple on 2-dimensional chromatogram

20. Isoleucine

    a. Rf on 2-dimensional chromatogram
    b. purple on 2-dimensional chromatogram

21. Leucine

    a. Rf on 2-dimensional chromatogram
    b. purple on 2-dimensional chromatogram

22. Methionine

    a. Rf on 2-dimensional chromatogram
    b. purple on 2-dimensional chromatogram

B. Amino acids definitely absent from B. dermatitidis in concentrations with the methods utilized.

   1. Tryptophan

      a. no spot with o-pthalaldehyde
      b. no red with isatin
      c. no new spot with Ehrlich's reagent

   2. Hydroxyproline

      a. spot fades with Ehrlich's reagent after ninhydrin; no purple red color

   3. Thiolhistidine

      a. no color with sulfonilic acid
      b. no spot with 2-dimensional chromatograph

   4. Sarcosine

      a. no red with vanillin

   5. Phenylalanine

      a. no blue after ninhydrin and dilute $NaHCO_3$
      b. no spot on 2-dimensional chromatograph

(6) The Effects of Ultraviolet Irradiation on Amino Acids.

Since glutamic acid, valine, alanine, aspartic acid, and glycine were readily distinguishable by previous Rf values, color reactions, densities, and separation, they were used to determine the effects of ultraviolet irradiation on the cells.

A. The effect of 20 minutes of ultraviolet irradiation.

Following a 20-minute exposure to ultraviolet, all 5 of the above-mentioned amino acids produced spots less dense than those of the control. Aspartic acid, glycine, and glutamic acid concentrations were the least affected. Alanine was extremely light and the valine spot

was almost undetectable. Also, there appeared to be better separation of amino acids from those cells which had been previously treated with ultraviolet light.

B. Amino acids not present in irradiated cells as demonstrated by 2-dimensional paper chromatography.

On 2-dimensional chromatograms, 8 amino acids were demonstrated from normal cells which were not found in irradiated cells (Table 10).

C. A comparison of the amino acids of freshly harvested normal cells with those which were non-irradiated and starved, irradiated and starved, and irradiated but not starved.

An appraisal was made of the amino acid concentrations observed on one-dimensional chromatograms. All of the amino acids were taken into consideration, but if any difficulty arose in determining which were the denser spots, glutamic acid was used as the key since its spot was the heaviest and the most easily distinguished. As was anticipated, the amino acids of non-irradiated, non-starved cells were the most concentrated. Spots from irradiated, non-starved cells were less dense than the control. When irradiated cells were starved at 37 C the amino acid content decreased with time.

D. A comparison of amino acids in irradiated cells and irradiated, illuminated cells.

No qualitative difference could be seen between the 2 types of cells. Glutamic acid may have been slightly darker in the illuminated cells.

TABLE 10

AMINO ACIDS NOT PRESENT IN IRRADIATED CELLS AS DEMONSTRATED
BY 2-DIMENSIONAL PAPER CHROMATOGRAPHY

Control	Irradiated	Not Present After Irradiation
1. Cysteine	1. Cysteine	1. Asparagine
2. Aspartic Acid	2. Aspartic Acid	2. Taurine
3. Glutamic Acid	3. Glutamic Acid	3. Theronine
4. Cystine	4. Cystine	4. Tyrosine
5. Asparagine	5. Glycine	5. Ornithine
6. Serine	6. Serine	6. Citrulline
7. Glycine	7. Alanine	7. Histidine
8. Taurine	8. Proline	8. Arginine
9. Threonine	9. Valine	
10. Tyrosine	10. Lysine	
11. Alanine		
12. Citrulline		
13. Ornithine		
14. Lysine		
15. Histidine		
16. Arginine		
17. Valine		
18. Proline		

(7) A Comparative Investigation of the Nucleic Acids in Normal, in Irradiated, and in Irradiated, Illuminated Cells.

A. The RNA and DNA concentrations of normal unirradiated cells (Tables 11-12, Figures 14-15).

The average concentration of RNA in unirradiated B. dermatitidis was 73.5 mu g/ mg dry weight. The average amount of DNA in unirradiated control cells was 7.0 mu g/mg dry weight.

B. The effect of ultraviolet irradiation on the concentrations of RNA and DNA (Tables 11-12 and Figures 14-15).

Ultraviolet irradiation caused a definite decrease in the concontrations of both RNA and DNA. The longer the ultraviolet light was administered, the greater the decrease in concentration of RNA. After 5 minutes the concentration of RNA dropped to 68.8 mu g/mg dry weight and to 55.1 mu g/mg dry weight after one hour. These concentrations differed by as much as 19.9 mu g/mg dry weight from normal unirradiated cells and represents about 26 per cent reduction from the initial concentration.

DNA showed an irregular but similar decrease in concentration following ultraviolet irradiation. The concentrations after 5, 15, and 30 minutes were 5.2, 5.4 and 5.3 mu g/mg dry weight, respectively. After one hour the concentration had dropped to 4.9 mu g/mg dry weight. These concentrations differed by as much as 2.1 mu g/mg dry weight from the unirradiated cells, and represent about 30 per cent reduction from the initial concentrations.

TABLE 11

THE CONCENTRATION* OF NUCLEIC ACIDS IN NORMAL, IRRADIATED,
AND IRRADIATED PHOTOREACTIVATED B. DERMATITIDIS

RNA				DNA			
Time	Control	U.V.	U.V. + P	Time	Control	U.V.	U.V. + P
5'	71.3	63.8	70.1	5' + 20'	7.0	5.2	6.5
15'	73.6	65.2	69.7	15' + 60'	7.0	5.4	6.2
30'	73.5	58.5	65.2	30' + 120'	7.0	5.3	5.9
60'	75.0	55.1	72.1	60' + 120'	7.0	4.9	6.7

*Mu g/mg dry weight.

C. The effect of illumination on nucleic acid concentrations in

irradiated cells (Tables 11-12, Figures 14-15).

When irradiated cellls were illuminated for a period equal to 4

times that of irradiation time, the RNA concentration in cells increased.

The amount of increase was indirectly proportional to the previous ef-

fects of the irradiation. Never did the amount of RNA recorded in

photoreactivated cells equal the controls. However, even after one

hour of irradiation, photoreactivation for 240 minutes increased the

level 17 mu g/mg dry weight and returned the RNA concentrations to 96

per cent of the normal.

DNA was also photoreactivable with concentrations increased as

much as 1.8 mu g/mg dry weight or 84 per cent of the normal value.

TABLE 12

MU G/MG DRY WEIGHT CHANGE OF NUCLEIC ACIDS IN
B. DERMATITIDIS

RNA					DNA				
Time	Con-trol	U.V.	U.V.+P	Ug loss after Photo	Time	Con-trol	U.V.	U.V.+P	Ug loss after Photo
5'	71.3	-2.5	+1.3	-1.2	5'+ 20'	7.0	-1.8	+1.3	-0.5
15'	73.6	-8.4	+4.5	-3.9	15'+ 60'	7.0	-1.6	+0.8	-0.8
30'	73.5	-15.0	+6.7	-8.3	60'+ 120'	7.0	-1.7	+0.6	-1.1
60'	75.0	-19.9	+17.0	-2.9	120'+ 240'	7.0	-2.1	+1.8	-1.3

(8) The Infectivity of B. Dermatitidis in Mice as Correlated with the Effect of Varied Doses of Ultraviolet Irradiation and the Concentrations of Cells.

A. Suspensions containing 1.5×10^5 cells per 0.5 ml.

Increased exposure of the cells to ultraviolet irradiation reduced the number of visible colonies recovered on SAB. When irradiated from zero to 11 minutes the concentration of cells was too heavy for adequate and accurate cell counts. After the 11-minute exposure the number of visible colonies decreased with the duration of the ultraviolet irradiation (Table 13, Figure 16).

