1967

The comparative pathogenicity of normal and inhibitor grown systemic pathogenic fungi

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THE COMPARATIVE PATHOGENICITY OF NORMAL AND INHIBITOR GROWN SYSTEMIC PATHOGENIC FUNGI

by

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B.A. Lewis and Clark College

Presented in partial fulfillment of the requirements for the degree of

Master of Science

UNIVERSITY OF MONTANA

1967

Approved by:

Chairman, Board of Examiners

Dean, Graduate School

JUN 7 1967

Date

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ACKNOWLEDGMENTS

The author wishes to express his sincere thanks and appreciation to Dr. John J. Taylor, Associate Professor of Microbiology, Department of Microbiology, University of Montana, for his advice, assistance and encouragement throughout the course of this investigation.

This work was supported by a grant to Dr. Taylor from National Institutes of Health (E3078).

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CHAPTER I

INTRODUCTION

Blastomyces dermatitidis, *Sporotrichum schenckii* and *Histoplasma capsulatum* are fungi which cause systemic infections in man and animals. These organisms display temperature-dependent cultural dimorphism. Salvin (1949) noted that incubation of cultures of *B. dermatitidis* at room temperature (25 C) resulted in typical mycelial growth, while incubation at 37 C resulted in yeast-like cultures. Kligman and Baldridge (1951) found similar results for *S. schenckii* and *H. capsulatum*. Other factors involved in dimorphism, such as growth media, sulfhydryl compounds, irradiation, age and nutritional factors have been reviewed by Scherr and Weaver (1953) and Scherr (1957).

Natural infections caused by these fungi generally result from the inhalation, ingestion or implantation of spores produced by the mycelial phase. Following implantation of spores in the host animal, they germinate into the yeast-like, parasitic form. *B. dermatitidis* is the etiological agent for North American Blastomycosis (Blastomycosis, Gilchrist's Disease). The infection, which is a chronic granulomatous and suppurative disease, usually originates as a respiratory infection and then can disseminate to other organs, mainly those of the pulmonary, cutaneous, osseous, genitourinary and central nervous systems. Sporotrichosis is most commonly a localized, subcutaneous, lymphatic infection resulting from implantation of *S. schenckii* spores. The disseminated form of the infection is much less common and involves lesions of the oral and nasal mucosa, orchitis, mastitis, pulmonary

involvement, periostitis and osteomyelitis. In rare instances involvement of visceral organs may occur. Histoplasmosis (Darling's Disease), caused by H. capsulatum, generally results from inhalation or ingestion of the spores and is manifested by a primary, granulomatous lesion. The disease is usually respiratory in origin and may result in an asymptomatic, acute or chronic pulmonary infection. Disseminated forms of the disease often involve the reticuloendothelial system resulting in granulomatous lesions of the lungs, spleen, adrenal, liver, kidney and lumphatic tissue. The skin and central nervous system may also be involved.

Experimentally, Rowley and Huba (1955) found they could induce laboratory infections in mice with H. capsulatum and that there was no apparent difference in the death-producing effects of either the intravenous (I.V.) or intraperitoneal (I.P.) route of infection. Similarly, Mackinnon (1959) working with B. dermatitidis found that induced infections in white mice resulted in metastasis similar to that found in man suffering from a natural infection. He obtained similar results in animals inoculated by the intramuscular, I.P. and I.V. routes. Kleigman and Baldridge (1951) found that I.P. injection of S. schenckii into mice resulted in orchitis within 10 days while, Denton et al., (1961), reported that mice could be experimentally infected with B. dermatitidis by the I.V. route. In addition, Mackinnon (1951) reported that mice could be experimentally infected with B. dermatitidis by the respiratory route. Later Cozad and Larsh (1962), using an aerosol, found that mice were infected by relatively small numbers (5-22) of H. capsulatum yeast-phase cells.

Although experimentally induced infections are possible with the three organisms, Brandsberg et al. (1963), demonstrated that various animals exhibit different levels of susceptibility when injected I.V. with B. dermatitidis. Having tested guinea pigs, golden hamsters, albino rabbits, white rats and white Swiss mice, they reported that mice and hamsters were the most susceptible. In addition, Lodmell (1963) found that female white mice of the Webster strain were more susceptible than males of the same strain.

Increased pathogenicity of H. capsulatum was noted by Howell and Kepkie (1950) when they found that cells suspended in hog gastric mucin produced a larger percent of deaths in mice than similar dosages suspended in saline. Similarly, Salvin (1954) reported 20 percent more deaths in mice injected I.P. with H. capsulatum suspended in gastric mucin than similar saline suspensions. Campbell and Saslow (1950) found that following I.P. injection of mucin-suspended H. capsulatum, they could consistently recover organisms 30 days following inoculation while, the same organism was only rarely isolated following injection of saline-suspended cells.

Studies of the pathogenic fungi with respect to their growth requirements have been investigated by several workers Holliday and McCoy (1955), working with B. dermatitidis, found that biotin was necessary to culture this organism, and that the degree of growth was proportional to the concentration of the biotin. Salvin (1949) reported that the yeast phase of H. capsulatum required accessory growth factors for development, namely, biotin and a reduced sulfur compound. Pine (1957) studying 11 strains of yeast-phase cultures of H. capsulatum

stated that thiamin was required for maximum rate of growth by 8 of the 11 strains while both biotin and thioctic acid were required by one strain. By contrast he found that deletion of any single vitamin or group of vitamins was without any apparent effect on the rate or amount of mycelial growth obtained at 25 C. Gilardi and Lafter (1962) concluded from their experiments that B. dermatitidis synthesized its own vitamins and that it utilized 21 carbon and 25 nitrogen compounds as the source of the respective elements.

Bernheim (1942) reported that both the natural and nonnatural isomers of amino acids increase the oxygen uptake of B. dermatitidis. Later Cozad et al. (1958), found that all the L-forms of amino acids they tested stimulated oxygen uptake of H. capsulatum, with the exception of L-arginine. None of the D-forms tested showed any effect upon the respiration of the fungus. In addition, Gilardi and Lotter (1962) discovered that if a liquid medium was supplemented with casamino acid and biotin, no additional amino acids or vitamins were necessary for growth of B. dermatitidis.

Short chain fatty acids were found to be readily oxidized by B. dermatitidis; however, it was found that long chain fatty acids inhibited oxygen uptake even with the addition of other substrates. (Bernheim, 1942). Similarly, Cozad et al. (1958), found that the lower fatty acids from acetate through butyrate stimulated respiration while sodium oleate, one of the higher fatty acids, inhibited oxygen uptake. Later Gilardi and Loffer (1962) also reported that "Tween 80" inhibited respiration, indicating that the oleic acid of this substance could be the inhibitory factor. This inhibition was found by Pine (1954) to be easily reversed

by the addition of albumin or starch. It was postulated by Pine that albumin could function by binding heavy metals, react with mercaptan or serve as a source of -SH groups. However, the most probable function of albumin and starch is to bind excess faty acids and/or supply stimulatory peptides.

Carbohydrates also induce various effects on the oxygen uptake of pathogenic fungi. Levine and Novak (1950) reported that the hexoses, glucose and mannose stimulated oxygen uptake in B. dermatitidis while, galactose and fructose did not. Among the pentoses studied only xylose stimulated oxygen uptake while incubation with ribose, arabinose and rhamnose showed no increase in respiration over that of the endogenous. Cozad et al. (1958), found that with H. capsulatum, glucose, mannose, fructose, xylose and maltose stimulated oxygen uptake while, lactose, sucrose and arabinose were without effect. Galactose was also found to stimulate oxygen uptake in H. capsulatum (Garrison, 1960).

The optimum hydrogen ion concentrations for respiration was found to vary by different workers. Pine (1954) reported that a pH of 6.5 was optimum for the in vitro growth of yeast phase H. capsulatum and that a pH of 7.0 resulted in complete inhibition of growth after 2 to 4 days. He suggested, however, that this inhibitory effect was due not to the pH but rather to the inactivation of available -SH groups. In addition, a rather wide pH range of 5 to 8 was noted for the endogenous respiration of H. capsulatum. Exogenous glucose oxidation, however, showed a definite optimum at pH 7.0. Endogenous and substrate oxidation by B. dermatitidis was found by Levine and Novak (1950) to have an optimum pH of 6 to 8. Furthermore, they pointed out that this range

includes pH values normally found in animal tissue and would assure a hydrogen ion concentration conducive to parasitic existence.

The effects of ultraviolet irradiation have been studied extensively with bacterial cells and to a lesser extent with fungal cells. Dimond and Duggar (1941) found that susceptibility to irradiation among various nonpathogenic fungi was quite varied. They concluded that a combination of differences in pigmentation, size of spores and number of nuclei may have accounted for this variation. In addition, they noted that older cells appeared less susceptible to the effects of irradiation than younger cells. Also, it is generally accepted that the ploidy state of an organism is an important factor in its resistance to radiation (Pomper and Atwood, 1955). Bacq and Alexander (1961) stated that of the yeasts, the diploid organisms are much more resistant to ultraviolet and ionizing radiations than haploid cells. The mortality curve of diploid yeasts is sigmoid in nature while the haploid yeasts exhibit a straight line. Studying the effects of irradiation, Rothstein (1957) found that respiration and fermentation were not as sensitive to ultraviolet irradiation as was cell division. He also observed that under cettain conditions a marked stimulation of respiration was noted. Kelner (1953) found that the endogenous respiration of Escherichia coli was not affected by irradiation even at high dosages. Exogenous respiration was not apparently affected at first; however, the oxygen uptake gradually declined with longer periods of exposure. In addition, Heinmets and Kathan (1954) found that the oxygen uptake of bacterial cells was only slightly inhibited if the irradiated cells were tested in the presence of glucose. With B. dermatitidis, Lodmell (1963)

demonstrated that glucose stimulated the oxygen uptake of both fresh and starved cells, but that ultraviolet irradiation decreased the respiration of both.

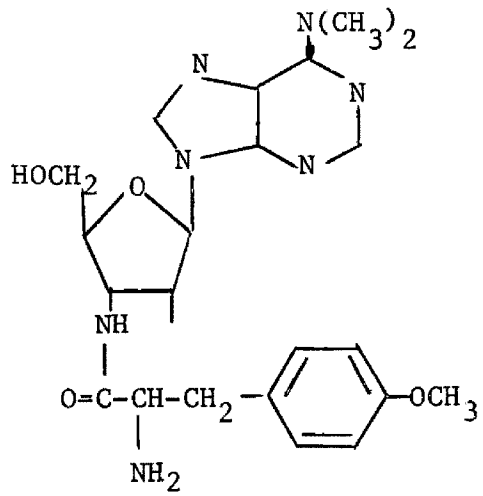
Ultraviolet irradiation just sufficient to block cell division has been shown to have little effect on the synthesis of DNA and RNA and protein. However, as the dose is increased, inhibitions occur and DNA synthesis appears to be the most sensitive (Rothstein, 1959). Barner and Cohen (1956) postulated that ultraviolet absorbed by the DNA molecule resulted in the breaking of a critical bond (s) with the formation of toxic products. Rothstein (1959) found that when DNA is irradiated in solution with ultraviolet light at 254 mμ, loss of transforming activity and changes in the physical-chemical properties of DNA could be detected. Working with D. pneumoniae and B. subtilis he stated that the loss of biological activity depended on the genetic marker under investigation as well as on its size and molecular weight of the DNA being irradiated.

Beukers and Berends (1961) reported that ultraviolet irradiation of thymine resulted in its conversion to mixed dimers. Later Kleuwen (1962) found that ultraviolet irradiation was mainly absorbed purine and pyrimidine bases resulting in dimerization which prevented uncoiling of the helix structure of the DNA molecule.

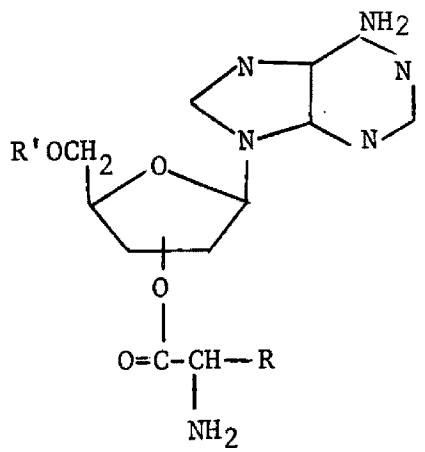
Recovery of irradiated cells has been shown to occur better on media not considered optimum for growth of the unirradiated cells. Nevell (1955) and Roberts and Aldous (1959) found that best recovery was effected on chemically defined media rather than on nutrient agar or an enriched medium containing concentrations of animal or vegetable extracts.

Similarly, Ramage (1961) found with Shigella sonnei that the medium which supported the best growth was poorest for recovery of ultraviolet irradiated cells. In contrast, Lodmell (1963) reported that irradiated cells of B. dermatitidis required a rich medium (Blood agar, SAB, Brain Heart Infusion) for growth and recovery.

Puromycin, previously known as tetracycline and antimycin is an antibiotic produced by Actinomyces alboniger (Porter et al., 1952).



PUROMYCIN



The amino acid-bearing end of tRNA. R represents the remainder of the amino acid residue, R' represents the remainder of the RNA polymer (Nathans and Neidle, 1963).

The structure was determined by Waller *et al.* (1953), and shown to consist of an amino-nucleoside linked to the amino acid p-methoxy-phenylalanine. Inspection of the structure shows that it can be regarded as an analog of adenosyl-phenylalanine; specifically of the terminal groups of the phenylalanyl-transfer RNA (tRNA) (Darken, 1964).

This antibiotic has a broad range of biological activity, inhibiting the growth of bacteria, protozoa, parasitic worms, algae plants and mammalian cells (Nathans, 1964). In spite of this range of biological activity, broad clinical use of puromycin was never realized, owing primarily to its nephrotoxicity. In addition, the antibiotic appears to be readily absorbed by virtually all tissue and is rapidly excreted by the kidneys (Sherman *et al.*, 1954-1955). Although puromycin lacked widespread clinical use, it has proved of great value in investigative work as a specific, reversible inhibitor of protein synthesis (Darken, 1964). The observations of Creaser (1955), first indicated that puromycin inhibits protein synthesis in bacteria. He noted that this inhibition was manifested by complete absence of induced B-galactosidase synthesis in *Staphylococcus aureus*. Further evidence for the inhibitory effect of puromycin on protein synthesis was established in 1959 by Yarmolinski and de la Haba. Its suppression of protein synthesis *in vivo* was first demonstrated by Gorski *et al.* (1961).

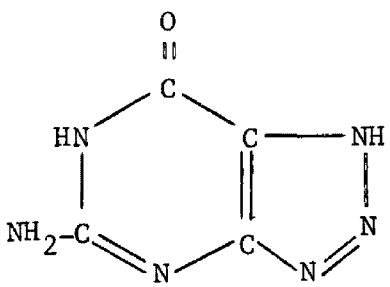
Takada *et al.* (1960), noted that in growing bacteria, the overall rate of protein synthesis was markedly reduced in the presence of puromycin; however, RNA and DNA syntheses continued at an approximately normal rate. Subsequent removal of the drug resulted in restoration of protein synthesis and growth. In *E. coli* 15T, Sells (1964) found that

the antibiotic inhibits protein formation while allowing ribosomal and transfer RNA to accumulate. In reticulocytes, puromycin promotes a rapid nonenzymatic release of soluble proteins from the ribosomes (Zamecnik, 1962 and Morris et al., 1962). Zamecnik (1962) suggested that puromycin might act by interrupting the synthesis of hemoglobin at the ribosomal level. The antibiotic could thus act as a substituted amino acyl-tRNA to block completion of the protein chains at various points and result in the separation of the incomplete peptide chain from the ribosomes; each chain carrying a terminal puromycin molecule (Brockman and Anderson, 1963).

Yarmolinski and de la Haba (1959) found that puromycin not only inhibited incorporation of C^{14}-leucine into protein in a cell free preparation from rat liver, but also inhibited the transfer of C^{14}-leucine from C^{14}-leucyl-tRNA to protein in the same system. Although the transfer of the amino acyl residues from tRNA to the ribosomes was strongly reduced, Yarmolinski and de la Haba (1959) found that the activation of amino acids and the formation of amino acyl-tRNA was not affected. This work was later confirmed by von der Decken and Hultin (1960) using C^{14}-valyl-tRNA. Nathans and Lipmann (1961) also confirmed this work with a cell-free system of ribosomes of E. coli.

The structural requirements for puromycin inhibition of amino acid transfer was studied by Nathans and Neidle (1963) by examining the effects of analogs and isomers of puromycin in the E. coli ribosomal system. Their major conclusions with regard to the structural specificity of puromycin were: 1) Both the amino nucleoside and amino acid are required (Yarmolinski and de la Haba previously observed that removal of

the purine base resulted in loss of activity) 2) The amino nucleoside must be substituted at the 3'-position 3) The amino acid must be of L-configuration 4) Aromatic amino acid derivatives are by far the most potent, where as derivatives with certain amino acids are inactive 5) Compounds with a dipeptide side chain are inactive. Great differences in the activity of puromycin-like compounds with different amino acids were noted by Nathans and Neidle. They found that substitution with glycine or proline led to analogs with no activity, whereas substitution with leucine gave slight activity and with phenylalanine or tyrosine, marked activity. Nathans and Neidle state that the basis for this specificity is unknown, but could reflect a tighter binding of the aromatic amino acyl derivative to the inhibited site.



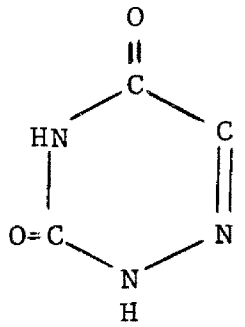
8-AZAGUANINE

It was demonstrated by Roblin et al. (1945), that the guanine analog, 8-azaguanine produced marked but reversible inhibition of growth among a variety of bacteria. Kidder and Dewey (1949) found that a similar inhibition occurred with the guanine-requiring organism, Tetrahymena. Similarly, Kidder et al. (1949), Sugiura et al. (1950), and Finkelstein and Thomas (1951), reported that the development of some viruses was also inhibited by this purine analog. Mangalo and Wachsmal (1961), stated that

the addition of 8-azaguanine to exponentially growing cells of Bacillus megaterium resulted in inhibition of growth after a lag period of approximately 30 minutes. In addition, they noted that this inhibitory effect could be reversed by addition of purines and their derivatives, but not by uridine, thymidine or cytidine. It was also reported by Kidder et al. (1952), Smith and Matthews (1956) and Hollinshead (1963) that 8-azaguanine is especially bactericidal under conditions where active growth is taking place. Mangalo and Wachsman (1961) found that growth of B. megaterium in the presence of a complete amino acid mixture either before or during exposure to the analog, increased the rate of killing. Kidder et al. (1952), also found that the inhibition ratio is unusual inasmuch as approximately 50 molecules of the natural purine must be added to the culture medium to overcome the effect of 1 molecule of the inhibitor.