These mice which were heavily infected were much different in physical appearance from the uninfected mice. These differences included a roughed fur, lesions, weeping eyes, slenderness, and a tendency to
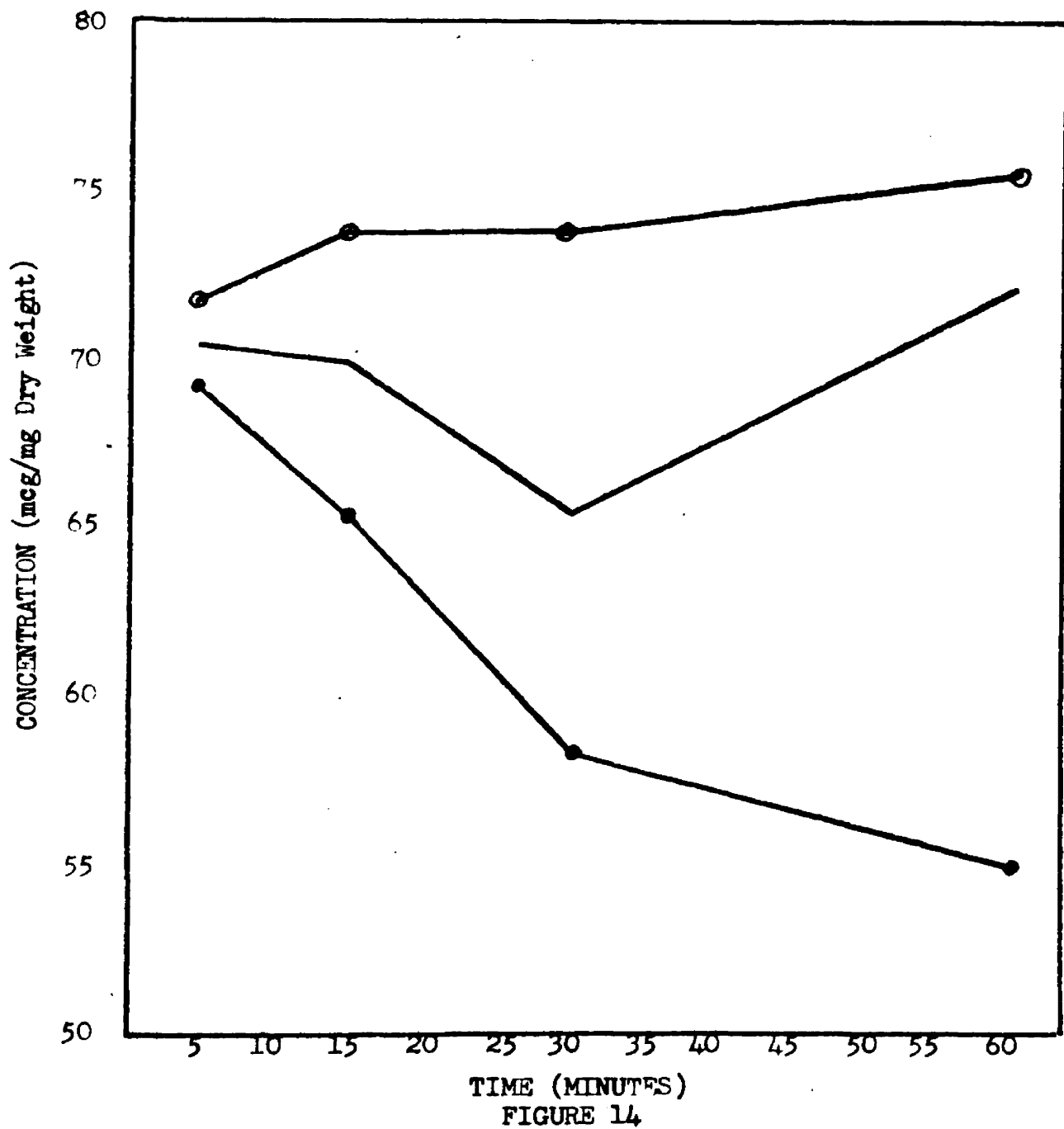
Concentration of RNA in normal, irradiated, and irradiated, illuminated Blastomyces dermatitidis: O -normal, ●-irradiated, — irradiated and illuminated
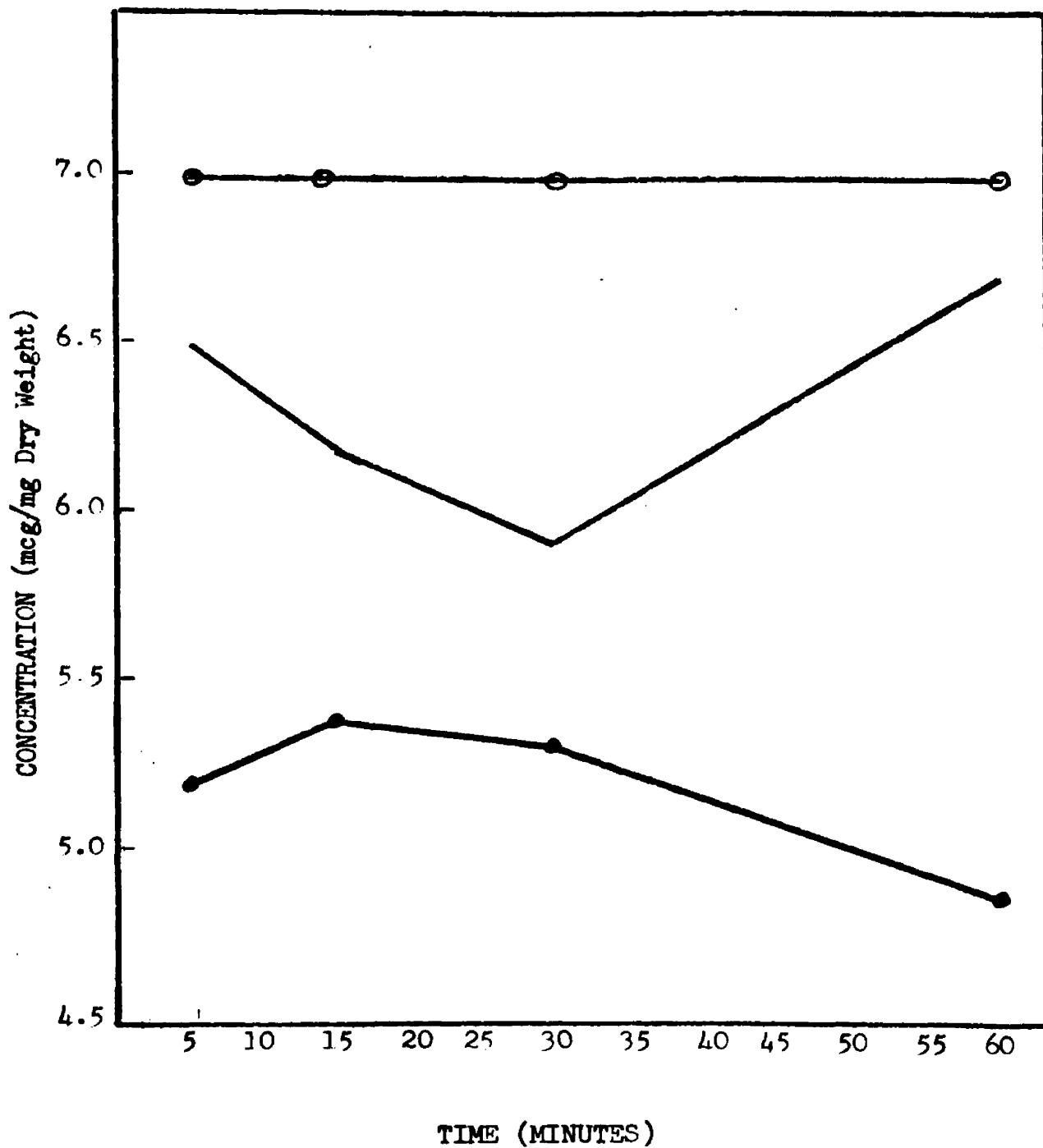
FIGURE 15

Concentration of DNA in normal, irradiated, and irradiated,
illuminated Blastomyces dermatitidis: O - normal, ●-irradiated,
━irradiated and illuminated

TABLE 13

VISIBLE COLONIES RECOVERED ON SAB AFTER VARYING
EXPOSURES OF ULTRAVIOLET

AVG. # COLONIES / 5 PLATES		U.V. 5' THROUGH 60'	
Time	1.5×10^5	1.0×10^5	0.5×10^5
Control	TMC	TMC	TMC
5'	TMC	5,106	3,717
8'	TMC	3,551	2,830
11'	TMC	2,651	2,328
14'	5,566	2,253	1,603
17'	3,817	1,085	1,266
20'	132	56	59
25'	64	5	25
30'	44	4	20
40'	26	7	8
60'	5	4	6

sleep continuously. (See Plate I).

The number of mice infected by intraperitoneal inoculation of
B. dermatitidis was also correlated with the duration of ultraviolet
exposure (Table 14). Four mice were infected with unirradiated cells.
Inocula exposed longer than 17 minutes did not produce infections in
this investigation.

There was a total of 14 mice infected in this investigation, 6
females and 8 males. These numbers were much lower than the investi-
gations which follow. Those SAB slants which were positive after
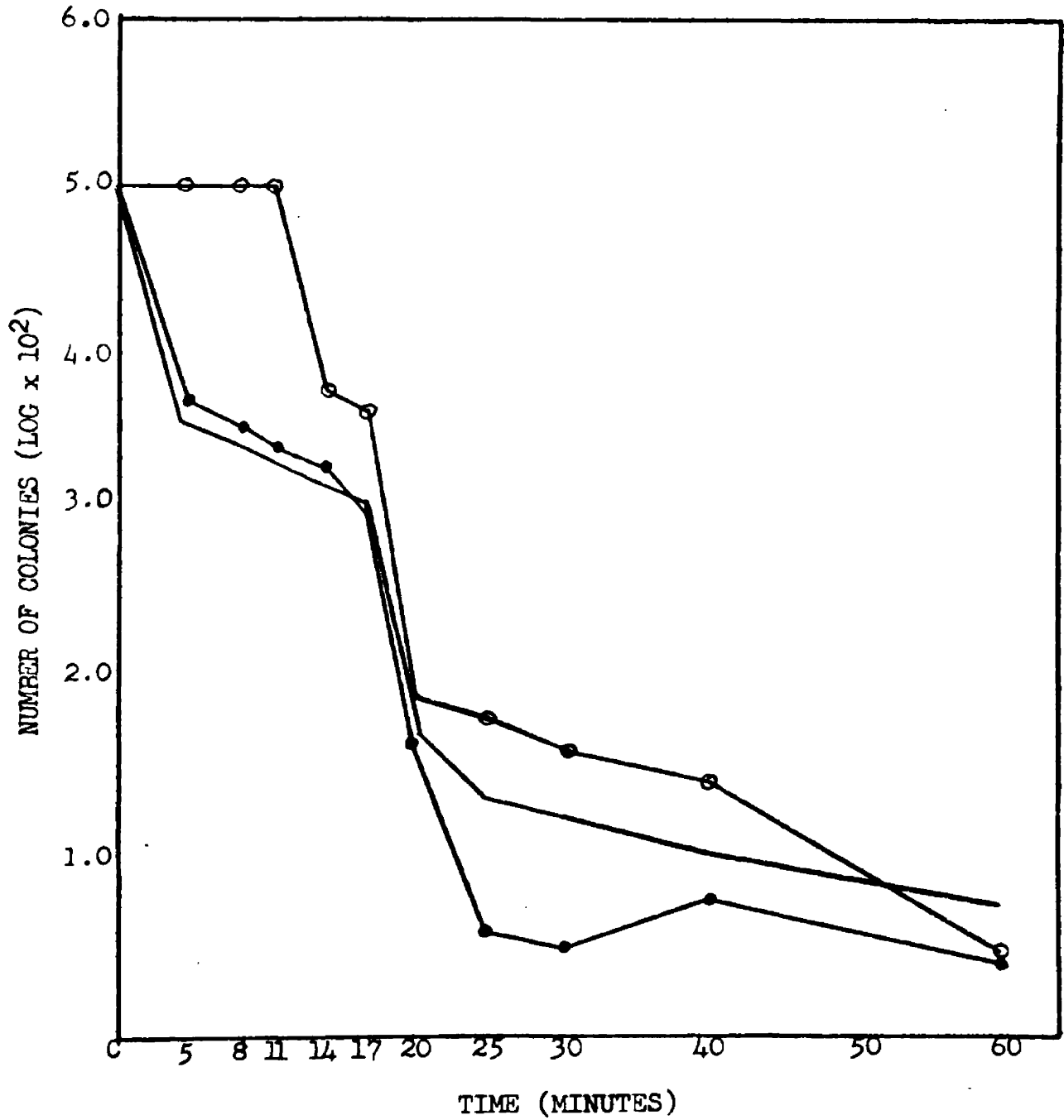
FIGURE 16

Visible colonies recovered on SAB after irradiation for various lengths of time: O·1.5 x 10^5, ●·1.0 x 10^5, —0.5 x 10^5

TABLE 14

MICE INOCULATED WITH 1.5×10^5 CELLS

Time	Mice from which Blastomyces was recovered on SAB	Mice showing lesions, Blastomyces not recovered on SAB	Mice not show-ing lesions on liver, Blasto-myces recovered on SAB	Total number of Mice infected by either lesions on liver or recov-ery on SAB
C	Male, 4	None	Male, 4	Male, 4
5'	Female, 3	None	Female, 3	Female, 3
8'	Male, e	None	Male, 1	Male, 3
11'	Female, 1	None	Female, 1	Female, 1
14'	None	None	None	None
17'	Female, 2 Male, 1	None	Female, 1	Female, 2 Male, 1
20'	None	None	None	None
25'	None	None	None	None
30'	None	None	None	None
40'	None	None	None	None
60'	None	None	None	None

inoculation with macerated liver and lung tissue became so between 5 and 17 days. After 17 days there were no additional positive slants.

B. Suspensions containing 1.0×10^5 cells per 0.5 ml.

As observed with the 1.5×10^5 concentration, the increased ex-posure to ultraviolet irradiation reduced the number of visible colonies recovered on SAB. The only colonies which were too concentrated to count were the controls. Increasing the exposure time from 5 minutes to 60 minutes produced decreasing numbers of colonies. As before, the

greatest single decrease in colony count was between 17 and 20 minutes (Table 13, Fibure 16).

The periods of irradiation between 5 and 17 minutes produced similar numbers of infection. Five mice were infected with normal cells, 3 mice after 5 minutes of exposure, 4 after 8 minutes, 4 after 11 minutes, 3 after 14 minutes and 5 after 17 minutes. Both 20- and 40-minute exposures infected 2 mice. If a colony recovered on SAB was formed by a single cell only and if other variables were considered, 7 cells were capable of producing an infection.

A total of 28 males were infected in this investigation, 9 males and 19 females (Table 15). As before, the slants of SAB which were positive became so after 5 to 17 days.

C. Suspensions containing 0.5×10^5 cells per 0.5 ml.

Results were similar to those observed in part B. The number of visible colonies recovered on SAB decreased with the length of ultra-violet irradiation. The greatest decline in colony count was as before, between the 17- and 20-minute exposures (Table 13, Figure 16).

Normal cells produced the greatest number of infections. Cells exposed between 5 and 25 minutes produced approximately the same number of infections. Two mice were infected by cells irradiated 30 minutes and only one by cells irradiated 40 minutes. If a colony on a SAB plate was produced by a single cell, as proposed previously 8 cells were capable of producing an infection. A total of 28 mice were infected, 10 males and 18 females (Table 16). All positive slants were recorded between 5 and 17 days.

TABLE 15

MICE INOCULATED WITH 1.0 x 10^5 CELLS

Time	Mice from which Blastomyces was recovered on SAB	Mice showing lesions on liver, Blastomyces not recovered on SAB	Mice not show-ing lesions on liver, Blasto-myces recovered on SAB	Total number of Mice infected by either lesions on liver or recovery on SAB
C	Female, 5	None	None	Female, 5
5'	Female, 1	Female, 2	Female, 1	Female, 3
8'	Male, 2	Male, 2	Male, 1	Male, 4
11'	Female, 2	Female, 2	None	Female, 4
14'	None	Male, 3	None	Male, 3
17'	Female, 2	Female, 3	None	Female, 5
20'	None	Male, 2	None	Male, 2
25'	None	None	None	None
30'	None	None	None	None
40'	Female, 1	Female, 1	None	Female, 2
60'	None	None	None	None

(9) Determination of the Minimal Infective Dose in Mice and the Rela-
tionship of Sex to Susceptibility.