One explanation proposed for the inhibition by 8-azaguanine is that it is incorporated into nucleic acid, forming an abnormal-inhibitory product (Kidder and Dewey, 1949). The first direct evidence for this incorporation was reported b Mitchell et al. (1950), when they obtained some evidence from tracer studies indicating the incorporation of this analog into the nucleic acids of both tumor tissue and nontumor tissue of tumor-bearing mice. In 1959, Chantrenne and Devreux stated that in the presence of 8-azaguanine the protein synthesis of Bacillus cereus is strongly inhibited and the formation of constitutive penicillinase is completely destroyed. These workers also found that low concentrations of the analog which were sufficient to block penicillinase formation did not change the rate of DNA synthesis. However, higher concentrations

caused an initial slight inhibition which became much stronger after about one doubling of the DNA. In addition, they found that 8-azaguanine increases the rate of RNA formation while being incorporated into the RNA molecule; as much as 40 percent of the RNA guanine was replaced by the analog.



6-AZAURACIL

The uracil analog, 6-azauracil was found by Handschmacher and Welch (1956) to be a competitive antagonist of uracil and uridine in a strain of Lactobacillus bulgaricus, able to utilize uracil for growth and a noncompetitive inhibitor of growth on orotate. In addition, they found that 6-azauracil not only inhibits those organisms dependent upon exogenously supplied pyrimidines, but also others in which derivatives of uracil are synthesized endogenously. Schindler and Welch (1957) reported that the uracil analog was also an inhibitor of several experimental tumors, including sarcoma 180 in mice. In contrast, however, 6-azauracil appears to exhibit no in vitro inhibitory effects on tissue cultures of HeLa cells or mouse fibroblasts.

Handschumacher (1957) and Schindler and Welch (1957) reporting on studies on nucleoside and nucleotide metabolites of the analog isolated from the culture medium or from the inhibited cells, suggest that 6-azauracil might first be converted to phosphorylated derivatives or its

riboside in order to become an effective inhibitor. Schindler and Welch (1957) working with the sarcoma 180 cells in tissue culture, found that 6-azauracil in concentrations as high as 5 mM was completely inactive as an inhibitor. This finding coupled with their observation of the *in vivo* inhibitory effect on sarcoma 180 cells in mice led them to suggest that the *in vivo* observation might be attributable to a metabolite of the analog formed by the liver or other normal tissue. A possible metabolite, they suggest, could be the phosphorylated riboside, 6-azauridine, since this derivative is also formed by certain microorganisms and inhibits the growth of 6-azauracil-resistant strains that emerge when *Streptococcus faecalis* is grown in the presence of 6-azauracil.

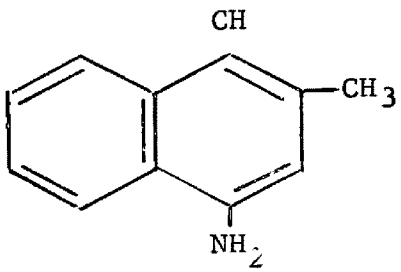
Sokoda (1958) found that orotic acid, orotidine 5'-phosphate and uridine 5'-phosphate accumulate in the culture medium of *E. coli* B following incubation with 6-azauracil.

Shigiura (1962) reported that the inhibitory capacity of 6-azauracil was enhanced with the exogenous addition of adenine and adenine derivatives, while Otsuji and Takagi (1958) found that exposure of *E. coli* K-12 in salt-glucose medium to 1 µg/ml of the uracil analog caused inhibition of growth.

Habermann (1960) states that with *E. coli* and *B. cereus*, after addition of 6-azauracil a temporary blocking of growth and RNA synthesis was observed. Concommitantly, a gradual cessation of protein synthesis was noted; however, Habermann suggests that this is secondary and is not directly inhibited.

Otsuji and Takagi (1958) suggest that the inhibitory effect of 6-azauracil on intact cells is not entirely directed toward formation of

"abnormal" nucleic acid and protein formation. Rather, a definite accululation of N-acetyl amino sugar esters noted 30 minutes after the addition of the uracil analog suggests, in part, an interference with utilization or function of uridine nucleotide compounds especially in the synthesis of cell walls.



VITAMIN K-5 (SYNKAMIN)
2 methyl-4 amino-1 hydroxynaphthalene

Vitamin K-5 (Synkamin) is a synthetic preparation used therapeutically for the prevention or correction of hypoprothrombinemia due to Vitamin K deficiency. In contrast to the natural Vitamin K, which is fat soluble and dependent on the presence of bile for the adequate absorption, Vitamin K-5 is water-soluble and is readily absorbed in the absence of bile (Merck Index, 7th Edition).

In addition ot its therapeutic value, particularly in children, Vitamin K-5 has also been shown to have antibacterial properties. Fosdick et al. (1942), reported that small amounts of synthetic Vitamin K (2-methyl-1, 4-naphthoquinone) prevented the production of acid when saliva-glucose mixtures were incubated. Armstrong and Knutson (1943) concluded that this effect of 2-methyl-1, 4-naphthoquinone was due to the quinone structure of the compound and found that the same results could be obtained, in varying degrees, with other naphtho-, tolu- and benzoquinones. Later the antibiotics Fumigatin and Actinomycin were

reported by Waksman and Woodruff (1942) to exhibit antibacterial properties and also, that they contained a quinone group.

Testing the vitamin analog against coagulase-positive strains of Staphylococcus, Armstrong et al. (1943), concluded that the antibacterial properties of Vitamin K-5 could be explained by its ready oxidation to the corresponding 1-4 naphthoquinone.

Comparing the antibacterial activity of three analogs of Vitamin K, Vitamin K-5, Hykinone (2-methyl-1, 4-naphthoquinone sodium bisulfite), and Synkayvite (tetrasodium 2-methyl-1, 4-naphthohydroquinone), Kimler (1950) found that the Vitamin K-5 analog was the most effective, while the other two were nearly devoid of antibacterial properties. He concludes that this was due, for the most part, to the presence of the free amino group at the fourth carbon. Similarly, Swartzman (1948) found that Vitamin K-5 was an effective antibacterial agent against both gram positive and gram negative organisms. He found the compound strongly effective against gram positive bacteria in the presence of both casein hydrolysate and blood serum, and against gram negative organisms in synthetic media. He also reported that the antibacterial activity of Vitamin K-5 was related to air oxidation and that excessive oxidation brought about a loss of activity. He suggested that the antibacterial activity of the vitamin analog was due to an intermediate state of oxidation by air which possibly takes place immediately upon dissolving the substance. However, he also states that the presence of an amino group plays an important role in its antibacterial activity.

In spite of this biological activity, it appears that Vitamin K-5 is not toxic to individuals or animals and that side effects are

negligible when given in appropriate doses. However, Allison (1955) reported that excessive doses of the vitamin analog could have the potential of causing hyperbilirubinemia, hemolytic anemia and kernicterus. Laurance (1955) also reported that 6 newborn children developed clinical signs of kernicterus and died after receiving Vitamin K-5 for 3 days. Moore and Sharman (1959) found that in mice and rats deficient in Vitamin E, an intense hemoglobinurea resulted following intramuscular injection of the vitamin analog. The effect, however, was temporary and both animals and their urine appeared normal following 24 hours. Experimentally, Skimkin (1941) noticed that I.P. injections of Vitamin K-5 in low concentrations in mice produced no observable toxicity except for a slight discoloration of the urine. In high concentrations, however, marked hemorrhagic extravastion in the renal tubules and in the liver occurred and if death occurred, it was due to respiratory failure.

CHAPTER II

STATEMENT OF PROBLEM

This investigation was undertaken to study the relative pathogenicity of normal- and inhibitor-grown systemic pathogenic fungi and the possible relationship of nucleic acid content, respiration and ultraviolet sensitivity to infectivity and viability.

CHAPTER III

MATERIALS AND METHODS

I. GENERAL MATERIALS AND METHODS

(1) Organisms Employed.

Two strains of Blastomyces dermatitidis were used in this investigation: strain 6046, obtained from the Rocky Mountain Laboratory, U.S.P.H.S., Hamilton, Montana; and strain LBa, obtained from the Carolina Biological Supply Company, Burlington, North Carolina. Two additional organisms were also included in this study: Histoplasma capsulatum, strain 10230, obtained from the American Type Culture Collection, Washington D.C.; and Sporotrichum schenckii obtained from the Communicable Disease Center, U S P.H.S., Atlanta, Ga.

(2) Buffer and Saline Solutions

The sterile phosphate-buffered saline (SPBS) used throughout this investigation consisted of 180 ml of 0.1M K_2HPO_4, 120 ml of 0.1M KH_2PO_4, 700 ml of distilled water and 8.5 g NaCl. The pH of this solution remained 7.0 after sterilization at 121 C for 15 minutes.

Sterile and nonsterile phosphate-buffered solutions used in preparing media and in experimental studies consisted of 180 ml of 0.1M K_2HPO_4, 120 ml of 0.1M KH_2PO_4 and 700 ml distilled water. Following addition of the medium, the solutions were autoclaved.

Unbuffered saline solutions were prepared by adding 8.5 g NaCl to one liter of distilled water.

(3) Culture Media and Maintenance of Cultures.

Stock cultures of all organisms were grown and maintained on Sabouraud Dextrose Agar (SAB) slants, pH 7.0 (Baltimore Biological Laboratory). Yeast phase cultures were incubated at 37 C and were transferred every 10 to 14 days. Stock cultures were checked periodically for contamination by microscopic examination.

Cultures for experimental use were grown in Bacto-Eugon broth (Difco). The phosphate buffered medium was dispensed in 150-ml aliquots into 1000-ml erlenmeyer flasks and sterilized in the autoclave for 15 minutes at 121 C. Following inoculation of the media, the cultures were placed on a platform shaker and incubated at 37 C. The shakers were adjusted so as to just break the surface and allow for aeration of the liquid medium. Organisms cultured in this manner are referred to throughout this thesis as "normal" cells.

(4) Preparation of Cells.

A variety of cell suspensions and concentrations were used throughout this investigation. However, initial procedures in preparing the suspensions were conducted in the following manner. Five-day yeast phase cells were transferred in 10-ml aliquots to sterile tubes and centrifuged at 2,000 r.p.m. in an International Clinical Centrifuge for 10 minutes. The cells were then washed four times with 5-ml aliquots of SPBS. If higher concentrations of cells were necessary, additional aliquots from the culture flasks were added following the first centrifugation. The cell suspensions were then diluted to a volume of 50 ml. This 50 ml of suspension was placed in

a Waring blender and agitated at low speed for 10 to 15 minutes at approximately 8 C to assure separation of all cell aggregates. After blending, aliquots were removed and their optical densities adjusted with a Coleman Junior Spectrophotometer at 600 mμ. In some cases, further treatment of the cell suspension was necessary as warranted by the experiment in question.

Mycelial phase cells were filtered aseptically on Whatman No. 1 filter paper disks in a sterile Buchner funnel. The cells were stirred periodically during filtration with sterile glass rods. The washing procedure was repeated four times after which the cells were transferred to sterile vials and resuspended in 0.85 percent SPBS. In most cases the cell suspensions were further treated as required by the experiment and as described under Experimental Methods.

(5) Plate Counting Method.

Phosphate-buffered SAB at pH 7.0 was used as the plate count medium Standard plate counting methods were employed using the Quebec Colony Counter for counting colonies on the plates. When possible, all colonies on the plates were counted.

(6) Ultraviolet Light Source and Procedure for Irradiation.

The source of ultraviolet light was a Mineralight, Model SL 2537 short wave lamp. Factory rated, the maximum output of the lamp was approximately 2,537 Å at 5 watts. The distance from the mercury tube to the bottom of the 100-ml beaker was 15.5 cm in all experiments. This distance established a dose rate of 18.5 ergs/mm^2 per second as measured

by the method of Jagger (1961). Cells which were to be irradiated were suspended in SPBS and the optical density adjusted to 0.525 at 600 mμ. Aliquots of 20 ml were transferred to sterile 100-ml beakers. The output of the lamp was allowed to equilibrate for 5 minutes before irradiating the cells. During irradiation the suspensions were constantly rotated on a Yankee Rotator at 180 r.p.m. A cabinet was constructed to enclose the rotator and lamp and all work was conducted in a darkened room. Following irradiation 1-ml and 0.1-ml of 1:10 and 1:100 dilutions of the cell suspensions were plated. All plates were incubated in a moist chamber at 37 C for 7 days.

(7) Mice.

Mice used throughout this investigation were 21- to 26-day old females of the CFW strain originally obtained from the Rocky Mountain Laboratory, U.S.P.H.S., Hamilton, Montana. The colony was maintained in the Microbiology animal quarters, University of Montana.

II. EXPERIMENTAL METHODS AND PROCEDURES

(1) Culture Methods for Inhibitor Grown Cells.

The inhibitors used in this study were the analogs 8-azaguanine (Nutritional Biochemicals Corporation), 6-azauracil (N.B.C.) and the antibiotic, puromycin (N.B.C.). The analogs were added to separate flasks containing phosphate-buffered Bacto-Eugon broth and then autoclaved for 15 minutes at 121 C. Swinney filtered Puromycin was added aseptically to previously autoclaved phosphate-buffered Bacto-Eugon broth. The

concentrations of these agents for the organisms grown are summarized in Table 1. Organisms grown in the presence of 8-azaguanine, 6-azauracil or puromycin are referred to as "inhibitor grown cells" throughout this thesis. Following inoculation of the culture media the flasks were incubated in the same manner as the normal cells.

(2) Studies to Determine Tolerance Levels to the Inhibitors.

A. Tolerance Levels of Microorganisms to the Inhibitors.

Yeast phase cells of all the organisms were employed. Melted phosphate-buffered Eugon agar was dispensed in 10-ml aliquots into test tubes and incubated in a 55 C water bath. Two-fold dilutions of 8-azaguanine and 6-azauracil were made starting, in each case, with a concentration of 50 mg/ml. The two-fold dilutions were carried through seven tubes resulting in a low concentration of 0.39 mg/ml. The tubes were then capped and sterilized at 121 C for 15 minutes. Following sterilization the media were poured into sterile petri plates and allowed to solidify. Two-fold dilutions of puromycin were also made by adding filter-sterilized puromycin aseptically to the first tube of each series of melted sterile Eugon agar at 50 C. The final concentration of puromycin in the first tubes was 200 µg/ml. Two-fold dilutions were carried through 6 tubes in each series, resulting in low concentrations of 3.125 µg/ml. The media were then poured into sterile petri plates.

Five-day yeast phase cell suspensions in SPBS were prepared as previously described. The optical densitites in each case was adjusted to 0.525 at 600 mµ. All plates were inoculated with 1-ml aliquots and incubated in moist chambers at 37 C for 10 days. The highest concentration

of the inhibitors showing appreciable growth were used for further studies.

B. Tolerance Levels of Mice to Vitamin K-5.

Several methods of preparing the Vitamin K-5 solution were attempted resulting in varying degrees of success. The procedure finally employed was to suspend Vitamin K-5 in previously sterilized 0.15M phosphate buffer. Though some flocculation occurred, the suspension was constantly agitated to provide a uniform suspension. Two-fold dilutions of the Vitamin K-5 solutions were used for initial screening. The highest concentration was 10 mg/ml. Two-fold dilutions were carried through 6 series, resulting in a low concentration of 0.3125 mg/ml. Ten mice were injected with Vitamin K-5 in each series, and 10 received sterile phosphate buffer as control. All mice were injected with 1-ml doses intraperitoneally. Surviving mice were reinjected 48 hours later with the same concentration. This was repeated every 48 hours for as long as the mice survived or until a maximum of 15 doses had been administered. All mice were autopsied upon death.

(3) Analytical Methods for Nucleic Acids.

The methods employed for analysis of RNA and DNA were similar to those used in "Methods of Biochemical Analysis" (Volkin and Cohn, 1954). Also employed were procedures used by Morse and Carter (1949). Cells were prepared as previously described with distilled water being used for the final 3 washings. The final volume of the cell suspension was adjusted to 10 ml and 1 ml from each preparation was removed and placed in separate tared crucibles for dry weight determinations. Aliquots of

5 ml and an equal volume of No. 10 Ballotini beads were then agitated in a Mickel disintegrator at maximum transit for 30 minutes at 4 C. The entire contents of the Mickle vial was then centrifuged for 10 minutes. The supernatant fluid was transferred to a test tube and treated, in an ice bath, with 10 percent trichloroacetic acid (TCA) for 30 minutes. Following centrifugation for 3 minutes in the cold, this procedure was repeated. The residue was treated with 1 ml of distilled water and 4 ml of 95 percent ethyl alcohol and centrifuged for 3 minutes. The residue was extracted and washed 3 times with 3:1 95 percent ethyl alcohol: diethyl ether at room temperature. Two ml of 0.3 N KOH was added to the residue and allowed to stand for 16 to 20 hours in a 37 C water bath. The solution was then neutralized with 6 N perchloric acid. An equal volume of 5 percent TCA was added and the entire mixture was centrifuged. This procedure was repeated and the combined supernatant fluids saved for RNA analysis. The remaining residue was suspended in 5 ml of 5 percent TCA, heated for 15 minutes at 90 C and centrifuged. The supernatant was decanted and the procedure repeated with 2.5 ml TCA at room temperature. Following centrifugation the supernatant fluids were combined and saved for DNA analysis.