Twenty-five thousand cells inoculated intraperitoneally into mice
produced an infection rate of 40 per cent in males and 80 per cent in
females. When similar inocula or their dilutions were plated on SAB
agar there were always fewer colonies formed than expected (Table 17).
Neither the number of cells counted in the inoculum nor the number of
colonies recovered on SAB gave indication of the percentage of infections

TABLE 16

MICE INOCULATED WITH 0.5×10^5 CELLS

Time	Mice from which Blastomyces was recovered on SAB	Mice showing lesions on liver, Blastomyces not recovered on SAB	Mice not showing lesions on liver, Blastomyces recovered on SAB	Total number of Mice infected by either lesions or recovery on SAB
C	None	Female, 5	None	Female, 5
5'	Male, 2	None	None	Male, 2
8'	Male, 2	Male, 2	None	Male, 4
11'	None	Female, 4	None	Female, 4
14'	Male, 1	Male, 1	None	Male, 2
17'	Female, 3	Female, 1	None	Female, 4
20'	None	None	None	None
25'	Female, 2	Female, 2	Female, 1	Female, 4
30'	None	Male, 2	None	Male, 2
40'	None	Female, 1	None	Female, 1
60'	None	None	None	None

(Table 17). For example, an inoculum which produced an average of 117 visible colonies on SAB after 5 days also produced an infection rate of 60 per cent. With another inoculum, which produced 390 colonies, the infection rate was only 30 per cent.

Female mice appeared to be much more susceptible than the males with all inocula.

TABLE 17

MINIMAL INFECTIVE DOSE AND SEX SUSCEPTIBILITY

Cells Counted in Inoculum	Colonies Recovered on SAB	Per cent of Males infected	Per cent of Females Infected
25,000	3,971	40	80
12,500	1,979	40	40
6,250	1,042	20	80
3,125	390	20	40
1,562	271	20	40
781	117	40	80
390	88	20	40
195	68	40	80
97	31	20	40
48	16	20	80
TOTALS		28	60

(10) <u>Mice Inoculations and the Effect of Illumination on Irradiated
Cells</u> (Table 18).

Illumination of irradiated cells caused an increase in the per-
centage of infections. Illuminations of irradiated cells resulted in an
increase of visible colonies formed on SAB. Female mice were more sus-
ceptible to unirradiated, irradiated, and irradiated, illuminated cells
than the males. In only one instance did the illuminated cells have as
high an infection rate as the normal cells. The length of exposure and

TABLE 18

THE EFFECT OF ILLUMINATION ON INFECTION

Treatment of Cells	Colonies Recovered on SAB			% Infected with control cells		% Infected with irradiated cells		% Infected With Irradiated and Illuminated Cells	
	C	U.V.	U.V. +ILL	Male	Female	Male	Female	Male	Female
17 Minutes Irradiation and 60 Minutes Illumination	T M C	64	100	80	100	40	60	60	80
20 Minutes Irradiation and 80 Minutes Illumination	T M C	44	85	60	80	40	60	60	80
25 Minutes Irradiation and 100 Minutes Illumination	T M C	34	96	80	100	40	80	60	80

and subsequent period of illumination did not affect the percentage of infections to any great extent.

CHAPTER V

DISCUSSION AND CONCLUSIONS

In any investigation of this type the methods employed will have
shortcomings. The determination of the number of cells present in any
given dilution and plate counts were the most probable sources of error.
The hemocytometer was utilized for cell counts since the average Blasto-
myces cell is approximately 5-7 mu or the same size as the average red
blood cell. This method has been itilized before by Salvin (1949),
Rowley and Huber (1955), and Young (1958). In the cell counts, if a
cell had a bud it was tabulated as 2 cells. It was not known if the
buds were dislodged from the cells while being processed in the Waring
blender. However, in this investigation it was noted that the number
of cells counted per ml never equalled the number of visible colonies
recovered on the culture plates. Previous work in this laboratory has
shown that one cell of Sporotrichum schenckii can produce one colony on
blood, SAB, BHI and eugon agars. SAB was used for all plate counts in
this investigation because it is a routinely recommended medium for the
cultivation of human pathogenic fungi. Other reasons for its use were
that it provided good growth, consisted of a relatively simple known
composition, and was convenient. Also, there was not enough blood to
make the quantity of blood agar plates required. Another possibility
for error in plate counts is that a typical Blastomyces colony is large,
rough, and irregular. Two cells may have grown in such a close proxim-
ity their separate detection was impossible. Also, since colony counts

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were made, for convenience, after 5 days of incubation more cells may have grown into colonies with a more prolonged incubation. Other disadvantages inherent in the methods employed will be discussed later.

The growth of B. dermatitidis varied a great deal on different media and the literature indicated that this should be expected.

TABLE 19

THE NITROGEN CONTENT OF VARIOUS MEDIA
USED FOR THE GROWTH OF B. DERMATITIDIS

Media	Nitrogen Source	Amount of N	Total Amount of Nitrogen	Ratio of Nitrogen to Carbon
1. Blood	Proteose peptone Veal heart infusion Defibrinated whole blood	1.437 g/1* 3.97 g/1 1.25 g/1	6.657 g/1	7.0
2. BHI	Bacto-peptone Calf brain infusion Beef heart infusion	1.616 .097 1.98	3.693 g/1	3.75
3. Eugon	Tryptose Soytone	1.5 g/1 1.3 g/1	2.8 g/1	0.85
4. SAB	Neopeptone	1.433 g/1	1.433 g/1	0.18
5. Fluid SAB	Casitone Peptamin	0.7 g/1 0.7 g/1	1.4 g/1	0.058
6. SAB 80	Neopeptone	1.433 g/1	1.433 g/1	0.18**
7. Mycological	Soytone	1.4 g/1	1.4 g/1	0.058
8. PDA	Potato infusion	0.4 g/1	0.4 g/1	0.03

*Nitrogen concentrations in trademarked products were taken from Difco Manual (1960). Other values were calculated from data in Hawk, Oser, and Summerson (1954).

**Lack of continuity in N/C ratio was the result of the incorporation of "Tween 80" which has been shown to be inhibitory (Gilardi and Laffer, 1962).

Although inorganic nitrogen sources were not used in this investigation for the growth of B. dermatitidis, Levine and Ordal (1946) grew the organism on a simple glucose ammonium salts medium. Salvin (1949) also found there was usually slight growth of B. dermatitidis on a medium containing ammonium salts as sole nitrogen source, but Gilardi and Laffer (1962) found ammonium salts capable of maintaining good growth of this organism.

Organic nitrogen was a more important factor in the growth of B. dermatitidis in this investigation. Organic nitrogen of an animal origin was apparently of much more value than that from a plant source. This could mean that the naturally occurring carbohydrates in soytone, for example, were either inhibitory to growth or else the plant peptides and proteins were hydrolyzed less easily.

Although Bullen (1949) maintained B. dermatitidis on a simple peptone medium, the ratio of available nitrogen to available carbon seemed to be the most important factor in the growth of this organism. The higher the ratio, the better the growth appeared to be. Blood agar, for example, contained the highest N/C ratio, in addition to a high total percentage of organic nitrogen, and whole blood as an enrichment factor and was the best medium for growth. This does not contradict the results of Salvin (1949) who stated that although growth of B. dermatitidis occurred with or without carbohydrate in the medium, the addition of 5 g of glucose per liter did increase the growth of yeast phase cells about 20 per cent.

The growth of irradiated cells was extremely variable on the different media. Basically, these media with the highest ratio of nitrogen to carbon were the best for growth. It was apparent that

even though the cells had been irradiated, they needed a rich medium

for growth and recovery from the ultraviolet irradiation. However,

this has not been the case with ultraviolet irradiated bacteria. Work

with these organisms has shown that growth on a rich medium was some-

what less than that on a so-called "suboptimal" medium. Wainwright and

Nevill (1955) stated that the number of survivors of E. coli was reduced

when ultraviolet irradiated cells were incubated on a peptone rather

than a poor medium. Alper and Gillies (1958a, 1958b) agreed by showing

that survival of irradiated E. coli B decreased if peptone was added

to nutrient agar and that oxoid blood agar (a rich medium) was poorer

for recovery than nutrient agar. These investigators believed that

inhibition of colony formation after irradiation was due in part to an

injury which led to imbalance in the synthetic processes of the cell,

so that "restoration" would be brought about by "suboptimal" growth

conditions. However, more recently, Weatherwax (1960) was observed

exactly the opposite result. He found that E. coli B grown on "sub-

optimal" media after irradiation were subject to starvation. This

starvation promoted the liberation from cells treated with ultraviolet

of a labile toxic material which killed neighboring cells (sensitized

by ultraviolet and starvation) which normally would have survived.

Since the generation time and metabolic rate of B. dermatitidis are

much slower than those of E. coli the richer media may have been of

more value. The imbalance to the synthetic processes brought about by

irradiation could have been restored in a much longer period and the

cells would not have needed the "suboptimal" condition for colony for-

mation. By the time they were ready to form colonies, the condition

of the cells could have been such as to warrant "optimal" conditions.

However, none of the media utilized to grow <u>Blastomyces</u> was considered to be "optimal" since even normal cells appeared to be inhibited to come extent. This was previously demonstrated in the differences between hemocytometer and plate colony counts. Even though the irradiation killed the more sensitive cells, those which survived were probably altered and no longer normal. For example, the SAB 80 medium was better for growth of irradiated than of normal cells. It was possible that the oleic acid was no longer inhibitory or that the cells were capable of utilizing it in their altered condition. Mycological agar and PDA were poor for the growth of both normal and of irradiated cells. These media had the lowest N/C ratio.

Thus, irradiated cells of <u>B. dermatitidis</u> were not inhibited by "suboptimal" conditions in this investigation. The inability of the ultraviolet irradiation to alter <u>completely</u> the conditions of the cell for growth could be explained by the multinuclear structure of each cell and the thickness of the cell wall. The irradiation may have damaged several of the nuclei, but if one remained unharmed it could still permit the cell to grow and require the same conditions for growth as before.

The recovery of cells photoreactivated at both 37 C and 8 C was best on blood agar. The other media varied in effectiveness according to the temperature of reactivation, but all the media provided a higher percentage of recovery following reactivation at 8 C than either the irradiated or the irradiated cells which were photoreactivated at 37 C. This was in contrast to the results of Kelner (1949) who stated that the rate of recovery of ultraviolet irradiated <u>Streptomyces</u> <u>griseus</u> increased directly with photoreactivation temperatures up to about 50 C.

He assumed that the substances which rendered the cell nonviable by irradiation were ozone and peroxides which were eliminated by decomposition was assumed to occur more rapidly at elevated temperatures. Buzzel (1956), who also investigated the effects of heat on photoreactivation of irradiated E. coli B, stated that the primary irradiation action was to transform cell metabolites into "poison". This "poison" had to diffuse away before heat could act on a sensitive recovery site. Since the cell wall and cell membrane of B. dermatitidis are so thick, it may be possible that the "poison" remained inside the cell, unable to diffuse out, and the heat could not act on the recovery site. Or, perhaps heat produced an additive effect to the irradiation damage, and illumination, regardless of its intensity, could have no effect. Rupert (1962) demonstrated this by showing that heat stabilized the complex formed by irradiation in the cell, and that the stabilized condition was eliminated by illumination. This produced repair and subsequent liberation of the photoreactivation enzyme. Previous experimentation with B. dermatitidis in this laboratory has shown it to be so sensitive to heat that exposure at 55 C for 15 minutes killed the cell suspensions tested. Since B. dermatitidis is so heat sensitive, it appeared that the complex formed by irradiation was stabilized by the 37 C temperature and that the 8 C temperature allowed elimination of this complex. The illumination at the lower temperature could then produce repair and liberation of the photoreactivating enzyme, which resulted in better growth on all media.

As before, those media with the highest ratios of nitrogen to carbon, the highest total concentrations of nitrogen, and the most accessory enrichment factors were the best for growth of B. dermatitidis.

Glucose increased the oxygen uptake by both fresh and starved cells of B. dermatitidis. The endogenous respiration of both types of cells was similar. Bernheim (1942) stated that washed suspensions of this organism oxidized glucose, mannose, lactate, and pyruvate when tested manometrically and that separate enzyme systems were involved. Nickerson (1949) found that B. dermatitidis demonstrated exogenous oxidation of acetate and glucose as well as oxidative assimilation of these substrates and that respiration rates rapidly increased with their addition. Levine and Novak (1956a) agreed that glucose and mannose stimulated respiration. But they stated that the introduction of fatty acids with a double bond decreased the oxygen uptake. The similarity in the oxygen uptake of fresh and starved cells, after the addition of glucose, indicated that the 2 types of cells were able to utilize this substrate in a comparable manner. Since this starvation period, of 7 days at 8 C, had no apparent effect on the basic metabolic pathways of the cells, they were probably in a resting state. This fact was verified by the comparison of the similar endogenous respiration rates of the cells.

The unirradiated fresh and starved cells, with glucose, had approximately twice as much oxygen uptake as their respective irradiated cells. It was also noted that the oxygen uptake by both the fresh and starved irradiated cells were comparable with their respective unirradiated cells for 40 minutes, at which time the irradiated values decreased rapidly. From these results it appeared that the ultraviolet caused an inhibition of respiratory enzyme synthesis and that the preformed respiratory enzymes were not destroyed. Thus, the respiration

was normal for a short time after irradiation while the preformed res-
piratory enzyme (s) was utilized, and then it declined when it was
necessary for the cells to synthesize additional respiratory enzyme
(s). These results were similar to those of Giese and Swanson (1953)
who stated that exogenous respiration of irradiated E. coli paralleled
that of the control for a period after irradiation and then declined.
Billen et al. (1953) supported this observation with x-irradiated E.
coli. They proposed that adaptive enzyme formation was prevented by
irradiation and that inhibition of enzyme synthesis rather than destruc-
tion of preformed enzyme was responsible. Although Giese and Swanson
(1947) reported an increased endogenous respiration by yeast cells fol-
lowing ultraviolet irradiation and Heinmets and Kathan (1954) found
oxygen uptake by E. coli in the presence of glucose was only slightly
affected by ultraviolet, neither of these observations were made with
B. dermatitidis. These differences were probably due to the manometric
procedures and to the length of time in which results were recorded.

Photoreactivation of fresh cells which were previously irradiated
resulted in approximately twice the oxygen uptake of those fresh irra-
diated cells which were not photoreactivated. However, the oxygen up-
take of these photoreactivated cells did not equal that of the cells
which were unirradiated. Respiration of irradiated starved cells was
no different after illumination. From these results it appeared that
7 days of starvation effected a utilization or other reduction of the
accessory ultraviolet-absorbing materials within the cells. This left
only the vital enzymes and mechanisms necessary to maintain normal
metabolic activities. Since these are primarily contained within the
nucleus, if it was "hit", the cells would die or be damaged to such an

extent that repair would seem impossible. Apparently when fresh cells

containing those accessary absorbing materials were irradiated, the

damage which was done to the nucleus was not complete, and repair was

impossible. This view is in agreement with Sarachek (1958) who stated

that ultraviolet-induced respiratory deficiency in Saccharomyces was

photoreversible, and that the nucleus was the site of radiation damage.

Pittman et al. (1959) agreed since they thought that the site of ultra-

violet damage affecting respiratory competence in yeasts was in the

nucleus. But, they also stressed the point that photoreactivability

of a given ultraviolet lesion did not imply by itself that the reactiv-

able damage was nuclear, since both nuclear and cytoplasmic damage have

been shown to be photoreversible. This latter point was also correlated

with the inability of starved cells to be photoreactivated. Apparently,

the starvation promoted liberation from cells treated with ultraviolet

of a labile toxic material. This material was probably formed in the

cytoplasmic portion of the cell. Since the starvation promoted its re-

lease other constituents from the cytoplasm were probably also released,

and this cytoplasmic material which was capable of photoreactivation,

(Pittman, 1959) was absent. The cytoplasmic material was present in

fresh cells. Therefore, its photoreactivation, along with that of the

enzyme systems of the nucleus, would restore the respiratory efficiency

of the fresh cells to almost normal, as was shown to occur in B. derma-

titidis.