A. RNA Analysis.

Stock RNA reagent was prepared by dissolving 13.5 g ferric ammonium sulfate and 20 g recrystalized orcinol in 500 ml of distilled water. Prior to use, 2.5 ml of this stock reagent was added to 41.5 ml of concentrated HCl and diluted to 50 ml with distilled water. In the test for RNA, 3 ml of the serially diluted test solution (containing 4-40

µg pentose) was mixed with 9 ml of reagent. The mixture was heated in a boiling water bath for 20 minutes and then cooled to room temperature. Optical densities of the solution were determined at 660 mµ. The readings obtained were compared with a reference curve prepared with solutions containing known RNA (Nutritional Biochemicals Corporation) concentrations.

B. DNA Analysis.

The DNA reagent was prepared by dissolving 1.0 g of diphenylamine in 98 ml of redistilled glacial acetic acid and 2 ml of concentrated H_2SO_4. For the analysis of DNA, 7.5 ml of the reagent was added to 3 ml of the test solution. The mixture was heated for 10 minutes in a boiling water bath then cooled to room temperature. Optical densities were determined at 540 mµ and the experimental values compared with a reference curve prepared with known concentrations of DNA (N.B.C.).

(4) Studies to Determine the Effects of Irradiation on Normal and Inhibitor Grown Cells.

Five-day yeast phase normal and inhibitor grown cells were prepared in the manner previously described and were suspended in SPBS. The optical density of each cell suspension was adjusted to a reading of 0.525 at 600 mµ. The time of irradiation ranged from 0 to 60 minutes with samples taken at intervals of 1, 2, 3, 4, 5, 10 and 15 minutes and every 10 minutes thereafter. Normal and puromycin-grown cells were plated on SAB buffered with 0.1M phosphate buffer at pH 7.0. Normal and analog-grown cells were recovered on SAB and were also plated on SAB media containing the normal purine and pyrimidine. The concentrations

of the guanine and uracil in the plate count media were the same as that of their analogs in the respective culture media. This step was included to test for possible chemcial reversibility of the irradiation effects on analog-grown cells. All plates were incubated in a moist chamber at 37 C for 7 days.

(5) Manometric Methods.

A. Studies to Determine the Respiration of Normal and Inhibitor Grown Cells.

Conventional manometric techinques were employed (Umbriet, Burris and Stauffer, 1949). The main compartment of the flask was charged with 1 ml of 0.1M substrate solution in 0.15M phosphate buffer. The substrates employed were: glucose (Allied Chemical); monosodium glutamate (N.B.C.); ribose (N.B.C.); fructose (Fisher Scientific Company) and xylose (Fisher Scientific Company). The endogenous system was prepared in the manner indicated above; however, 0.15M phosphate buffer was used in place of the substrate-buffer solution. The center well was charged with 0.2 ml of 20 percent KOH. A piece of filter paper was folded and placed in the center well in order to aid absorption of the carbon dioxide evolved. Yeast phase cells from a 5-day broth culture were washed as previously discussed and after the final washing suspended in 0.85 percent saline. The suspension was adjusted to an optical density of 0.525 at 600 mμ and a 1-ml aliquot pipetted into one of the side arms. The water bath temperature was maintianed at 37 C $\pm$ 0.2 C. The flasks were allowed to equilibrate for 20 minutes with the stopcocks open. All connections were then reexamined for leakage. The levels of the Brodie solution in the closed end of the manometers were adjusted to the 150

mm mark, the stopcocks closed and the shaker started. The system was then allowed to equilibrate for 5 minutes before the first reading was recorded. The cell suspension was then tipped into the main flask compartment. The sidearms were rinsed with some of the solution from the main compartment in order to mix the cells and the substrate. Readings were recorded every 30 minutes for the first hour then every 60 minutes following. Thermobarometer and endogenous systems were manipulated in the same manner as the experimental systems throughout each experiment. One control system using normal cells was always employed when studies involved the use of inhibitor grown cells.

B. Studies to Determine the Effect of Vitamin K-5 on Cellular Respiration.

Manometric techinques previously described were used throughout this series of experiments. The second sidearm was charged with 1 ml of a 0 15M phosphate-buffered solution of Vitamin K-5. All experiments were accomplished using a Vitamin K-5 concentration of 1.5 mg/ml The vitamin solution was tipped into the main compartment 2 hours after addition of the cells to the substrate. Control and thermobarometer systems received 1 ml of 0.15M phosphate buffer in lieu of the vitamin solution. A second endogenous system was also employed using the Viatmin K-5 solution. The average length of time for each experiment was 9 hours.

(6) Studies to Determine the In Vivo Effect of Inhibitors on Virulence.

A. In Vivo Studies with Normal and Inhibitor Grown Cells.

Five-day yeast phase cells were harvested, washed and suspended in SPBS as previously described. Normal and inhibitor-grown cells were treated in the same manner. After the cells were blended, the suspension was initially adjusted to an optical density of 0.825 at 600 mμ. The final concentration was then adjusted to 4.0×10^6 cells/ml by actual hemocytometer count. The suspensions were then diluted with an equal volume of sterile 5 percent gastric mucin adjuvant, pH 7.0. This resulted in a final concentration of 2.0×10^6 cells/ml. The suspensions were mixed by shaking and 0.5-ml aliquots were pipetted from each and plated on SAB, pH 7.0. These were used to establish the viability of the cells inoculated. One ml of the suspensions was inoculated intraperitoneally in each of 10 mice. The mice were checked on the average of 2 to 3 times daily and any dead ones were removed and autopsied. At the end of 8 weeks, the remaining mice were etherized and autopsied. Any visible lesions on the liver, spleen, lungs or diaphragm were removed aseptically and placed on SAB agar slants. If lesions were not visibly present, the liver, lungs and spleen of each animal were removed and macerated with 5 ml of SPBS. One-ml aliquots of these suspensions were plated on SAB plates and incubated at 37 C for 2 to 3 weeks. Livers and lungs showing visible lesions were also taken from each test group and placed in 10 percent buffered formalin. After fixing for 2 to 3 days the tissues were washed for 1 hour in running water then dehydrated through an ethyl alcohol series. Following complete dehydration in absolute ethyl alcohol, the

tissues were cleared for 48 hours in methyl salicylate. The tissues were then rinsed in toluene and imbedded in paraffin. Sections were made using an American Optical microtome model 815. Thickness of the sections varied between 8 and 15 microns. The sections were stained with the Hematoxylin-Eosin method and the Periodic Acid-Schiff reaction.

B. In Vivo Studies with Normal Grown Cells and Vitamin K-5.

Five-day normal yeast phase cells were prepared to a final concentration of 1.0×10^5 cells/ml as described in Part A. The experiment was divided into 6 experimental groups of 10 mice for each organism employed.

GROUP I. (CONTROL)

The mice were injected intraperitoneally (IP) with 0.5-ml doses of a 1.0 mg/ml Vitamin K-5 solution every 48 hours until a total of 6 mg of the vitamin had been administered. The mice were then injected IP, with a 1.0-ml aliquots of 2.5 percent gastric mucin in lieu of the cell suspension. Following injection of the adjuvant, 4 additional 0.5-mg doses of the vitamin were administered at 48 hour intervals.

GROUP II. (CONTROL)

Mice in this group received sterile 0.15M phosphate buffer in lieu of the Vitamin K-5 solution. The schedule was the same as that described for Group I. In place of the cell suspension, these mice were injected with 1.0 ml of 2.5 percent gastric muein.

GROUP III. (CONTROL)

Sterile 0.15M phosphate buffer was administered to the mice in lieu of the Vitamin K-5 solution. The schedule of injections was the same as

that described for Group I. Cells suspended in the adjuvant were injected intraperitoneally in 1.0-ml doses and this was followed by 4 additional doses of sterile 0.15M phosphate buffer at 48 hour intervals.

GROUP IV. (CONTROL)

The mice in this group were injected with the cell suspension; however, they did not receive Vitamin K-5 or 0.15M phosphate buffer.

GROUP V (EXPERIMENTAL)

The mice were injected IP with 0.5-ml doses of 1.0 mg/ml Vitamin K-5 solution every 48 hours until a total of 6 mg of the vitamin had been administered. The cells, suspended in the gastric mucin adjuvant, were then injected IP in 1.0-ml aliquots. Following injection of the cells, 4 additional 0.5-mg doses of the vitamin were administered at 48 hour intervals.

GROUP VI. (EXPERIMENTAL)

Mice in this group did not receive Vitamin K-5 prior to the IP injection of the cells Four hours following injection of the cells, 0.5 mg of the vitamin was administered IP. This was followed by injection of 0.5-mg doses at 48-hour intervals until a total of 6 mg had been administered.

C *In Vivo Studies with Bacterial Cells and Vitamin K-5.*

Cultures of *Streptococcus pyogenes* and *Salmonella typhosa* were maintained on Brain Heart Infusion agar slants (Difco) at 37 C. Cell suspensions were prepared as previosly described for the yeast

suspensions. The final concentration of the cells was adjusted to 1.0 x 10^6 cells/ml. Aliquots of 1 ml were injected IP into each mouse. Mice were treated prior to cellular inoculation with Vitamin K-5 as previously described for Group V. However, following inoculation of the bacterial cells, no additional injections of the vitamin were administered.

Positive determinations of an infected mouse, for all studies, were made by recovery of characteristic growth in culture, microscopic examination of stained tissue section.

CHAPTER IV

RESULTS

(1) Tolerance Levels of the Microorganisms to the Inhibitors.

Tolerance levels of the organisms varied with the inhibitor employed. The results summarized in Table 1 show that S. schenckii exhibited the greatest overall tolerance to the inhibitors. It was also noted that the soluble pigment normally evident after 7 to 10 days incubation became markedly noticeable after 2 to 3 days incubation in the presence of the inhibitors. H. capsulatum exhibited the lowest overall level of tolerance resulting in a 2- to 4-fold difference from that of S. schenckii. A distinct variation was noted between the two strains of B. dermatitidis. Though the maximum concentration for 8-azaguanine was 2.5 mg/ml for both strains, a 2-fold difference in tolerance level resulted with 6-azauracil and puromycin.

Table 1
Maximum* Tolerance Level of the Organisms
to the Inhibitors

Organism	6-azauracil	8-azaguanine	Puromycin
B. dermatitidis strain 6046	3.12 mg/ml	2.50 mg/ml	50 µg/ml
B. dermatitidis strain Lba	1.56 mg/ml	2.50 mg/ml	25 µg/ml
S. schenckii	6.26 mg/ml	2.50 mg/ml	50 µg/ml
H. capsulatum	1.56 mg/ml	1.56 mg/ml	25 µg/ml

* Concentrations higher than those indicated completely inhibited growth.

(2) Tolerance Level of Mice to Vitamin K-5.

All mice injected with 10 mg/ml in a single injection of Vitamin K-5 died within 5-15 minutes after administration. Within 10-30 minutes after injection, 100 percent of those mice receiving 5 mg/ml in a single dose were also dead. Mice receiving 2.5 mg/ml survived the first dose; however, all died within 30 minutes after administration of the second similar injection 48 hours later. All mice injected with 1.25 mg/ml survived the first two doses; however, within 3 hours after the third dose had been administered, 60 percent had died. The surviving mice all died within 30 minutes following the fourth injection. All mice receiving 0.625 mg/ml and 0.313 mg/ml doses survived a series of 10 injections of these quantities given at 48-hour intervals. Marked toxicity to Vitamin K-5 was observed in those mice receiving the 0.625 mg/ml doses. Mice injected with 0.312 mg/ml doses appeared to remain normal except that all passed reddish brown urine. Upon autopsy, no noticeable organic disorders could be observed in any of the mice injected with the vitamin analog. Liver, kidney and spleen coloration remained normal. The spleen was not distended. All mice showing toxemia to the vitamin analog exhibited extreme hyperactivity, scratching, labored breathing and convulsions. The experiment was repeated with 0.5 mg/ml concentrations and resulted in no apparent toxicity to the mice after 15 injections although reddish brown urine was observed.

(3) The Effect of Inhibitors on the Concentration of Nucleic Acids. (Figures 1, 2, 3.)

The total RNA concentration of normally grown organisms ranged from an average of 68.2 μg/mg dry weight for S. schenckii to 86.6 μg/mg

dry weight for B. dermatitidis strain LBa. The concentration difference between B. dermatitidis strains LBa and 6046 averaged 5.8 µg/mg dry weight, strain LBa having consistently the higher concentration. H. capsulatum averaged 74.4 µg/mg dry weight.

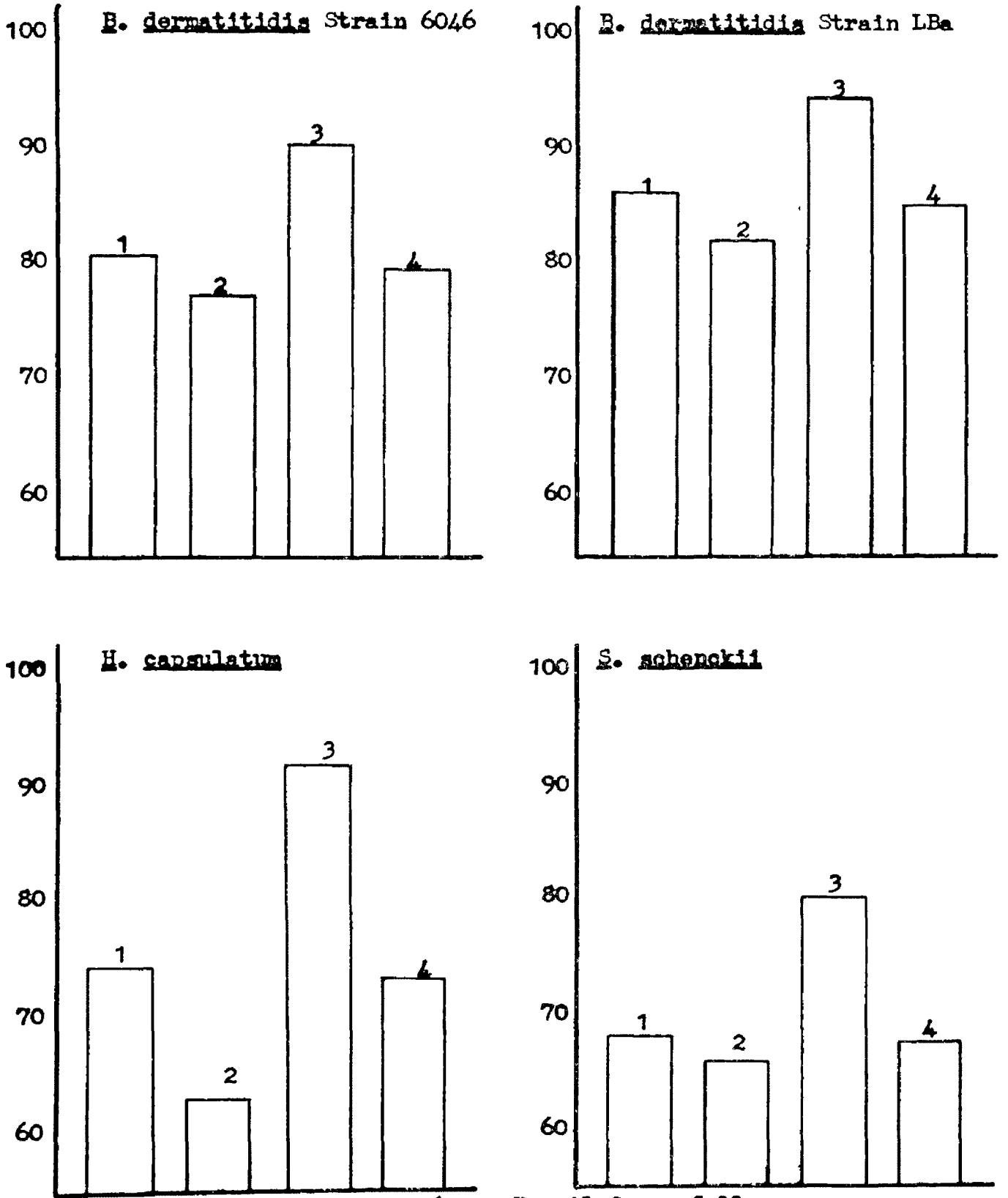
The effect of puromycin on the total RNA concentration was negligible with all values being within ± 1.1 µg of the normal cells. The uracil analog produced only slight changes from the normal cells. Net changes of the 6-azauracil grown cells from the normal cells ranged from 2.3 µg/mg dry weight decrease for S. schenckii to 6.4 µg/mg decrease for H. capsulatum. This represents a change of 3.7 to 8.6 percent from the normal. B. dermatitidis strains LBa and 6046 exhibited similar slight decreases in RNA concentration. The respective changes represented 4.8 and 4.2 percent decreases.

The organisms grown in the presence of 8-azaguanine showed consistent increases in RNA concentration. H. capsulatum exhibited the greatest change of 17.6 µg/mg or a 23.8 percent increase. The net changes for B. dermatitidis strains LBa and 6046 represented a respective increase of 9.5 and 11.8 percent. S. schenckii showed an increase of approximately 17.8 percent.

The DNA concentration of the normal cells ranged from 8.3 µg/mg dry weight for H. capsulatum to 6.2 µg/mg dry weight for S. schenckii. B. dermatitidis strains LBa and 6046 averages 7.2 µg/mg dry weight and 6.3 µg/mg dry weight respectively. The changes in DNA concentrations for inhibitor grown cells from that of the normal were negligible for all organisms and inhibitors employed.

Fig. 1

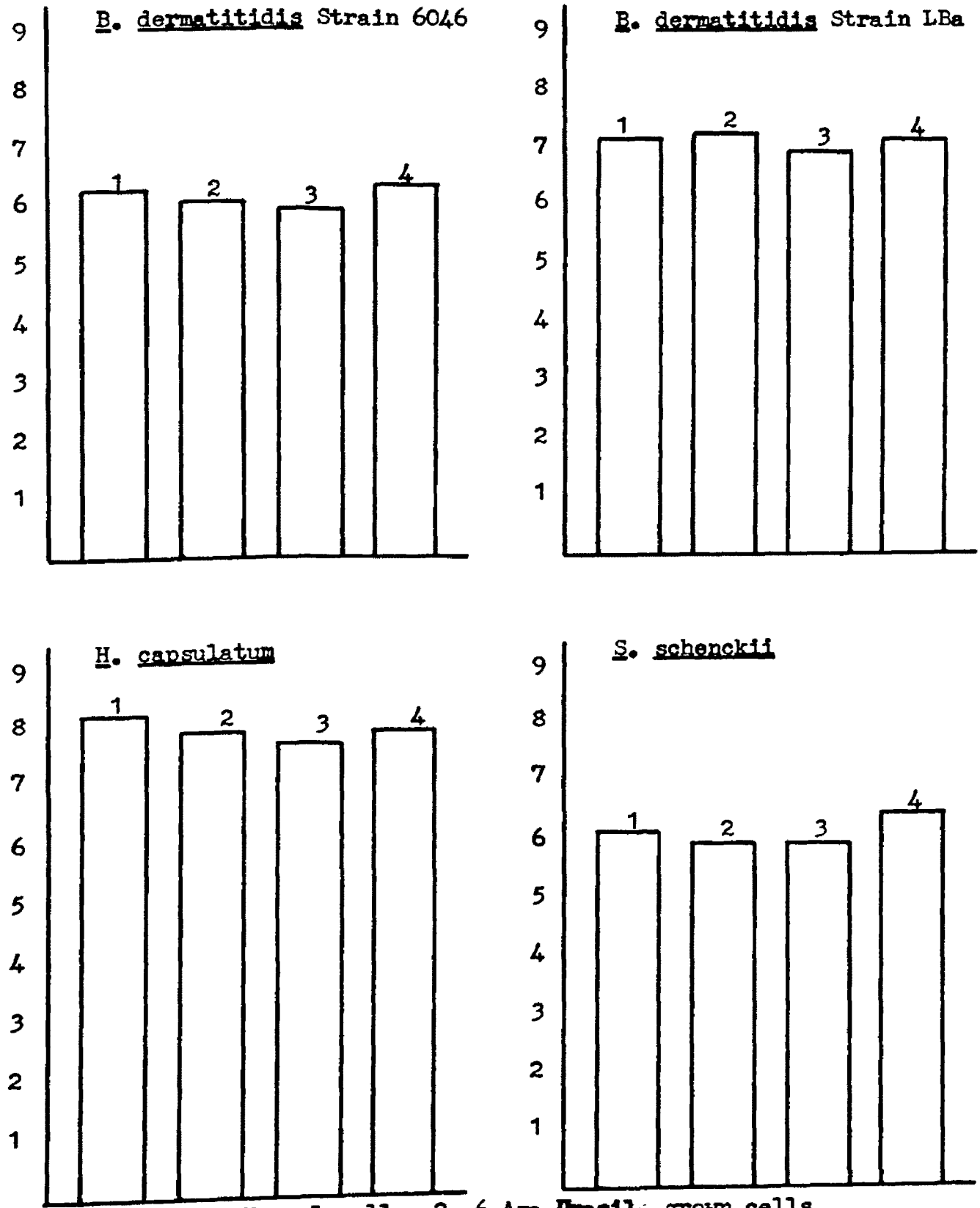
THE RNA CONCENTRATION OF NORMAL AND INHIBITOR GROWN CELLS
µg/mg Dry Weight



1- Normal Cells, 2- 6-Aza Uracil Grown Cells,
3- 8-Aza Guanine Grown Cells, 4- Puromycin Grown Cells.

Fig. 2

THE DNA CONCENTRATION OF NORMAL AND INHIBITOR GROWN CELLS
µg/mg Dry Weight



1- Normal cells, 2- 6-Aza Uracil grown cells,
3- 8-Aza Guanine grown cells, 4- Puromycin grown cells.

Fig. 3

THE CONCENTRATION OF NUCLEIC ACIDS IN NORMAL AND INHIBITOR GROWN CELLS
(µg/mg Dry Weight)

RNA CONCENTRATION

	Normal	6-Aza Uracil	8-Aza Guanine	Puromycin
B. dermatitidis 6046	80.8	77.4	90.4	79.7
Net Change		-3.4	+9.6	-1.1
B. dermatitidis LBa	86.6	82.4	94.7	85.5
Net Change		-4.2	+8.1	-1.0
H. capsulatum	74.4	62.8	92.0	73.6
Net Change		-6.4	+17.6	-0.8
S. schenckii	68.2	65.9	80.4	67.8
Net Change		-2.3	+12.2	-0.4

DNA CONCENTRATION

	Normal	6-Aza Uracil	8-Aza Guanine	Puromycin
B. dermatitidis 6046	6.3	6.1	6.0	6.4
Net Change		-0.1	-0.3	+0.1
B. dermatitidis LBa	7.2	7.3	7.0	7.2
Net Change		+0.1	-0.2	±0.0
H. capsulatum	8.3	8.0	7.8	8.0
Net Change		-0.3	-0.5	-0.3
S. schenckii	6.2	6.0	6.0	6.5
Net Change		-0.2	-0.2	+0.3

(4) The Effects of Ultraviolet Irradiation on Normal and Inhibitor Grown Cells.

Normally-Grown Cells.

Irradiated cells of normally-grown B. dermatitidis strain LBa exhibited little differences when recovered on SAB, SAB plus uracil and SAB plus guanine. Following 5, 15, and 45 minutes of irradiation recovery on SAB averaged 70.2, 46.8 and 6.8 percent of the 0 time control. Respective recovery values obtained with SAB plus uracil averaged 76.6, 40.2 and 8.7 percent. Similarly, percent recovery on SAB plus guanine after the same periods of irradiation averaged 73.3, 49.2 and 7.3

Percent recovery of B. dermatitidis strain 6046 after 5, 15 and 45 minutes exposure to ultraviolet averaged 62.3, 33.2 and 0.26, respectively, on SAB agar. On SAB plus uracil respective values were 67.1, 24.7 and 0.11 percent for the same time periods. Using SAB plus guanine as the recovery medium, respective survival rates averaged 58.6, 38.2 and 0.11 percent.

Irradiated cells of S. schenckii recovered on SAB agar exhibited average percent survival values of 92.2, 80.3 and 1.1, respectively at 5, 15 and 45 minutes. Respective values obtained using SAB plus uracil were 87.7, 81.1 and 0.62 percent. On SAB plus guanine percent recovery during the same time periods averaged 88.2, 75.1 and 2.6 percent.

Recovery of H. capsulatum cells following 5, 15 and 45 minutes irradiation produced respective values averaging 72.4, 34.2 and 0.31 percent of SAB medium. Using SAB plus uracil as the recovery medium, respective values averaged 70.2, 38.6 and 0.81 percent. On SAB plus

guanine survival rates following the same periods of exposure were 74.7, 30.0 and 1.3 percent.

6-Azauracil-Grown Cells.

Following 5, 15 and 45 minutes of irradiation recovery of B. dermatitidis strain LBa on SAB medium averaged 69.4, 42.5 and 1.4 percent of 0 time controls. Recovery on SAB plus uracil differed little with respective values of 71.1, 43.1 and 2.6 percent.

Percent recovery of B. dermatitidis strain 6046 after 5, 15 and 45 minutes exposure to ultraviolet averaged 57.7, 28.6 and 0.73 on SAB agar while on SAB plus uracil, values were 58.3, 29.7 and 0.96 percent, respectively.

Irradiated cells of S. schenckii recovered on SAB agar exhibited percent survival rates of 90.1, 77.1 and 0.71, respectively after 5, 15 and 45 minutes exposure. Respective values obtained using SAB plus uracil were 91.2, 71.2 and 0.71 percent.

Recovery of H. capsulatum cells on SAB following the same periods of irradiation were 70.1, 33.6 and 0.57 percent. On SAB plus uracil, recovery values averaged 71.6%, 35.1% and 0.82%, respectively.

8-Azaguanine-Grown Cells.

Irradiated cells of B. dermatitidis strain LBa exhibited percent survival rates of 62.1, 42.3 and 0.14, respectively, following 5, 15 and 45 minutes irradiation. Recovery was accomplished on SAB medium. On the "enriched" medium of SAB plus guanine, respective rates were 71.2, 43.2 and 1.1 percent.

Percent recovery of B. dermatitidis strain 6046 after the same periods of irradiation, averaged 46.6, 19.7 and 0.09 on SAB medium, while using SAB plus guanine values averaged 51.1, 24.4 and 1.0 percent.

Results of S. schenckii cells following 5, 15 and 45 minutes irradiation and recovered on SAB averaged 81.1%, 41.1% and 0.16%, respectively. Using SAB plus guanine as the recovery medium, respective percent survival rates were 85.6, 45.1 and 0.56.

Irradiated cells of H. capsulatum recovered on SAB following similar periods of exposure were 64.7%, 28.3% and 0.11%, while on the enriched medium respective values averaged 68.3, 30.1 and 0.22 percent.

Puromycin-Grown Cells.

Cells grown in the presence of puromycin were found to be the most sensitive to ultraviolet irradiation.

Table 2
Percent Survival of Puromycin-Grown Cells
After Varying Exposure to Ultraviolet.

TIME (Minutes)	A	B	C	D
0	100.00	100.00	100.00	100.00
1	96.10	94.20	97.20	90.20
2	80.40	71.60	82.20	65.60
3	41.10	43.20	46.30	35.10
4	17.60	12.70	20.10	14.20
5	3 70	1.60	9.70	1.60
10	0.82	0.43	0.61	0.12
15	0.27	0.11	0.07	0.09
25	0.07	-----	-----	-----
35	-----	-----	-----	-----

A. B. dermatitidis Strain LBa; B. B. dermatitidis Strain 6046; C. S. schenckii; D. H. capsulatum.

Table 3

PER CENT SURVIVAL* OF NORMAL AND INHIBITOR GROWN E. DERMATITIDIS STRAIN LBa AFTER VARYING EXPOSURE TO ULTRAVIOLET

	NORMALLY-GROWN			6-AZAURACIL-GROWN		8-AZAGUANINE-GROWN	
Time (Minutes)	SAB	SAB URACIL	SAB GUANINE	SAB	SAB URACIL	SAB	SAB GUANINE
0	100.00	100.00	100.00	100.00	100.00	100.00	100.00
1	99.30	95.20	100.00	97.70	98.20	97.20	97.40
2	96.10	95.80	93.20	97.10	97.60	96.40	97.10
3	92.20	94.40	91.60	92.70	90.40	92.10	91.60
4	87.10	90.60	87.40	82.10	85.30	83.50	86.30
5	70.20	76.60	73.30	69.40	71.70	62.10	71.20
10	60.10	57.70	58.20	59.70	62.40	50.40	53.70
15	46.80	40.20	49.20	42.50	43.10	42.30	43.20
25	29.90	27.30	32.60	24.80	21.80	12.10	18.10
35	15.10	17.70	16.40	7.80	8.30	2.10	4.30
45	6.10	8.70	7.30	1.40	2.60	0.14	1.10
55	2.10	3.30	3.90	0.48	0.83	0.05	0.17
60	0.25	0.47	0.32	0.09	0.12	—	—

* Percent recovery calculated from 0 time controls.

Table 4

PER CENT SURVIVAL* OF NORMAL AND INHIBITOR GROWN B. DERMATITIDIS STRAIN 6046 AFTER VARYING EXPOSURE TO ULTRAVIOLET

	NORMALLY-GROWN			6-AZAURACIL-GROWN		8-AZAGUANINE-GROWN	
Time (Minutes)	SAB	SAB URACIL	SAB GUANINE	SAB	SAB URACIL	SAB	SAB GUANINE
0	100.00	100.00	100.00	100.00	100.00	100.00	100.00
1	99.20	100.00	98.60	98.30	97.30	99.40	99.60
2	98.60	96.60	98.80	98.60	97.70	96.70	98.50
3	96.30	93.70	95.40	97.20	96.40	96.20	97.70
4	80.40	77.30	85.20	80.10	81.10	78.90	82.60
5	62.30	67.10	58.60	57.70	58.30	46.60	51.10
10	51.10	48.80	46.10	44.10	47.80	30.20	37.30
15	33.20	24.70	38.20	28.60	29.70	19.70	24.40
25	14.20	10.20	12.30	10.30	7.60	6.70	11.10
35	3.20	4.70	7.60	1.30	2.70	0.88	1.70
45	0.26	0.11	0.10	0.73	0.96	0.09	1.00
55	0.05	0.10	—	0.04	0.05	—	—
60	—	—	—	—	—	—	—

* Per cent recovery calculated from 0 time controls.

Table 5

PER CENT SURVIVAL* OF NORMAL AND INHIBITOR GROWN S. SCHENCKII AFTER VARYING EXPOSURE TO ULTRAVIOLET

	NORMALLY-GROWN			6-AZAURACIL-GROWN		8-AZAGUANINE-GROWN	
Time (Minutes)	SAB	SAB URACIL	SAB GUANINE	SAB	SAB URACIL	SAB	SAB GUANINE
0	100.00	100.00	100.00	100.00	100.00	100.00	100.00
1	99.20	100.00	100.00	98.70	97.20	96.20	97.10
2	98.60	96.30	93.10	98.10	96.10	92.10	93.10
3	98.20	93.90	95.70	97.20	96.00	90.30	91.30
4	95.10	91.20	90.60	96.30	95.80	87.70	87.50
5	92.20	87.70	88.20	90.10	91.10	81.10	85.60
10	85.70	81.50	84.40	85.90	84.20	60.10	63.20
15	80.30	81.10	75.10	77.10	71.20	41.10	45.10
25	52.40	61.40	48.50	60.20	57.50	21.50	27.30
35	6.20	10.70	4.10	3.10	4.20	1.10	3.80
45	1.10	0.62	2.60	0.71	0.71	0.60	0.36
55	0.93	0.17	0.13	0.14	0.09	0.01	0.10
60	0.42	0.10	0.09	0.01	0.03	—	—

* Per cent recovery calculated from 0 time controls.

Table 6

PER CENT SURVIVAL* OF NORMAL AND INHIBITOR GROWN H. CAPSULATUM AFTER VARYING EXPOSURE TO ULTRAVIOLET

	NORMALLY-GROWN			6-AZAURACIL-GROWN		8-AZAGUANINE-GROWN	
Time (Minutes)	SAB	SAB URACIL	SAB GUANINE	SAB	SAB URACIL	SAB	SAB GUANINE
0	100.00	100.00	100.00	100.00	100.00	100.00	100.00
1	98.70	96.70	98.10	99.30	99.10	97.30	98.40
2	94.20	95.40	97.80	96.20	98.30	91.70	93.00
3	90.40	93.30	95.30	94.10	89.70	83.20	87.10
4	81.20	87.60	84.20	83.30	84.00	82.70	86.10
5	72.40	70.20	74.70	70.10	71.60	64.70	68.30
10	51.60	47.30	44.10	48.20	46.20	35.90	37.40
15	34.20	38.60	30.00	33.60	35.10	28.20	30.10
25	24.60	19.10	28.80	25.00	25.70	10.20	31.10
35	7.30	11.40	8.60	4.20	6.10	2.20	10.20
45	0.31	0.81	1.30	0.57	0.82	0.11	0.22
55	0.26	0.08	0.19	0.06	0.08	—	0.07
60	0.19	0.08	0.11	0.02	0.05	—	0.02

* Per cent recovery calculated from 0 time controls.

B. dermatitidis strain LBa exhibited survival rates of 41.1, 3.7 and 0.27 percent following 3, 5, and 15 minutes exposure. B. dermatitidis strain 6046 averaged respective values of 43.2, 1.6 and 0.11 percent. Percent survival rates for S. schenckii following the same periods of irradiation were 46.3, 9.7 and 0.07 percent. Respective values of H. capsulatum averaged 35.1, 1.6 and 0.09 percent.

(5) The Oxygen Uptake of Normal and Inhibitor-Grown Systemic Pathogenic Fungi.
(Figures 4, 5, 6, 7; Tables 7, 8, 9. 10, 11)

Normally Grown Cells

A distinct difference was noted between B. dermatitidis, strains LBa and 6046 when ribose was used as the substrate. B. dermatitidis strain LBa exhibited slight but consistent oxygen uptake. Two hours after the start of the experiments, an average of 1.60 µl/mg dry weight was recorded. At the terminal point of 6 hours the oxygen uptake was 7.28 .l/mg dry weight. These figures represent net values of oxygen uptake, with the endogenous rates subtracted.

Throughout repeated experiments, B. dermatitidis strain 6046, in the presence of ribose, exhibited no oxygen uptake. All recorded values for B. dermatitidis strain 6046 were within ± 1.12 µl of the normal endogenous rate. Similarly, no exogenous oxygen uptake could be detected for S. schenckii when ribose was used as the substrate. Values obtained for S. schenckii were within ± 1.67 µl of the endogenous rate. H. capsulatum showed slight uptake with average net values of 1.87 µl/mg dry weight at 2 hours, 4.44 at 4 hours and 4.95 µl at 6 hours.

Exogenous respiration was clearly evident with all organisms using monosodium glutamate, glucose, fructose and xylose. With monosodium glutamate as the substrate, respective net terminal values in µl/mg dry weight were 23.80, 17.58, 36.19, and 19.35 for B. dermatitidis strain LBa, B. dermatitidis strain 6046, S. schenckii and H. capsulatum. Average net exogenous values at 6 hours, expressed also in µl/mg with glucose were 28.84, 24.85, 33.47 and 15.24, respectively for B. dermatitidis strain LBa, B. dermatitidis strain 6046, S. schenckii and H. capsulatum. Respective net oxygen uptake exhibited using fructose were 24.39 µl, 20.94 µl, 26.66 µl, and 18.40 µl per mg dry weight.

With the exception of ribose, each organism exhibited the lowest rate of oxygen uptake when the pentose sugar, xylose, was employed. Average µl of oxygen uptake per mg dry weight with xylose were 20.51, 17.98, 22.26 and 13.61 for B. dermatitidis strain LBa, B. dermatitidis strain 6046, S. schenckii and H. capsulatum respectively.

6-Azauracil-Grown Cells.