In the investigation of the amino acids present in B. dermatiti-

dis 4 one-dimensional and 2 2-dimensional paper chromatographic proce-

dures were compared. Of the one-dimensional systems used only the

pyridine-acetic acid-water and butanol-pyridine-water were of significant

value. The phenol system was not good because of the heavy streaking
it produced and the slowness of descent. Mizell and Simpson (1961)
warned that phenol should not be used on chromatograms because phenol
of sufficient purity was hard to obtain, the purification of obtainable
phenol is laborious, that the amino acid spots have a tendency to "tail"
and that some of the common amino acids were not separated by it. In
addition, this investigator observed that after development of the
chromatograms, the phenol left the paper dark brown. This interferred
with the identification of those spots which were very light. The
butanol-ammonia system was less satisfactory than the phenol. As Smith
(1958) found, there was not an adequate spread of the spots and the Rf
determinations were virtually impossible to make. Of the 2 one-dimen-
sional systems which were of value, the butanol-pyridine-water solvent
was the better. Smith found this solvent adequate for separation since
a variety of amino acids were distributed with a great degree of separ-
ation between spots. The Rf values established by Smith were nearly
the same as those in the present investigation. Pyridine-acetic acid-
water was also a good solvent for separation and similar Rf values
were found as those established by Block et al. (1958).

Of the 2 2-dimensional solvent systems used, the butanol-pyridine-
water followed by phenol-ammonia (Smith, 1958) was of little value.
Disadvantages of this system were numerous and similar to those stated
previously for a phenol system. The second 2-dimensional system of
n-butanol-acetic acid-water followed by n-butanol-methyl ethyl ketone-
water (Mizell and Simpson, 1961) was excellent. The color reactions
were specific, the spots when compared to the map prepared by Mizell
and Simpson were in similar positions and the Rf values were similar to

those previously obtained.

Regardless of the system used decomposition of amino acids must be expected during chromatography on paper. Of the 9 amino acids Moses (1962) investigated, the extent of decomposition during chromatography varied from zero with tyrosine to over 14 per cent with leucine. Generally, glycine, phenylalanine, and leucine showed the greatest degree of breakdown. This decomposition could be minimized by the distillation of phenol before use and pretreatment of the paper with oxalic acid. In the present investigation, leucine was present in a very low concentration. Phenylalanine was undetectable. Partial or complete decomposition may well have accounted for these observations. The concentrations of the other amino acids were probably more than observed, though no comparative quantitative determinations were made before and after chromatography.

This investigation has shown the presence of 22 amino acids in B. dermatitidis. These and the results of similar determinations made by other authors are listed in Table 20.

Those amino acids which were present in the highest concentrations in this investigation were: (1) alanine, (2) aspartic acid, (3) glutamic acid, (4) glycine, and (5) valine. These determinations were estimated qualitatively by visual observation of the various densities of the respective spots. Similar results were noted in quantitative determinations by other investigators. Shiio et al. (1962) also found these amino acids in the highest concentrations in Brevibacterium flavum with the exception of serine, which he stated was as concentrated as the others. Serine was found to be of the least concentrated amino acids in B. dermatitidis.

TABLE 20

THE AMINO ACIDS PRESENT IN B. DERMATITIDIS AND
VARIOUS OTHER ORGANISMS

Organism	Amino Acids Identified	Reference
1. Blastomyces dermatitidis	proline, asparagine, aspartic acid, alanine, valine, glutamic acid, arginine, histidine, ornithine, citrulline, cystine, cysteine, serine, glycine, threonine, taurine, tyrosine, B-alanine, lysine, iso-leucine, leucine, and methionine.	This investigation (1963)
2. Cholera and cholera-like vibrios	diaminopimelic acid, cystine, lysine, histidine, arginine, aspartic acid, serine, glycocoll, hydroxyproline, glutamic acid, threonine, alanine, proline, 2-aminobutyric acid, tyrosine, tryptophan, methionine, valine, phenylalanine, leucine, isoleucine.	Pustovalova (1962)
3. Globisporus streptomycini	Cystine, histidine, lysine, arginine, aspartic acid, glutamic acid, serine, glycocoll, threonine, alanine, tyrosine, proline, methionine, valine, phenylalanine, leucine, norleucine.	Prokofieva-Belgovakaya and Demyanovaskaya (1957)
4. Penicillium chrysogenum	alanine, aspartic acid, glycine, glutamic acid, histidine, proline, valine	Milnikova and Surikova (1957)

Pustovalova (1962) found that the content of aspartic and glutamic acids, alanine, leucine, and isoleucine exceeded the concentration of the other amino acids by as much as 2 to 3 times. Leucine and isoleucine were not present in high concentrations in B. dermatitidis as determined in this investigation. However, he also found that arginine and hydroxyproline were low in concentrations. This was true of arginine in B. dermatitidis but hydroxyproline was not detected by methods employed in this investigation. Das Gupta and Nandi (1956), while

working with 3 species of _Polyporus_, identified the most common amino

acids as aspartic and glutamic acids, serine, glycine, alanine, valine,

methionine, and leucine. The amino acids identified in this investiga-

tion and those identified by others were quite comparable, although

some variation was found (Table 20). Reasons for differences could

have been in the methods employed and application of recently developed

tests for identification. An example of this was shown by Pustovalova

(1962) who treated his chromatographic paper with 8-hydroxyquinoline

dissolved in n-butanol-acetic acid-water in order to aid in the separ-

ation of the amino acid spots. Moses (1962), as stated previously,

discovered decomposition of amino acids during paper chromatography.

This decomposition was decreased by treatment of the paper with oxalic

acid. The paper used in this investigation was not treated. Also, the

absence of certain amino acids on chromatograms prepared in this inves-

tigation may have indicated an insignificant amino acid concentration

which was undetectable by the methods employed. For the most part,

those amino acids present in the highest concentration as well as those

which were present in much lower concentrations in other investigations

were the same as those found in _B. dermatitidis_.

The amino acids in ultraviolet irradiated cells were less con-

centrated than in unirradiated cells. Aspartic acid, glutamic acid and

glycine were altered the least. The alanine spot was lighter than those

and the valine spot was almost undetectable. Two-dimensional chromato-

grams showed, in addition, that arginine, asparagine, citrulline, histi-

dine, ornithine, taurine, threonine, and tyrosine disappeared from

irradiated cells. The chromatograms of the irradiated cells demons-

trated better separation of the amino acid spots than those of the

unirradiated cells. This was probably due to the decrease in concentration of some of the amino acids and the complete elimination of others, as demonstrated by the 2-dimensional chromatogram.

Of the 8 amino acids which were undetectable after irradiation, arginine, citrulline, and histidine were basic animo acids. From this it appeared that the basic amino acids were the best altered by ultraviolet irradiation.

It appeared from the data of this investigation that undissociated amino acids may absorb less ultraviolet irradiation than those which were dissociated. Therefore, the greater the charge, the more the absorption and the zwitterion, or neutral form, would be affected the least. This was demonstrated by the 2-dimensional chromatogram and the amino acids which disappeared after irradiation. Seven of these 8 were either positively or negatively charged and only one was neutral. The fact that the basic amino acids appeared to be more affected than the acidic amino acids was probably due to the concentration of these acids present in the cell. Since the acidic amino acids, glutamic and aspartic acids, were present in such high concentrations they never disappeared after irradiation. However, the basic amino acids, arginine, histidine, and citrulline, were present initially in very low concentrations and, because they were highly charged, the irradiation reduced them to undetectable concentrations. These results are in agreement with Nakanishi (1958). He stated that an addition of urea to a tyrosine solution brought about a decrease in the absorption of ultraviolet by tyrosine. This demonstrated the suppressing effect of the urea on the dissociation of the -OH groups as well as -COOH groups.

It was also interesting to note that of the 3 common amino acids

which were not detected in this investigation, tryptophan and phenylala-
nine contained 6-membered cyclic rings and the third, hydroxyproline,
contained a 5-membered cyclic ring. This would seem to demonstrate that
these ring structures, with their double bonds, were easily broken by
chemical physical factors.