The effect of growing cells in the presence of the uracil analog was most evident when glutamate was subsequently employed as the substrate, and to some lesser extent when fructose was used. With glutamate as the substrate, reduction in the oxygen uptake by the inhibitor-grown B. dermatitidis strain LBa amounted to approximately 39.5 percent from the normal, at the 6 hour mark. B. dermatitidis strain 6046 exhibited an average terminal decrease of approximately 50.0 percent, S. schenckii 43.9 percent and H. capsulatum 36.4 percent. When fructose was used as

Table 7

THE OXYGEN UPTAKE OF NORMAL AND INHIBITOR-GROWN H. CAPSULATUM AND B. DERMATITIDIS STAIN LBa WITH RIBOSE AS THE SUBSTRATE

H. capulatum

	Normally Grown	Azauracil Grown	Azaguanine Grown	Puromycin Grown
Time (Hours)	O_2 * Uptake	O_2 * Uptake	O_2 * Uptake	O_2 * Uptake
2.0	1.87	1.05	0.60	0.64
4.0	4.44	1.45	0.91	1.06
6.0	4.95	2.74	1.47	1.29

B. dermatitidis strain LBa

2.0	1.60	—— **	——	——
4.0	4.79	——	——	——
6.0	7.28	——	——	——

* µl/mg dry weight, minus the endogenous.

** No oxygen uptake detected.

the substrate, B. dermatitidis strain LBa exhibited an average terminal decrease of 34.8 percent, B. dermatitidis strain 6046 38.2 percent, S. schenckii 31.6 percent and H. capsulatum 44.0 percent. Reductions in oxygen uptake of 6-azauracil-grown cells were not as great, when compared with the normal, with the substrates glucose and xylose. Average terminal decreases with glucose were 13.5, 18.3, 22.2, and 22.2 percent for B. dermatitidis strain LBa, B. dermatitidis strain 6046, S. schenckii and H. capsulatum, respectively. With xylose the respective reductions were 11.4, 7.7, 19.6 and 26.3 percent. Calculation of average slopes (Table 8-11) revealed similar results.

The organisms which normally show slight oxygen uptake with ribose were also tested. Results obtained showed a reduction in oxygen uptake of approximately 45 percent for H. capsulatum. Throughout repeated experiments, no oxygen uptake could be detected for 6-azauracil-grown B. dermatitidis strain LBa, B. dermatitidis strain 6046 or S. schenckii with this substrate.

8-Azaguanine-Grown Cells.

Cells grown in the presence of 8-azaguanine exhibited distinct reductions in oxygen uptake from that of the normally grown cells. Although uptake rates were consistently lower than that of the cells grown with the uracil analog, an optimum substrate(s) was not clearly definable as was the case in the former group. Results obtained using glutamate showed terminal reductions from the normal of 63.9, 56.5, 62.2 and 56.5 percent for B. dermatitidis strain LBa, B. dermatitidis strain 6046, S. schenckii and H. capsulatum, respectively. Values representing

percent reductions obtained with fructose were 51.1, 61.9, 45.1 and 49.5 for the same respective organisms. The rather slight differences in oxygen uptake on glucose noted between normal and 6-azauracil-grown cells were markedly increased with the 8-azaguanine-grown cells. Average reductions in net oxygen uptake at 6 hours were 42.2 50.0, 44.2 and 35.1 for B. dermatitidis strain LBa, B. dermatitidis strain 6046, S. schenckii and H. capsulatum, respectively. Similarly, a greater decrease was noted with the substrate xylose. Respective reductions from the normal were: B. dermatitidis strain LBa, 47.0 percent; B. dermatitidis strain 6046, 45.5 percent; S. schenckii, 47.2 percent and H. capsulatum, 50.6 percent. Warburg studies with ribose were negative for all the organisms tested.

Puromycin-Grown Cells.

Puromycin grown cells exhibited the lowest overall degree of oxygen uptake. Net changes during the three 3-hour periods of each experiment were also lowest for this group. As was the case for 8-azaguanine-grown cells, it was difficult to establish an optimum substrate(s) from those tested. However, it appears from the tables and figures indicated above that monosodium glutamate generally stimulated the least amount of exogenous oxygen uptake. Results obtained using glutamate showed terminal reductions of 78.0, 67.2, 80.2, and 74.4 percent, respectively for B. dermatitidis strain LBa, B. dermatitidis, S. schenckii and H. capsulatum. Respective changes with fructose amounted to 62.2, 67.6, 59.8 and 57.9 percent.

Fig.4

THE OXYGEN UPTAKE OF NORMAL AND INHIBITOR GROWN B. DERMATITIDIS STRAIN LBa

µl/mg Dry Weight*

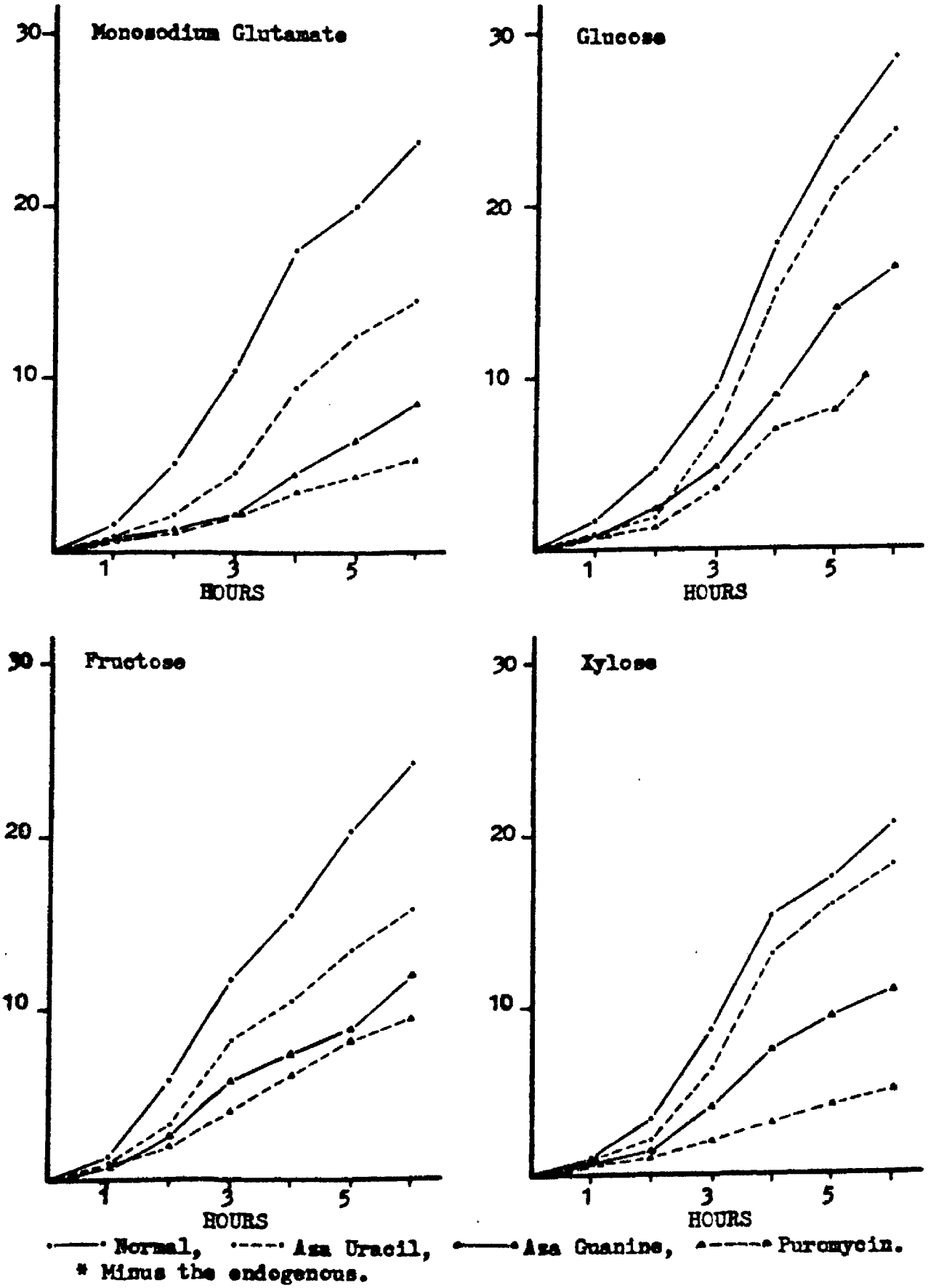
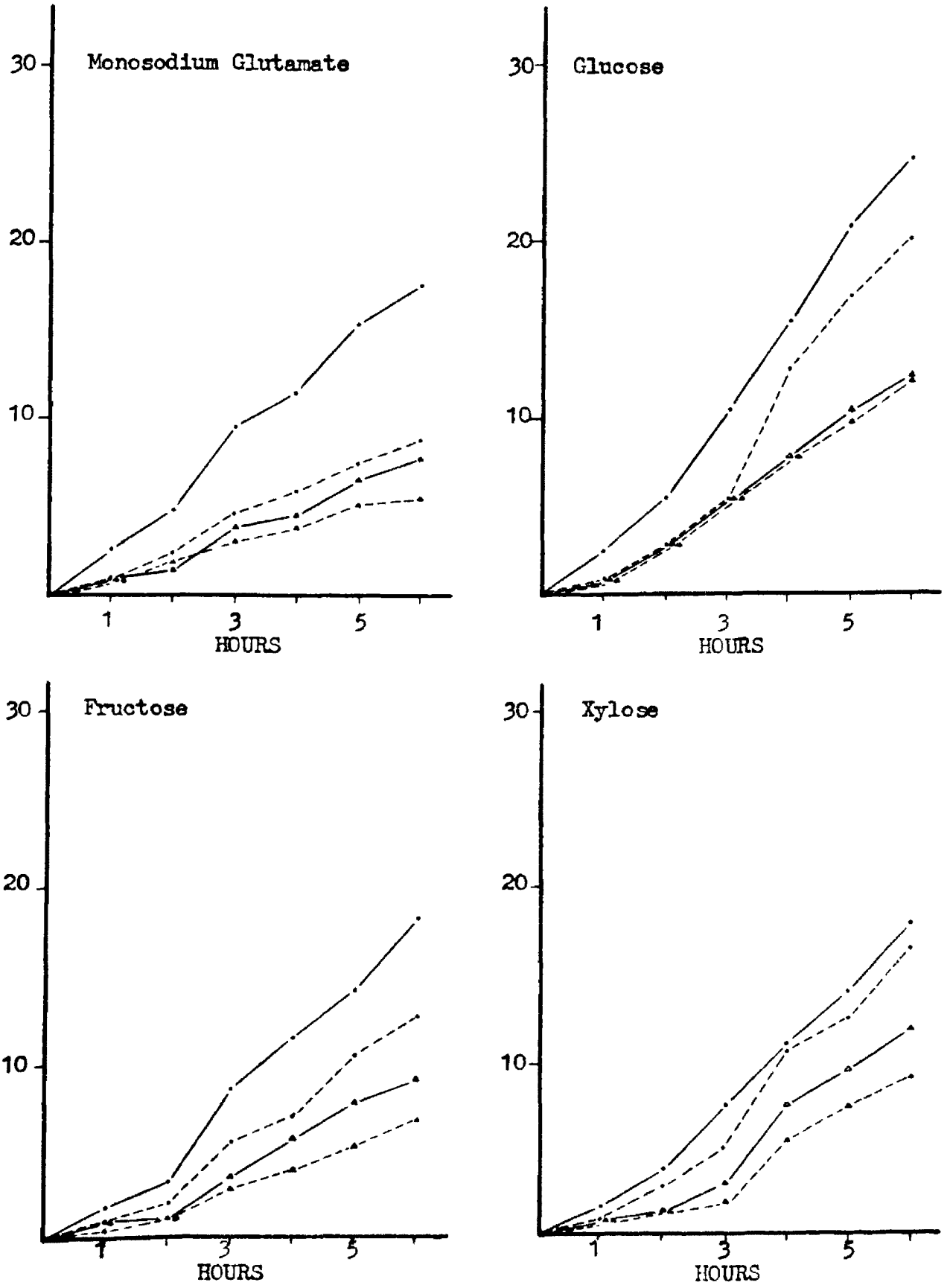


Fig. 5

THE OXYGEN UPTAKE OF NORMAL AND INHIBITOR GROWN
B. DERMATITIDIS STRAIN 6046
µl/mg Dry Weight*

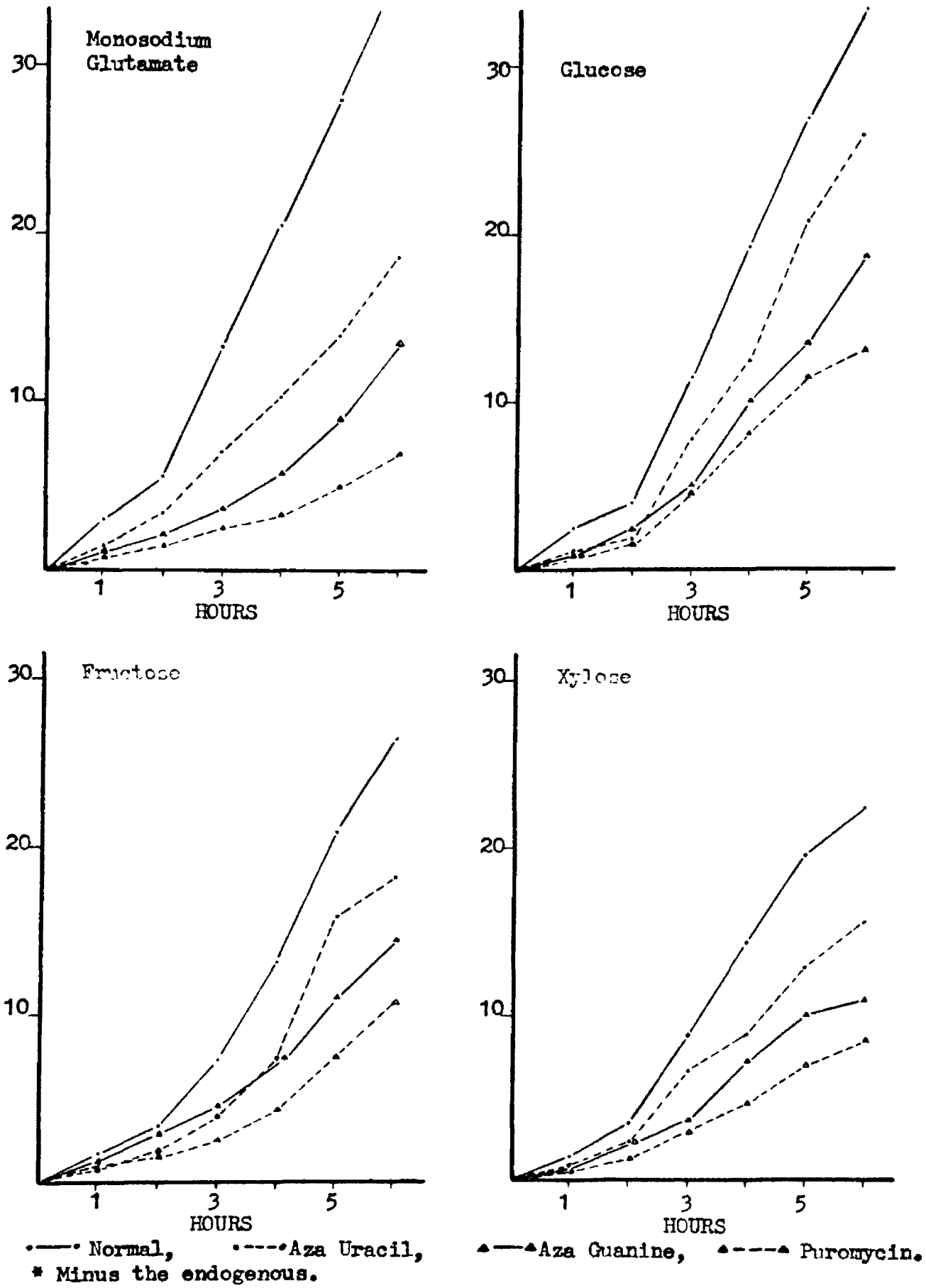


* Minus the endogenous.

Fig. 6

THE OXYGEN UPTAKE OF NORMAL AND INHIBITOR GROWN *S. SCHENCKII*

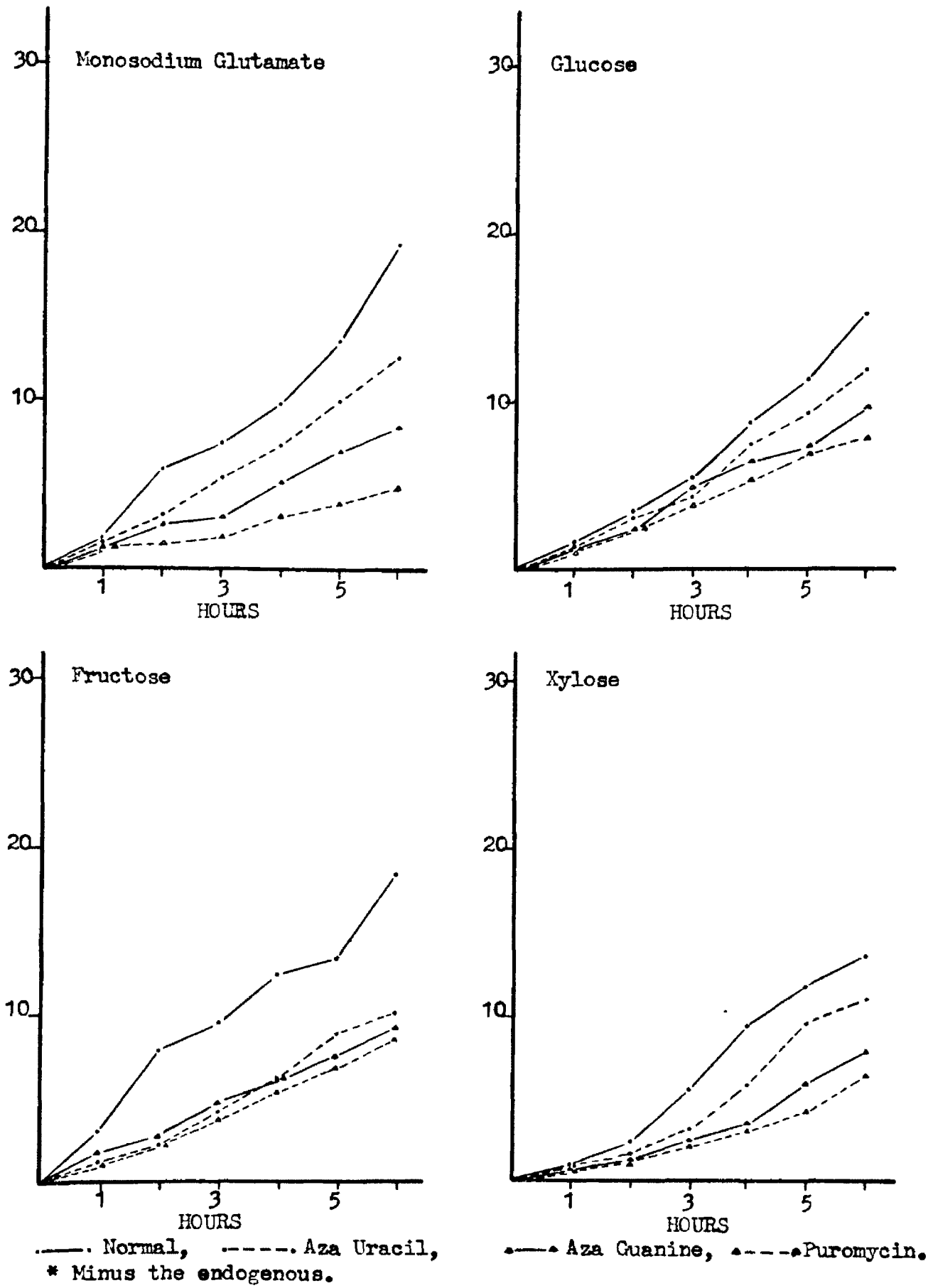
µl/mg Dry Weight*



* Minus the endogenous.

Fig. 7

THE OXYGEN UPTAKE OF NORMAL AND INHIBITOR GROWN
H. CAPSULATUM
µl/mg Dry Weight*



* Minus the endogenous.

Table 8

A Summary of the Data Presented in Figures 4

Blastomyces dermatitidis, strain LBa

Normal Grown Cells

	Endogenous		Monosodium Glutamate		Glucose		Fructose		Xylose	
Time period (Hours)	O_2 Uptake	** Slope	O_2* Uptake	** Slope	O_2* Uptake	** Slope	O_2* Uptake	** Slope	O_2* Uptake	** Slope
0-2	2.70	1.35	5.16	2.58	4.83	2.41	5.72	2.86	3.31	1.65
2-4	2.02	1.01	12.37	6.18	13.12	6.56	9.79	4.89	11.82	5.91
4-6	1.29	0.64	6.27	2.13	10.89	5.44	8.88	4.44	5.38	2.69

6-Aza Uracil Grown Cells

0-2	2.57	1.28	2.10	1.05	1.99	0.99	3.28	1.64	2.17	1.08
2-4	2.19	1.09	7.38	3.69	13.07	6.53	7.31	3.65	10.87	5.43
4-6	1.76	0.88	5.12	2.56	9.58	4.79	5.33	2.66	5.18	2.59

8-Aza Guanine Grown Cells

0-2	3.16	1.58	1.24	0.62	2.37	1.18	2.61	1.30	1.46	0.73
2-4	1.25	0.62	3.17	1.58	6.62	3.31	4.83	2.41	5.91	2.95
4-6	1.16	0.58	3.93	1.96	7.66	3.83	4.70	2.85	3.50	1.75

Puromycin Grown Cells

0-2	2.80	1.40	1.12	0.56	1.27	0.63	1.99	0.99	1.14	0.57
2-4	1.20	0.60	2.21	1.10	5.69	2.84	4.25	2.12	1.98	0.99
4-6	1.10	0.55	1.92	0.96	3.15	1.57	3.20	1.60	2.94	1.47

* Change in oxygen uptake during the designated time period in µl/mg dry weight, minus the endogenous.

** $S = \frac{Y_2 - Y_1}{t_2 - t_1}$

Table 9

A Summary of the Data Presented in Figures 5

Blastomyces dermatitidis, strain 6046

Normal Grown Cells

	Endogenous		Monosodium Glutamate		Glucose		Fructose		Xylose	
Time period (Hours)	O_2 Uptake	** Slope	O_2* Uptake	** Slope	O_2* Uptake	** Slope	O_2* Uptake	** Slope	O_2* Uptake	** Slope
0–2	3.72	1.86	4.70	2.35	5.58	2.79	3.25	1.62	3.93	1.96
2–4	1.76	0.88	6.85	3.42	10.01	5.00	8.27	4.13	7.09	3.54
4–6	1.60	0.80	6.03	3.01	9.26	4.63	9.42	4.71	7.96	3.98

6–Aza Uracil Grown Cells

0–2	3.35	1.67	2.33	1.16	2.92	1.46	2.13	1.06	2.93	1.46
2–4	2.22	1.11	3.39	1.69	9.79	4.89	4.94	2.47	7.96	3.98
4–6	1.79	0.89	3.08	1.54	7.46	3.73	5.27	2.63	5.63	2.81

8–Aza Guanine Grown Cells

0–2	3.29	1.64	1.33	0.66	2.90	1.45	1.27	0.63	1.40	0.70
2–4	1.40	0.70	3.28	1.64	4.88	2.44	4.43	2.21	4.57	2.28
4–6	1.29	0.64	2.99	1.49	4.68	2.34	3.27	1.63	3.96	1.98

Puromycin Grown Cells

0–2	2.96	1.48	1.85	0.92	2.84	1.42	1.15	0.57	1.57	0.78
2–4	1.32	0.66	1.91	0.95	3.74	1.87	2.77	1.38	3.90	1.95
4–6	1.14	0.57	1.86	0.93	3.91	1.95	2.85	1.42	2.55	1.27

* Change in oxygen uptake during the designated time period in µl/mg dry weight, minus the endogenous.

** $S = \frac{Y2 - Y1}{t_2 - t_1}$

Table 10

A Summary of the Data Presented in Figures 6

Sporotrichum schenckii

Normal Grown Cells

	Endogenous		Monosodium Glutamate		Glucose		Fructose		Xylose	
Time period (Hours)	O_2 Uptake	Slope **	O_2* Uptake	Slope	O_2* Uptake	Slope	O_2* Uptake	Slope	O_2* Uptake	Slope
0-2	2.81	1.40	5.47	2.78	3.87	1.93	3.44	1.72	3.65	1.82
2-4	2.06	1.03	14.99	7.49	15.48	7.74	9.78	4.89	10.67	5.33
4-6	1.42	0.71	15.73	7.86	14.12	7.06	13.44	6.72	7.94	3.97

6-Aza Uracil Grown Cells

0-2	3.17	1.58	3.27	1.63	1.70	0.85	1.65	0.82	2.43	1.21
2-4	2.38	1.19	7.10	3.55	10.89	5.44	6.02	3.01	6.43	3.21
4-6	1.64	0.82	9.94	4.97	13.48	6.74	10.59	5.29	6.83	3.41

8-Aza Guanine Grown Cells

0-2	2.64	1.32	2.09	1.04	2.48	1.24	2.91	1.45	2.47	1.23
2-4	1.92	0.96	3.65	1.82	7.55	3.77	4.35	2.17	4.79	2.39
4-6	1.29	0.64	7.94	3.97	8.71	4.35	7.43	3.71	4.51	2.25

Puromycin Grown Cells

0-2	2.32	1.16	1.39	0.69	1.42	0.71	1.53	0.76	1.37	0.68
2-4	1.47	0.73	1.87	0.88	7.30	3.65	2.71	1.35	3.34	1.67
4-6	1.08	0.54	3.67	1.83	7.57	3.78	6.50	3.25	3.60	1.80

* Change in oxygen uptake during the designated time period in µl/mg dry weight, minus the endogenous.

** $S = \frac{y_2 - y_1}{t_2 - t_1}$

Table 11
A Summary of the Data Presented in Figures 7

Histoplasma capsulatum

Normal Grown Cells

	Endogenous		Monosodium Glutamate		Glucose		Fructose		Xylose	
Time period (Hours)	O_2 Uptake	Slope**	O_2* Uptake	Slope**	O_2* Uptake	Slope**	O_2* Uptake	Slope**	O_2* Uptake	Slope**
0-2	2.91	1.45	5.91	2.95	3.48	1.74	7.98	3.99	2.41	1.20
2-4	2.65	1.32	3.87	1.93	5.47	2.73	4.36	2.18	6.97	3.48
4-6	1.34	0.67	9.57	4.78	7.39	3.69	6.07	3.03	4.47	2.23

6-Aza Uracil Grown Cells

0-2	3.20	1.60	3.18	1.59	3.19	1.59	2.29	1.14	1.81	0.90
2-4	2.96	1.48	4.05	2.02	4.50	2.25	3.95	1.97	4.03	2.01
4-6	1.88	0.94	5.49	2.74	4.32	2.16	4.05	2.02	4.22	2.11

8-Aza Guanine Grown Cells

0-2	2.97	1.48	2.49	1.24	2.22	1.11	2.71	1.35	1.31	0.65
2-4	2.30	1.15	2.62	1.31	4.24	2.12	3.62	1.81	2.29	1.14
4-6	1.24	0.62	3.29	1.64	3.45	1.77	2.98	1.49	3.12	1.56

Puromycin Grown Cells

0-2	2.47	1.23	1.32	0.66	2.59	1.24	2.11	1.05	1.28	0.64
2-4	2.15	1.07	1.84	0.92	2.68	1.34	3.22	1.61	1.99	0.99
4-6	1.23	0.61	1.80	0.90	3.22	1.61	2.42	1.21	3.13	1.57

* Change in oxygen uptake during the designated time period in µl/mg dry weight, minus the endogenous.

** $S = \frac{y_2 - y_1}{t_2 - t_1}$

Only slight differences between puromycin-grown and 8-azaguanine-grown B. dermatitidis strain 6046 and H. capsulatum could be detected in the presence of glucose. Net changes from the normal were 57.8 and 47.5 percent, respectively. B. dermatitidis strain LBa and S. schenckii exhibited reductions of 64.9 and 51.2 percent. Oxygen uptake using the substrate xylose produced reductions ranging from the most marked difference of 75.3 percent for B. dermatitidis strain LBa to the lowest of 51.2 percent for B. dermatitidis strain 6046. S. schenckii and H. capsulatum exhibited changes of 62.5 and 53.0 percent. No oxygen uptake could be detected with any of the organisms using ribose as the substrate.

(6) The Effect of Vitamin K-5 on Cellular Respiration. (Table 12, 13 and 14; Figures 8, 9, 10, 11)

Vitamin K-5 had marked effect in reducing the oxygen uptake of the organisms tested. This inhibition was noted with all substrates employed. The rates of oxygen uptake for the control systems (those cells not exposed to the vitamin analog) were similar to those previously described as normally grown cells. Similarly, endogenous rates for those systems lacking the vitamin analog were similar to endogenous rates previously described. A slight but consistently higher rate of oxygen uptake was noted with endogenous systems for organisms tested in the presence of Vitamin K-5.

With the addition of the vitamin analog 2 hours after the start of each experiment, a period averaging 1 hour was noted before any inhibition of oxygen uptake was observed. Distinct inhibition became evident during the 3 to 6 hour period, though the degree of inhibition varied considerably among the various organisms and substrates.

Table 12

THE CHANGES IN OXYGEN UPTAKE OF NORMAL CELLS AND CELLS IN THE PRESENCE OF VITAMIN-K-5

H. capsulatum

Substrate	Ribose		Ribose Vitamin K-5	
Time Period (Hours)	O_2 * Uptake	Slope **	O_2 Uptake	Slope
0-3	3.89	1.29	3.63	1.21
3-6	1.55	0.51	-0.92	-0.31
6-9	3.10	1.03	-0.32	-0.11

B. dermatitidis strain LBa

0-3	2.75	0.92	3.06	1.02
3-6	4.54	1.51	-1.33	-0.44
6-9	6.15	2.05	-0.69	-0.23

* The change in oxygen uptake during the designated time period in ug.mg dry weight, minus the endogenous.

**
$$S = \frac{y_2 - y_1}{t_2 - t_1}$$

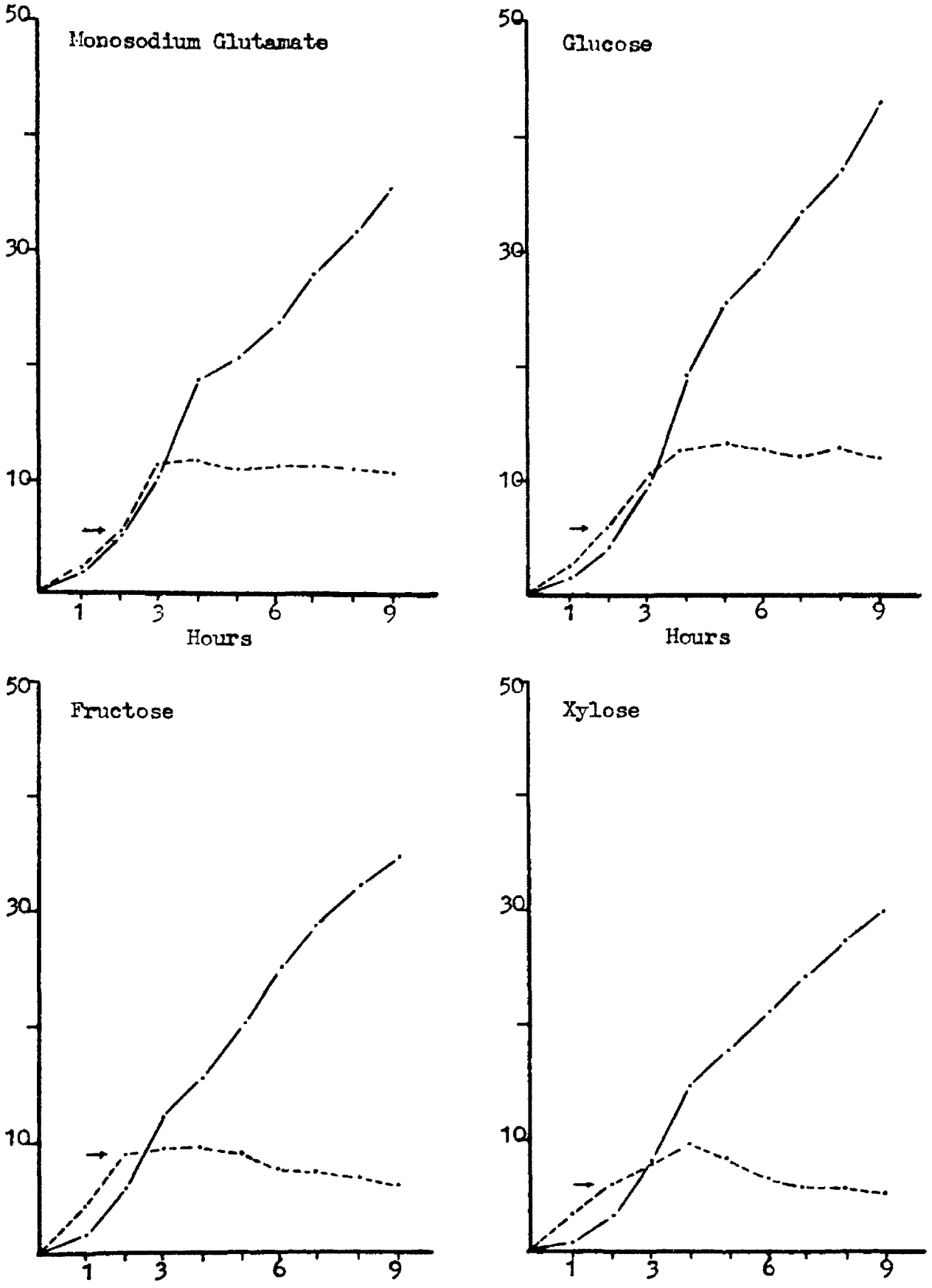
During this time, S. schenckii exhibited an average positive uptake rate with monosodium glutamate of 1.26 µlper hour. Similarly, B. dermatitidis strain 6046 showed an average hourly uptake of 0.47 µl. With fructose as the substrate, the slopes for the 3- to 6-hour period for B. dermatitidis strain 6046 was positive; however, it was negative for B. dermatitidis strain LBa. S. schenckii exhibited a distinct negative slope during this time period with xylose as the substrate, while B. dermatitidis strain 6046 showed a total oxygen uptake of 2.97 µl during this same period. Although all the results obtained indicated distinct inhibition beginning approximately 1 hour following addition of the vitamin analog, absolute values for oxygen uptake were variable with any given organism or substrate.

The final 3-hour period (hours 6 to 9) were generally similar for all the organisms and all the substrates. With the exception of H. capsulatum in fructose and xylose which exhibited slight positive slopes, all other results were plotted as negative slopes. Terminal differences in the net oxygen uptake varied from 56 percent reduction, from the normal, with B. dermatitidis strain 6046 on glutamate to 87 percent reduction with S. schenckii on glucose. B. dermatitidis strain 6046 showed the greatest reduction from the normal at the 9 hour mark with respective reductions of 56, 75, 70 and 59 percent on glutamate, glucose, fructose and xylose. B. dermatitidis strain LBa produced respective decreases of 69, 71, 83 and 82 percent. S. schenckii exhibited respective reductions of 65, 87, 82, and 78 percent. H. capsulatum produced respective 84, 83, 64 and 65 percent reductions from the normal.