The amino acids of B. dermatitidis were probably located in 2
distinct "pools" which were associated with protein synthesis. Accord-
ing to Zalaker (1961) Candida also contained these "pools". He concluded
that the exogenous amino acids entered the "expandable pool" where they
accumulated and that precursors for protein synthesis were taken directly
from the "internal pool". There was free exchange between the external
precursors and the "expandable pool", while the "internal pool" took up
amino acids only at the rate of their use in protein synthesis. The
ultraviolet damage to the amino acids was most likely to be in these
2 "pools". If they were inactivated by the ultraviolet while in the
"internal pool", their incorporation into ribonucleoprotein (RNP) would
be altered since this pool supplies the precursors for protein synthe-
sis. Thus, they would not be able to form a complex with soluble RNA
and be subsequently transferred to RNP (Takanami and Okamoto, 1960).
This transfer action would be catalyzed by an enzyme and Takanami and
Okamoto (1960) referred to it as "transferring enzyme". Another fact
which should be mentioned is that protein synthesis is dependent on RNA
synthesis. Since this investigation has shown a marked effect of ultra-
violet on RNA and amino acids, which are necessary for protein synthesis,
the effects of ultraviolet on protein synthesis could vary. Setlow and
Hanawalt (1960) stated that the effects of ultraviolet irradiation on
RNA and protein synthesis were the same. Also, that protein synthesis

was more sensitive than RNA synthesis due to damaged DNA specifying

"nonsense" RNA which could not sustain protein synthesis. Thus, the

damage to the amino acids of B. dermatitidis appears to have been due

to several factors. First, the DNA may have been damaged, specifying

"nonsense" RNA, which resulted in abnormal protein synthesis. Second,

the inactivated amino acids may not have been incorporated into RNP.

Or finally, that one or more of the enzymes responsible for these

mechanisms may have been damaged. It seems more probable though that

the actual damage to the amino acids was in the "internal pool" and

that because of this, the protein synthesis was affected in 2 ways.

First, by the inability to utilize these inactivated amino acids, and

secondly, by their disappearance from the "internal pool". The ultra-

violet apparently damaged the cell membrane and allowed the amino acids

or their irradiation products to diffuse from the cell into the surround-

ing medium. For example, this investigation has also shown that starva-

tion of B. dermatitidis at 37 C decreased the amino acid concentrations

in the cell and allowed large quantities of ammonia to accumulate in

the suspending liquid. This amino-acid decrease was more pronounced

if the cells were irradiated after starvation. It was interesting to

note, however, that starvation for one day decreased the amino acid

concentrations more markedly than did ultraviolet exposure without a

starvation period. Also, those cells which were starved for the long-

est period and then irradiated had the lowest concentration of amino

acids. Apparently, the starvation alone caused the cells to utilize

the amino acids in the 2 "pools" for synthesis of protein and for ful-

fillment of a nutrient carbon deficiency. With starvation the ultra-

violet irradiation produced an additive effect on amino acid concen-

trations allowing loss of certain materials from the cell. This may have accounted for the failure to demonstrate photoreactivation of amino acid levels in irradiated cells.

Ultraviolet irradiation produced a definite decrease in the concentrations of both RNA and DNA in B. dermatitidis. The longer the ultraviolet was administered, the greater was the decrease in the concentration of RNA. This decrease may have been due to a loss of intracellular RNA from the cells through the damaged cell membrane. It was likely that the greatest effect of the ultraviolet was to the "soluble" RNA which is responsible for protein synthesis. Drakulic et al. (1961) showed a loss of intracellular RNA from E. coli B. They also believed the effect of the ultraviolet was a disturbance in the metabolism of the RNA which consisted chiefly of a decreased incorporation of adenine into "particulate" RNA and a greater incorporation into the "soluble" RNA. Therefore, they concluded that after ultraviolet irradiation the "particulate" RNA synthesized did not find its protein partner and became soluble. Setlow and Hanawalt (1960) and Drakulic et al. (1961) stated that "particulate" RNA synthesis was constant in the absence of DNA synthesis. However, both the "soluble" RNA and protein syntheses increased since "particulate" RNA synthesis was determined in part by the number of intact DNA "units" in the cell. They also found that damage to one strand of DNA was sufficient for unit effect on protein synthesis, but both strands must be damaged for unit effect on RNA synthesis.

The concentration of DNA in ultraviolet irradiated B. dermatitidis decreased very rapidly. However, the length of exposure did not have an additive effect. Kanazir and Errera (1954) and Kelner (1954)

also observed this immediate but complete inhibition of DNA synthesis after ultraviolet irradiation may have been a result of several factors. The most probable reason was the interference in replication through alteration of the DNA structure (Kimball, 1957). This alteration may have weakened the stability of the helix structure from an interference in normal hydrogen bonding, or cross linkage of the DNA strands may have occurred with dimer formation of thymine (Marmur et al., 1961). The thymine may also have converted to IPT (irradiation produce of thymine) which was responsible for the lethal effects (Beukers and Berends, 1961). Since the purines and pyrimidines absorb the ultraviolet irradiation and prevent the uncoiling of the helix by dimerization, there may also have been a shift toward the keto form of the hydroxyl groups which caused a strengthening of hydrogen bonds and a prevention of the helix separation which prevented DNA synthesis (Klouwen, 1962).

Both RNA and DNA concentrations in B. dermatitidis increased in ultraviolet irradiated cells after photoreactivation. The increase was proportional to the length of photoreactivation although concentrations of the nucleic acids never equalled or surpassed that of the unirradiated cells. Mechanisms for the photoreactivability of the nucleic acids could be explained in various ways. It was believed that with E. coli the rejuvenation of the nucleic acids basically involved the repair of damaged DNA rather than increasing DNA synthesis, and that the subsequent synthesis of RNA depended on the former rather than the latter effect. But, photoreactivation of RNA synthesis could occur in the absence of DNA synthesis (Hanawalt and Buehler, 1960). Also, there may have been enzymes in the photoreactivable cells which combined with

ultraviolet lesions in DNA, absorbed light and repaired the ultraviolet damage to transforming DNA (Rupert, 1961, 1962).

Mice were highly susceptible to intraperitoneal inoculations of B. dermatitidis. Visible lesions were usually apparent on the liver and diaphragm of an infected animal and sometimes on either the large or small intestine. Working with Histoplasma capsulatum, however, Rowley and Huber (1955) found that gross lesions were not present in mice sacrificed 4-6 weeks after injection with 10 to 100 "groups" of cells. Two, 4, 8 or 10 x 10^6 (Tsubura et al., 1962) yeast cells of H. capsulatum killed 50-100 per cent of the mice 60 days after intraperitoneal injection. Since the maximum concentration of B. dermatitidis cells inoculated was 2 x 10^5 only 2 injected mice died before autopsy. At autopsy no signs of infection were observed in these mice.

Brandsberg et al. (1963) found white Swiss mice to be very susceptible to intraperitoneal inoculations of the mycelial phase of B. dermatitidis. They stated that the number of viable particles necessary to produce a high per cent of infections was in excess of 100. In this investigation 10 "viable particles" of the yeast phase could produce an infection. Although the details of the Brandsberg study are not presently available, this difference may be attributable to the use of mucin-suspended cells throughout this investigation. Howell and Kepkie (1950) also found that cells suspended in mucin produced a larger per cent of deaths than similar dosages suspended in saline. Salvin (1954) found that H. capsulatum suspended in mucin was 20 per cent more effective than saline suspensions. Campbell and Saslow (1950) stated that this same organism was rarely isolated from mice when it was injected intraperitoneally suspended in saline, but was consistently

isolated from mice when suspended in mucin.

Following injection of the yeast phase of B. dermatitidis Howard
and Herndon (1960) assumed that it was not affected by phagocytosis.
It seemed rather unlikely to them that this organism could be phagocy-
tized since the cells are often the same size as an average white blood
cell. Young (1958), however, found that the yeast phase of C. albicans
was more readily phagocytized than the mycelial phase and for that rea-
son he assumed the mycelial phase to be the infectious form. This or-
ganism when injected intraperitoneally into newborn mice affected the
liver and kidneys and strongly suggested a glycogen storage disease
(Mankowski, 1962). Though this point has not been developed by pre-
vious investigations it was noted that B. dermatitidis also established
infection in the liver.

According to Peck (1947), the lipid constituents of pathogenic
fungi have a significant role in the mechanism of the infectious dis-
ease. It was not known at the time whether this was a result of the
lipids as a whole or of a defined portion, such as the acetone-soluble
fraction of phospholipid or even of some small portion of these compon-
ents. Al-Doory and Larsh (1962) stated that the total lipids cannot be
considered the only responsible factor in the mechanism of the infec-
tious disease since some non-pathogenic fungi have more lipids than the
pathogens. Peck (1947) also reported that the absence of the carbohy-
drate-containing phospholipid in all but one of the nonpathogenic or-
ganisms was the most significant variation between these and the patho-
gens with respect to their free lipid.