Fig. 8

THE EFFECT OF VITAMIN K-5 ON THE OXYGEN UPTAKE OF B. DERMATITIDIS STRAIN LBa

µl/mg/ Dry Weight*

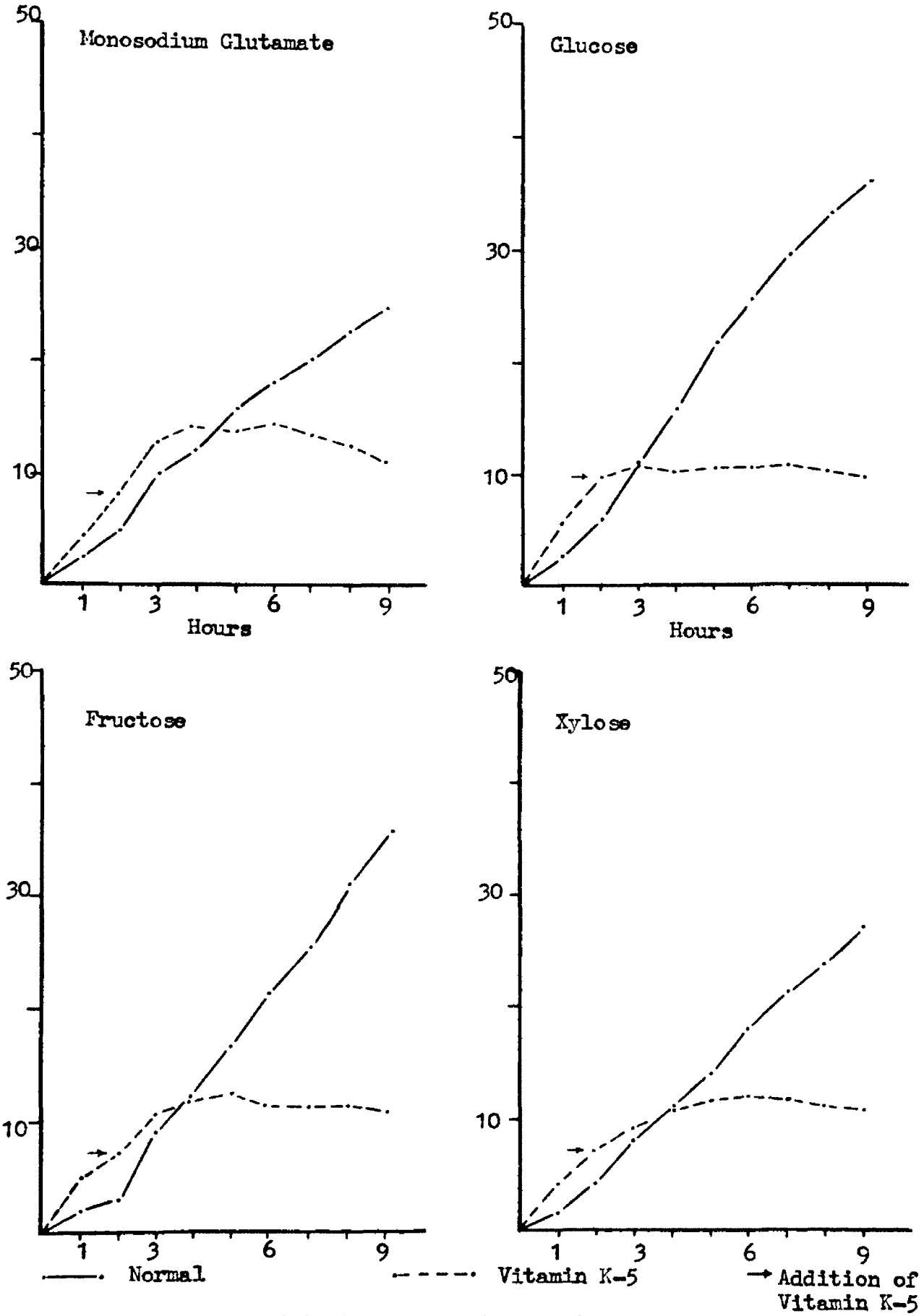


* The endogenous uptake has been subtracted.

Fig 9

THE EFFECT OF VITAMIN K-5 ON THE OXYGEN UPTAKE OF B. DERMATITIDIS STRAIN 6046

µl/mg Dry Weight*

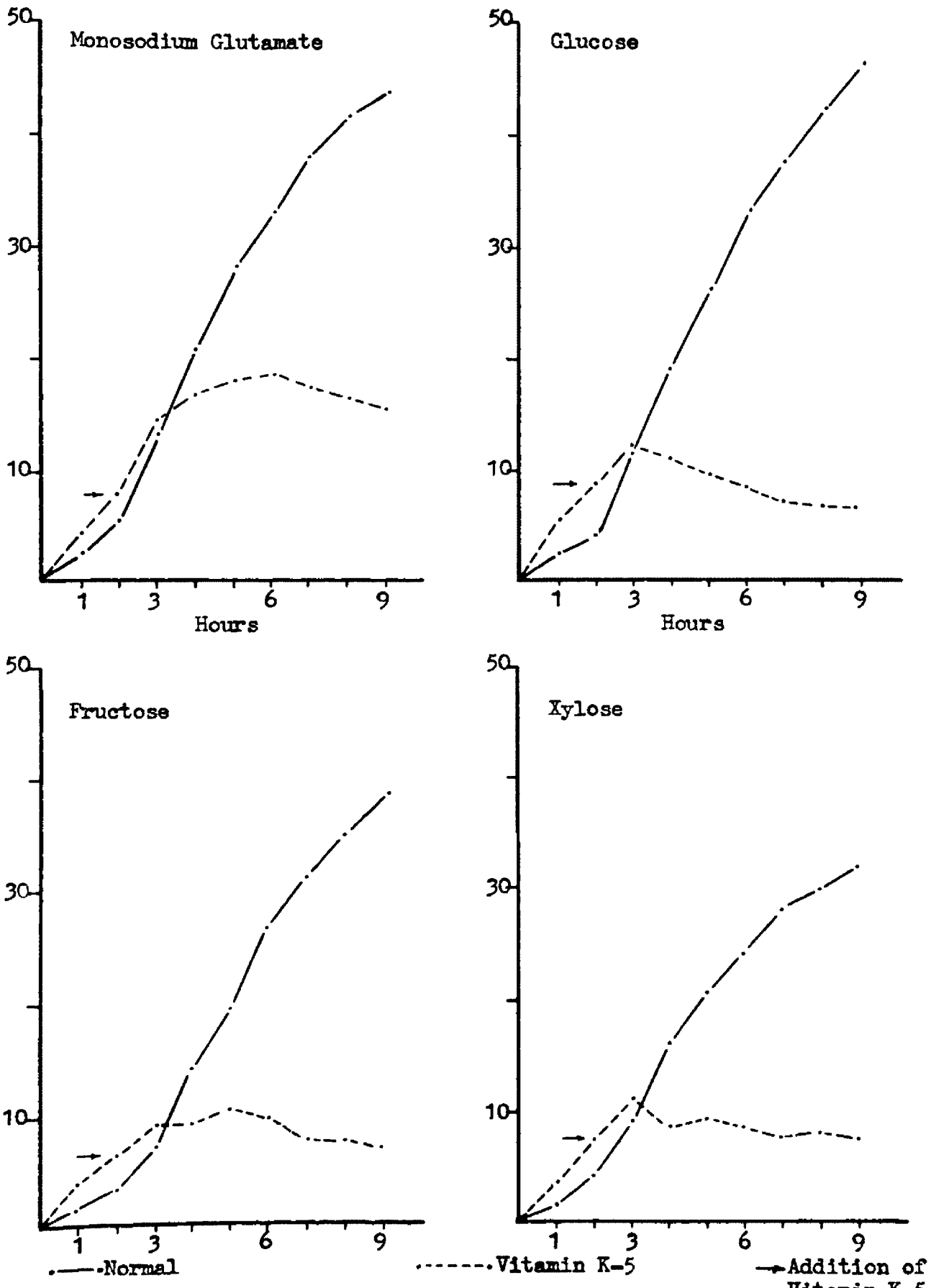


* The endogenous uptake has been subtracted.

Fig. 10

THE EFFECT OF VITAMIN K-5 ON THE OXYGEN UPTAKE OF S. SCHENCKII

µl/mg Dry Weight*

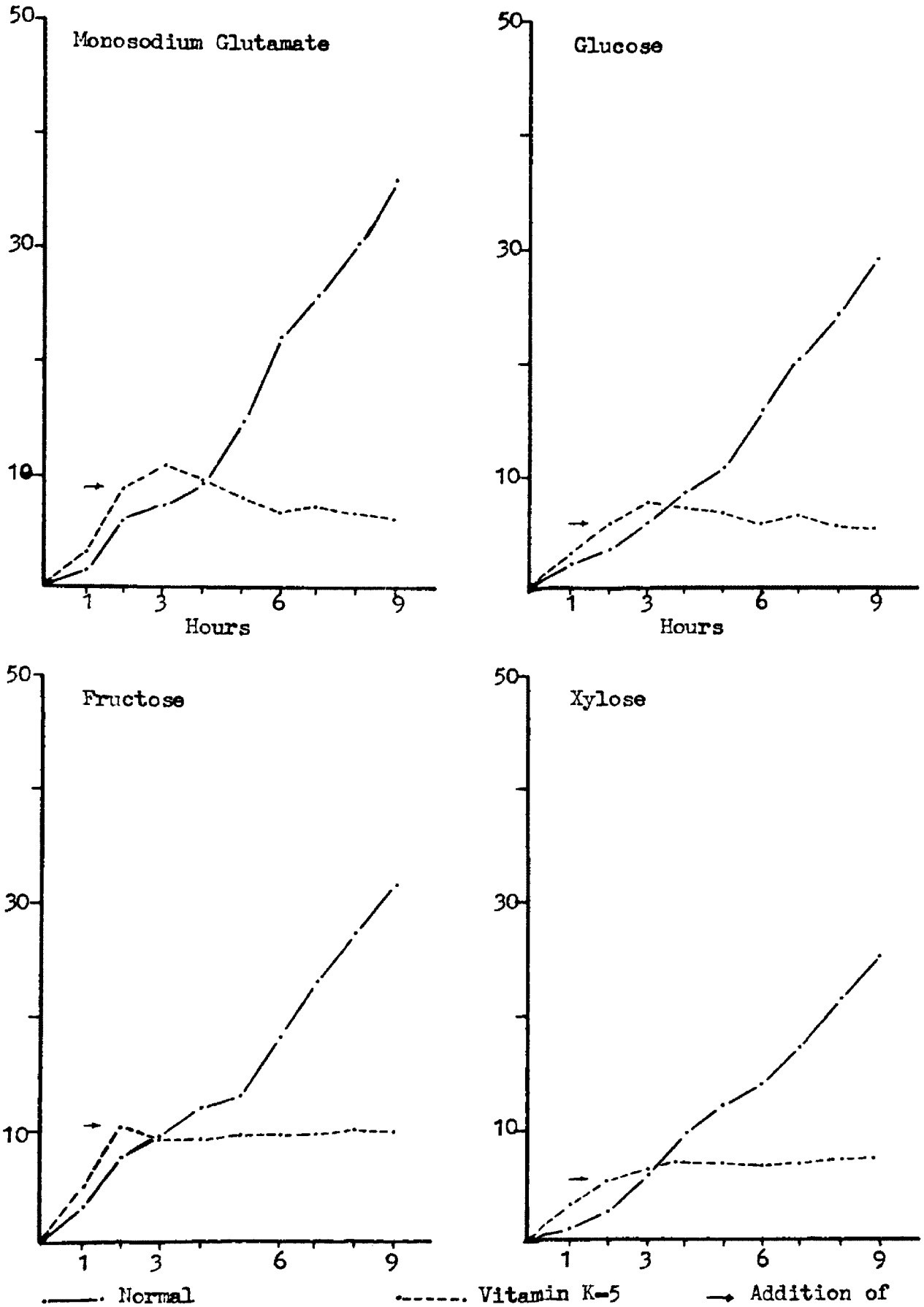


* The endogenous uptake has been subtracted.

Fig. 11

THE EFFECT OF VITAMIN K-5 ON THE OXYGEN UPTAKE OF *H. CAPSULATUM*

µl/mg Dry Weight*



* The endogenous uptake has been subtracted.

Table 13

A Summary of the Data Presented in Figures 8 and 9 .

B. dermatitidis strain 6046

Sub.	Endogenous		Monosodium Glutamate		Glucose		Fructose		Xylose	
Time period (hours)	O_2 Uptake	Slope**	O_2^* Uptake	Slope	O_2^* Uptake	Slope	O_2^* Uptake	Slope	O_2^* Uptake	Slope
0-3	4.08	1.36	10.08	3.36	11.05	3.68	9.12	3.04	8.03	2.68
3-6	1.42	0.47	7.93	2.64	14.58	4.86	12.39	4.13	10.06	3.35
6-9	1.27	0.42	6.93	2.31	10.53	3.51	14.15	4.71	9.06	3.02

B. dermatitidis strain 6046 with Vitamin K-5

0-3	5.74	1.91	13.08	4.36	10.76	3.59	10.77	3.59	8.99	3.00
3-6	2.12	0.71	1.40	0.47	- 0.07	-0.02	0.81	0.27	2.97	0.99
6-9	2.02	0.67	- 3.32	-1.10	- 0.89	-0.26	- 0.67	-0.22	- 1.24	-0.41

B. dermatitidis strain LBa

0-3	3.79	1.26	10.35	3.45	9.75	3.25	12.12	4.04	8.13	2.71
3-6	2.04	0.68	13.48	4.49	19.61	6.53	13.05	4.35	12.95	4.31
6-9	1.70	0.57	11.78	3.92	12.27	4.09	9.66	3.22	8.64	2.88

B. dermatitidis strain LBa with Vitamin K-5

0-3	4.19	1.36	11.49	3.83	10.75	3.65	9.25	3.08	7.86	2.62
3-6	2.54	0.85	- 0.27	-0.09	2.11	0.70	- 1.70	-0.63	- 1.32	-0.44
6-9	2.39	0.79	- 0.41	-0.13	- 0.88	-0.29	- 1.36	-0.45	- 1.21	-0.40

* Change in oxygen uptake during the designated time period in µl/mg dry weight, minus the endogenous.

** $S = \frac{y_2 - y_1}{t_2 - t_1}$

Table 14
A Summary of the Data Presented in Figures 10 and 11 .

H. capsulatum

Sub.	Endogenous		Monosodium Glutamate		Glucose		Fructose		Xylose	
Time period (hours)	O_2 Uptake	Slope**	O_2^* Uptake	Slope	O_2^* Uptake	Slope	O_2^* Uptake	Slope	O_2^* Uptake	Slope
0-3	3.52	1.17	7.81	2.34	5.72	1.90	9.54	3.18	5.72	1.90
3-6	2.24	0.74	14.71	4.90	10.19	3.36	8.70	2.70	8.09	2.69
6-9	2.19	0.73	14.11	4.70	13.30	4.43	13.27	4.42	11.20	3.73

H. capsulatum with Vitamin K-5

0-3	4.64	1.54	10.91	3.64	7.67	2.56	9.30	3.10	6.20	2.07
3-6	3.20	1.07	- 4.21	-1.40	- 1.06	-0.35	0.32	0.10	0.48	0.16
6-9	3.12	1.04	- 0.50	-0.17	- 0.26	-0.08	0.17	0.06	0.51	0.17

S. schenckii

0-3	3.12	1.04	13.33	4.44	11.47	3.82	7.21	3.40	9.31	3.10
3-6	2.60	0.87	19.76	6.59	11.73	3.91	19.53	6.51	14.81	4.93
6-9	1.56	0.52	10.79	3.59	13.37	4.46	12.13	4.04	7.87	2.62

S. schenckii with Vitamin K-5

0-3	3.82	1.27	14.69	4.89	12.78	4.26	9.29	3.09	11.21	3.73
3-6	3.39	1.13	3.78	1.26	- 4.27	-1.42	0.59	0.19	- 2.54	-0.83
6-9	2.83	0.94	- 2.81	-0.93	- 1.98	-0.66	- 1.72	-0.57	- 1.12	-0.37

* The change in oxygen uptake during the designated time period in µl/mg dry weight, minus the endogenous.

** $S = \frac{y_2 - y_1}{t_2 - t_1}$

The two organisms capable of metabolizing ribose produced trends similar to the other results (Table 12). The reductions from the normal at the 9 hour mark were 72 percent for H. capsulatum and 82 percent for B. dermatitidis strain LBa.

(7) The In Vivo Effect of Normal and Inhibitor-Grown Cells. (Tables 15, 16, 17, 18)

Normally Grown Cells.

Twenty-five percent of the mice injected with B. dermatitidis strain LBa were dead by the fifteenth day and 95 percent after 30 days. B. dermatitidis strain 6046 exhibited a somewhat lower rate with 15 percent dead at 15 days and 75 percent at 30 days. The mice inoculated with S. schenckii had rates averaging 45 percent at 15 days and a terminal level of 95 percent. H. capsulatum-injected mice exhibited rates similar to B. dermatitidis strain 6046 with 15 percent and 85 percent death at 15 and 30 days respectively.

6-Azauracil-Grown Cells.

Results obtained from this group did not differ appreciably from the normally grown cells Fifteen percent of the mice injected with B. dermatitidis strain LBa were dead after 15 days and 90 percent after 30 days. Again, B. dermatitidis strain 6046 exhibited lower rates than B. dermatitidis strain LBa with 5 percent and 70 percent of the mice dead after 15 and 30 days respectively. The mortality rate among mice injected with 6-azauracil-grown S. schenckii were nearly identical with that of the normal rate. Similarly, H. capsulatum exhibited little difference from its normally grown counterpart with 10 and 85 percent of the mice dead after 15 and 30 days respectively.

8-Azaguanine-Grown Cells

A distinct reduction in the mortality rates of the mice was observed when injected with 8-azaguanine-grown cells. B. dermatitidis strain LBa and H. capsulatum showed approximately 15 and 30 percent decreases, from the normal, at 15 and 30 days. B. dermatitidis strain 6046 exhibited decreases of approximately 5 and 30 percent at days 15 and 30. Mice injected with the analog-grown S. schenckii resulted in a 30 percent decrease at the 15 day mark and a 25 percent reduction at the terminal point of 30 days.

Puromycin-Grown Cells.

Puromycin-grown cells produced the lowest mortality rates in mice. A marked reduction in rates was observed with all the organisms employed. B. dermatitidis strain LBa inoculated mice showed an average reduced mortality level of 45 and 60 percent at 20 and 30 days. B. dermatitidis strain 6046 exhibited a significant change with a 25 percent reduction at 20 days and 65 percent at 30 days. S. schenckii-injected mice produced 10 percent dead at 20 days and 40 percent dead at 30 days. This represents average decreases of 60 and 55 percent at the indicated days. H. capsulatum grown in the presence of the antibiotic resulted in a reduction of 40 and 55 percent at days 20 and 30.