When compared with the references cited, the results of this
investigation have shown that B. dermatitidis was more likely to cause

an infection than certain other pathogenic fungi, and that fewer cells were needed to establish the infection in susceptible animals (Young, 1958, and Tsubura et al., 1962). According to Al-Doory and Larsh (1962) the relative lipid contents might explain this observation. These authors and other investigators found that the yeast phase of B. dermatitidis contained 10 per cent by weight more total lipids than either H. capsulatum or C. albicans. They also discovered that the lipid extract of pathogenic fungi produced the same tissue reactions when injected as did whole cells. It was apparent that more information is needed to provide a basis for a better evaluation of the degree in which lipids of pathogenic fungi participated in the role of pathological reactions accompanying infections. The application of purified fractions might reveal a better answer to the real role of lipids in the mechanism of each of the infectious processes. Since B. dermatitidis does not contain or produce toxins, the initial effect was probably due to the lipid content which caused an inflammatory response to infection by either a suppurative or granulomatous reaction, followed by normal cell division and growth. The actual destructive effects of the organism were probably related directly to the rapid proliferation of the fungus intracellularly and extracellularly rather than to the depletion of essential cell nutrients by the growth and metabolism of the organism (Howard and Herndon, 1960).

It was also noted in this investigation that female mice were more susceptible than males to intraperitoneal injections of B. dermatitidis. This was probably due to hormonal differences between the sexes.

Ultraviolet irradiated cells produced a lower percentage of

infections than unirradiated cells, and as before, the females were more susceptible than the males. Pershina and Vasil'Eva (1960) also found Shigella flexneri less virulent in mice after irradiation. Since previous experimentation with ultraviolet in this investigation has produced noticeable effects on enzymes, respiration, amino acids, protein synthesis, and nucleic acids the virulence could be expected to be lessened by either one or a combination of these effects.

Those cells which were photoreactivated after irradiation produced a higher percentage of infections than the irradiated cells. However, the per cent of infections of these photoreactivated cells never equalled that of the unirradiated cells. Since photoreactivation can restore or repair the syntheses or damage to cell constituents and cell metabolism, as demonstrated in this investigation, a restoration of virulence was not unexpected. This, however, is the first demonstration that virulence in human pathogenic fungi has been photoreactivable.

CHAPTER VI

SUMMARY

1. Ultraviolet-irradiated Blastomyces dermatitidis strain 6046 was studied and compared with normal cells.

2. The growth of B. dermatitidis varied a great deal on different media.

A. The growth of irradiated cells was extremely variable on the different media. A rich medium was needed for growth and recovery.

B. Recovery of cells photoreactivated at both 8 C and 37 C was demonstrated. There was higher percentage of recovery on all media following reactivation at 8 C.

3. Glucose increased the oxygen uptake by both fresh and starved cells of this organism. The endogenous respiration of both types of cells was similar.

A. Ultraviolet irradiation decreased the respiration rates of both fresh and starved cells.

B. Photoreactivation of irradiated fresh cells increased the oxygen uptake. Respiration of irradiated starved cells was no different after illumination.

4. Twenty-two amino acids were identified in B. dermatitidis strain 6046.

A. Eight of these 22 amino acids were undetectable after ultraviolet irradiation. The other amino acids were less concentrated after irradiation.

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B. Illumination of irradiated cells did not increase their amino acid concentrations.

5. The DNA and RNA concentrations of this organism were determined.

A. Ultraviolet irradiation decreased the concentrations of DNA and RNA.

B. RNA and DNA concentrations increased in ultraviolet irradiated cells after photoreactivation.

6. Mice were highly susceptible to intraperitoneal inoculations of B. dermatitidis. Females were more susceptible than male mice.

A. Ultraviolet irradiated cells were less virulent than normal cells.

B. Cells which were photoreactivated after irradiation were more virulent than irradiated cells.

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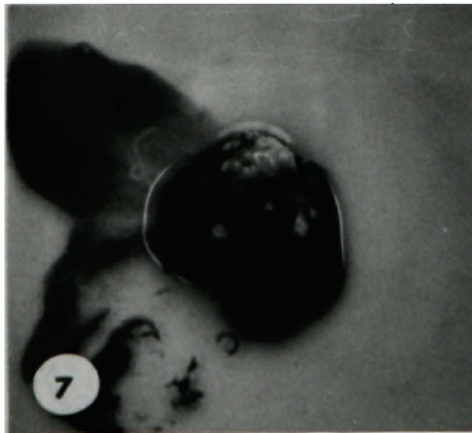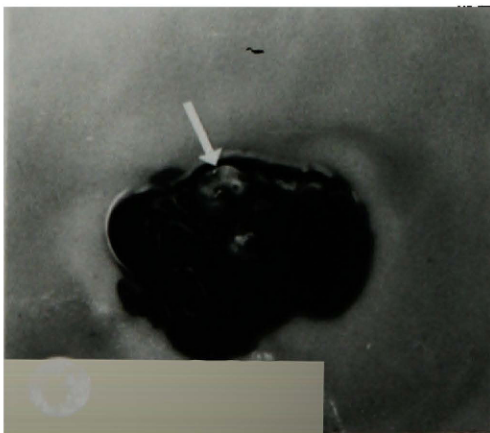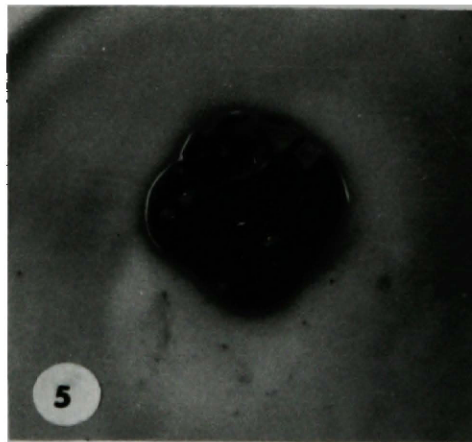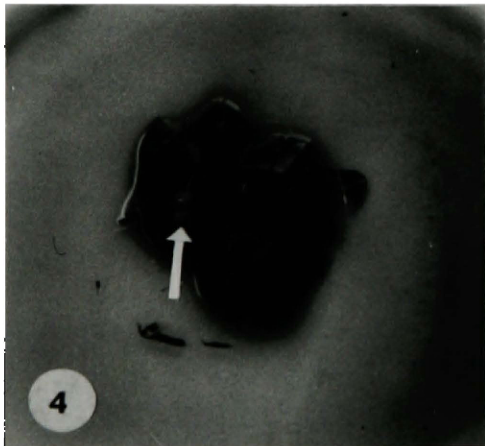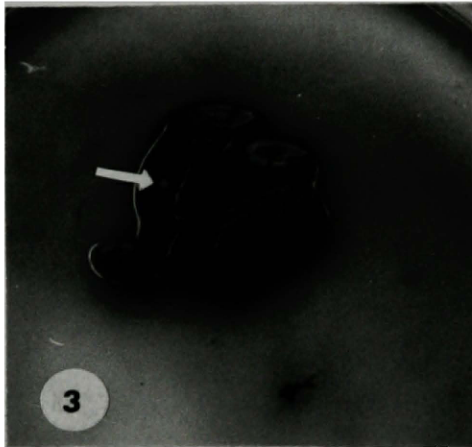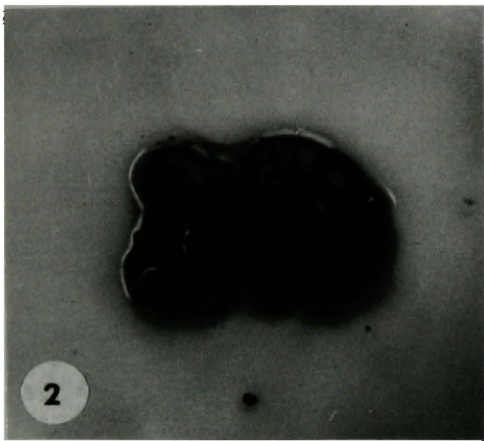
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