TABLE 15

The Comparative Virulence of Normal and Inhibitor Grown *E. dermatitidis*, strain LBa

Normal Grown Cells								6-Aza Uracil Grown Cells							
Exp.I			Exp. II					Exp. I			Exp. II				
D.A.I.*	No. Dead	% Dead	D.A.I.*	No. Dead	% Dead	Total	%	D.A.I.*	No. Dead	% Dead	D.A.I.*	No. Dead	% Dead	Total	%
5	0/10	0	5	0/10	0	0/20	0	5	0/10	0	5	0/10	0	0/20	0
10	0/10	0	10	0/10	0	0/20	0	10	0/10	0	10	0/10	0	0/20	0
15	4/10	40	15	1/10	10	5/20	25	15	0/10	0	15	3/10	30	3/20	15
20	6/10	60	20	4/10	40	10/20	50	20	5/10	50	20	4/10	40	9/20	45
25	9/10	90	20	6/10	60	15/20	75	20	8/10	80	20	6/10	60	14/20	70
30	9/10	90	30	10/10	100	19/20	95	30	9/10	90	30	9/10	90	18/20	90

8- Aza Guanine Grown Cells								Puromycin Grown Cells							
Exp. I			Exp. II					Exp. I			Exp. II				
D.A.I.*	No. Dead	% Dead	D.A.I.*	No. Dead	% Dead	Total	%	D.A.I.*	No. Dead	% Dead	D.A.I.*	No. Dead	% Dead	Total	%
5	0/10	0	5	0/10	0	0/20	0	5	0/10	0	5	0/10	0	0/20	0
10	0/10	0	10	0/10	0	0/20	0	10	0/10	0	10	0/10	0	0/20	0
15	0/10	0	15	0/10	0	0/20	0	15	0/10	0	15	0/10	0	0/20	0
20	3/10	30	20	2/10	20	5/20	25	20	1/10	10	20	0/10	0	1/20	5
25	5/10	50	25	3/10	30	8/20	40	25	2/10	20	25	1/10	10	3/20	15
30	6/10	60	30	7/10	70	13/20	65	30	4/10	40	30	3/10	30	7/20	35

* The number of days after inoculation of cells.

TABLE 16

The Comparative Virulence of Normal and Inhibitor Grown B. dermatitidis, strain 6046.

Normal Grown Cells								6-Aza Uracil Grown Cells							
Exp. I			Exp. II					Exp. I			Exp. II				
	No. Dead	% Dead	D.A.I.*	No. Dead	% Dead	Total	%	D.A.I.*	No. Dead	% Dead	D.A.I.*	No. Dead	% Dead	Total	%
5	0/10	0	5	0/10	0	0/20	0	5	0/10	0	5	0/10	0	0/20	0
10	0/10	0	10	0/10	0	0/20	0	10	0/10	0	10	0/10	0	0/20	0
15	2/10	20	15	1/10	10	3/20	15	15	0/10	0	15	1/10	10	1/20	5
20	3/10	30	20	3/10	30	6/20	30	20	2/10	20	20	5/10	50	7/20	35
25	7/10	70	25	6/10	60	13/20	65	25	7/10	70	25	7/10	70	14/20	70
30	7/10	70	30	8/10	80	15/20	75	30	7/10	70	30	7/10	70	14/20	70

8-Aza Guanine Grown Cells								Puromycin Grown Cells							
Exp. I			Exp. II					Exp. I			Exp. II				
	No. Dead	% Dead	D.A.I.*	No. Dead	% Dead	Total	%	D.A.I.*	No. Dead	% Dead	D.A.I.*	No. Dead	% Dead	Total	%
5	0/10	0	5	0/10	0	0/20	0	5	0/10	0	5	0/10	0	0/20	0
10	0/10	0	10	0/10	0	0/20	0	10	0/10	0	10	0/10	0	0/20	0
15	0/10	0	15	0/10	0	0/20	0	15	0/10	0	15	0/10	0	0/20	0
20	0/10	0	20	2/10	20	2/20	10	20	0/10	0	20	1/10	10	1/20	5
25	3/10	30	25	3/10	30	6/20	30	25	1/10	10	25	1/10	10	2/20	10
30	5/10	50	30	4/10	40	9/20	45	30	1/10	10	30	1/10	10	2/20	10

* The number of days after inoculation of cells.

TABLE 18

The Comparative Virulence of Normal and Inhibitor Grown H. capsulatum.

Normal Grown Cells								6-Aza Uracil Grown Cells							
Exp. I			Exp. II					Exp. I			Exp. II				
	No. Dead	% Dead	D.A.I.*	No. Dead	% Dead	Total	%	D.A.I.*	No. Dead	% Dead	D.A.I.*	No. Dead	% Dead	Total	%
5	0/10	0	5	0/10	0	0/20	0	5	0/10	0	5	0/10	0	0/20	0
10	0/10	0	10	0/10	0	0/20	0	10	0/10	0	10	0/10	0	0/20	0
15	1/10	10	15	2/10	20	3/20	15	15	1/10	10	15	1/10	10	2/20	10
20	5/10	50	20	6/10	60	11/20	55	20	4/10	40	20	7/10	70	11/20	55
25	8/10	80	25	8/10	80	16/20	80	25	7/10	70	25	8/10	80	15/20	75
30	8/10	80	30	9/10	90	17/20	85	30	7/10	70	30	10/10	100	17/20	85

8-Aza Guanine Grown Cells								Puromycin Grown Cells							
Exp. I			Exp. II					Exp. I			Exp. II				
	No. Dead	% Dead	D.A.I.*	No. Dead	% Dead	Total	%	D.A.I.*	No. Dead	% Dead	D.A.I.*	No. Dead	% Dead	Total	%
5	0/10	0	5	0/10	0	0/20	0	5	0/10	0	5	0/10	0	0/20	0
10	0/10	0	10	0/10	0	0/20	0	10	0/10	0	10	0/10	0	0/20	0
15	0/10	0	15	0/10	0	0/20	0	15	0/10	0	15	0/10	0	0/20	0
20	3/10	30	20	3/10	30	6/20	30	20	1/10	10	20	2/10	20	3/20	15
25	5/10	50	25	4/10	40	9/20	45	25	3/10	30	25	3/10	30	6/20	30
30	6/10	60	30	4/10	40	10/20	50	30	3/10	30	30	3/10	30	6/20	30

* The number of days after inoculation of cells.

TABLE 17

The Comparative Virulence of Normal and Inhibitor Grown *S. schenckii*.

Normal Grown Cells								6-Aza Uracil Grown Cells							
Exp. I			Exp. II					Exp. I			Exp. II				
	No. Dead	% Dead	D.A.I.*	No. Dead	% Dead	Total	%	D.A.I.*	No. Dead	% Dead	D.A.I.*	No. Dead	% Dead	Total	%
5	0/10	0	5	0/10	0	0/20	0	5	0/10	0	5	0/10	0	0/20	0
10	0/10	0	10	0/10	0	0/20	0	10	0/10	0	10	0/10	0	0/20	0
15	5/10	50	15	4/10	40	9/20	45	15	3/10	30	15	5/10	50	8/20	40
20	8/10	80	20	6/10	60	14/20	70	20	6/10	60	20	6/10	60	12/20	60
25	8/10	80	25	8/10	80	16/20	80	25	8/10	80	25	7/10	70	15/20	75
30	10/10	100	30	9/10	90	19/20	95	30	8/10	80	30	8/10	80	16/20	80

6-Aza Guanine Grown Cells								Puromycin Grown Cells							
Exp. I			Exp. II					Exp. I			Exp. II				
	No. Dead	% Dead	D.A.I.*	No. Dead	% Dead	Total	%	D.A.I.*	No. Dead	% Dead	D.A.I.*	No. Dead	% Dead	Total	%
5	0/10	0	5	0/10	0	0/20	0	5	0/10	0	5	0/10	0	0/20	0
10	0/10	0	10	0/10	0	0/20	0	10	0/10	0	10	0/10	0	0/20	0
15	1/10	10	15	2/10	20	3/20	15	15	0/10	0	15	0/10	0	0/20	0
20	5/10	50	20	5/10	50	10/20	50	20	2/10	20	20	0/10	0	2/20	10
25	5/10	50	25	6/10	60	11/20	55	25	3/10	30	25	2/10	20	5/20	25
30	7/10	70	30	6/10	60	13/20	65	30	5/10	50	30	3/10	30	8/20	40

* The number of days after inoculation of cells.

(8) The Effect of Vitamin K-5 Treatment on Mice Inoculated with Systemic Pathogenic Fungi.
(Tables 19, 20, 21, 22, 23)

GROUPS I AND II. (CONTROLS)

Control groups I and II (Table 20), gave consistent results throughout repeated experiments indicating that gastric mucin in combination with Vitamin K-5 (Group I), or phosphate buffer (Group II) have no observable effect on the mice. One hundred percent survival rates were noted for both groups and no toxicity was observed, except for the reddish brown urine previously described.

GROUP III. (CONTROL)

Mice of this group were injected with the cell adjuvant suspension and were administered sterile 0.15 M phosphate buffer in lieu of the Vitamin K-5 solution.

No deaths were recorded with B. dermatitidis strain LBa at 10 days. Fifteen percent of the mice were dead at 15 days with a terminal death count at 30 days of 55 percent. B. dermatitidis strain 6046 exhibited an average of 5 percent dead at 15 days and 35 percent at 30 days. Mice injected with H. capsulatum showed an average terminal death rate of 40 percent and S. schenckii an average of 50 percent dead.

GROUP IV. (CONTROL)

The mice in this experimental system were inoculated with cells; however, they were not injected with Vitamin K-5 or phosphate buffer. Mortality rates among this group of mice did not differ appreciably from those of Group III. B. dermatitidis strain LBa produced mortality rates of 10 and 60 percent 15 and 30 days following injection of the

cells. Again, B. dermatitidis strain 6046 exhibited a lower degree of virulence with 15 percent of the mice dead after 15 days and 40 percent dead at 30 days. S. schenckii-injected mice had average mortality rates of 5 and 45 percent at days 15 and 30, respectively. Mice receiving H. capsulatum cells exhibited no deaths 15 days after injection; however, an average terminal death rate of 30 percent was observed by the thirtieth day.

GROUP V. (EXPERIMENTAL)

The mice of this group, which received a total of 6 mg of Vitamin K-5 prior to inoculation of the cells and an additional 2.0 mg after inoculation, exhibited the most rapid and highest death rates. B. dermatitidis strain LBa produced the highest death rate in the mice with an average of 55 percent dead after 10 days and 100 percent by 25 days. An average of 5 percent of the mice injected with B. dermatitidis strain 6046 were dead the fifth day and 90 percent after 30 days. Twenty-five percent of those mice injected with H. capsulatum were dead by the tenth day and 90 percent by the thirtieth day. S. schenckii did not differ markedly from the other organisms killing an average of 5 percent of the mice after 10 days and 95 percent by 30 days.

GROUP VI. (EXPERIMENTAL)

This group of mice received a total of 6 mg of Vitamin K-5, with the first injection being administered 4 hours following inoculation of the cells. The death rates for the groups of mice in this experimental system did not differ appreciably from their counterparts in Group V. B. dermatitidis strain LBa-injected mice had the highest death rate with

an average of 50 percent dead after 10 days and 100 percent dead after 25 days. The two strains of B. dermatitidis averaged a difference of 30 percent at 10 days and 15 percent at 30 days. B. dermatitidis strain 6046 consistently exhibited the higher death rate. Of the mice injected with S. schenckii, no deaths were recorded at 10 days; however, 100 percent of the mice were dead at 30 days. H. capsulatum inoculated mice exhibited 5 percent dead at 10 days and 90 percent dead at 30 days.

(9) The Effect of Vitamin K-5 Treatment on Mice Injected with Bacterial Cells.

Vitamin K-5 treated mice injected with S. pyogenes exhibited somewhat higher death rates than untreated mice. Ten percent of the Vitamin K-5 treated mice were dead 3 hours following injection of the cells and 40 percent by 6 hours. Ninety percent were recorded dead at 9 hours and 100 percent at 10 hours. Of the untreated mice, 10 percent were dead at 8 hours, 50 percent at 18 hours and 100 percent at 35 hours. One hundred percent survival rates were recorded for the 3 control groups.

Vitamin K-5 treated mice injected with S. typhosa exhibited 10 percent dead 12 hours following cellular inoculation, 30 percent at 16 hours, 60 percent at 18 hours and 90 percent at 26 hours. One hundred percent of the untreated mice survived through 36 hours. All controls exhibited 100 percent survival throughout the experimental period.

Table 19

The Effect of Vitamin K-5 Treatment on Mice Inoculated With Systemic Pathogenic Fungi

Control Groups I and II

2.5% Gastric Mucin, Vitamin K-5: Groups I*		
DAI**	No. Dead	% Dead
5	0/10	0
10	0/10	0
15	0/10	0
20	0/10	0
25	0/10	0
30	0/10	0

2.5% Gastric Mucin, Phosphate Buffer: Group II*		
DAI**	No. Dead	% Dead
5	0/10	0
10	0/10	0
15	0/10	0
20	0/10	0
25	0/10	0
30	0/10	0

* These controls were set up with each series of experiments. Results were identical for all experiments.

** The number of days after inoculation of cell (gastric mucin).

Table 20

THE EFFECTS OF VITAMIN K-5 TREATMENT ON MICE INOCULATED WITH *B. DERMATITIDIS* STRAIN LBa

	Cells, Phosphate Buffer; Group III							Cells; Group IV						
	Exp. I			Exp. II				Exp. I			Exp. II			
	DAI *	No. Dead	% Dead	DAI *	No. Dead	% Dead	Total	%	DAI *	No. Dead	% Dead	DAI *	No. Dead	% Dead	Total	%
	5	0/10	0	5	0/10	0	0/20	0	5	0/10	0	5	0/10	0	0/20	0
	10	0/10	0	10	0/10	0	0/20	0	10	0/10	0	10	0/10	0	0/20	0
	15	1/10	10	15	2/10	20	3/20	15	15	1/10	10	15	1/10	10	2/20	10
	20	2/10	20	20	2/10	20	4/20	20	20	2/10	20	20	3/10	30	5/20	25
	25	6/10	60	25	5/10	50	11/20	55	25	6/10	60	25	5/10	50	11/20	55
	30	6/10	60	30	5/10	50	11/20	55	30	6/10	60	30	6/10	60	12/20	60

	Cells, Vitamin K-5 (Pre-inoculation); Group V							Cells, Vitamin K-5 (Post-inoculation); Group VI						
	Exp. I			Exp. II				Exp. I			Exp. II			
	DAI *	No. Dead	% Dead	DAI *	No. Dead	% Dead	Total	%	DAI *	No. Dead	% Dead	DAI *	No. Dead	% Dead	Total	%
	5	0/10	0	5	0/10	0	0/20	0	5	0/10	0	5	0/10	0	0/20	0
	10	6/10	60	10	5/10	50	11/20	55	10	6/10	60	10	4/10	40	10/20	50
	15	9/10	90	15	7/10	70	16/20	80	15	8/10	80	15	7/10	70	15/20	75
	20	10/10	100	20	9/10	90	19/20	95	20	10/10	100	20	9/10	90	19/20	95
	25	10/10	100	25	10/10	100	20/20	100	25	10/10	100	25	10/10	100	20/20	100
	30	10/10	100	30	10/10	100	20/20	100	30	10/10	100	30	10/10	100	20/20	100

* Number of days after inoculation of cells.

Table 21

THE EFFECTS OF VITAMIN K-5 TREATMENT ON MICE INOCULATED WITH B. DERMATITIDIS STRAIN 6046

Cells, Phosphate Buffer; Group III								Cells, Group IV							
Exp. I			Exp. II					Exp. I			Exp. II				
DAI *	No. Dead	% Dead	DAI *	No. Dead	% Dead	Total	%	DAI *	No. Dead	% Dead	DAI *	No. Dead	% Dead	Total	%
5	0/10	0	5	0/10	0	0/20	0	5	0/10	0	5	0/10	0	0/20	0
10	0/10	0	10	0/10	0	0/20	0	10	0/10	0	10	0/10	0	0/20	0
15	1/10	10	15	0/10	0	1/20	5	15	1/10	10	15	2/10	20	3/20	15
20	1/10	10	20	2/10	20	3/20	15	20	1/10	10	20	2/10	20	3/20	15
25	1/10	10	25	2/10	20	3/20	15	25	1/10	10	25	3/10	30	4/20	20
30	3/10	30	30	4/10	40	7/20	35	30	3/10	30	30	5/10	50	8/20	40

Cells, Vitamin K-5 (Pre-inoculation); Group V								Cells, Vitamin K-5 (Post-inoculation); Group VI							
Exp. I			Exp. II					Exp. I			Exp. II				
DAI *	No. Dead	% Dead	DAI *	No. Dead	% Dead	Total	%	DAI *	No. Dead	% Dead	DAI *	No. Dead	% Dead	Total	%
5	1/10	10	5	0/10	0	1/20	5	5	1/10	10	5	0/10	0	1/20	5
10	3/10	30	10	2/10	20	5/20	25	10	3/10	30	10	1/10	10	4/20	20
15	5/10	50	15	4/10	40	9/20	45	15	5/10	50	15	3/10	30	8/20	40
20	7/10	70	20	8/10	80	15/20	75	20	7/10	70	20	6/10	60	11/20	65
25	8/10	80	25	9/10	90	17/20	85	25	7/10	70	25	8/10	80	15/20	75
30	9/10	90	30	9/10	90	18/20	90	30	8/10	80	30	9/10	90	17/20	85

* Number of days after inoculation of cells.

Table 22

THE EFFECTS OF VITAMIN K-5 TREATMENT ON MICE INOCULATED WITH S. SCHENCKII

Cells, Phosphate Buffer; Group III								Cells; Group IV							
Exp. I			Exp. II					Exp. I			Exp. II				
DAI *	No. Dead	% Dead	DAI*	No. Dead	% Dead	Total	%	DAI *	No. Dead	% Dead	DAI*	No. Dead	% Dead	Total	%
5	0/10	0	5	0/10	0	0/20	0	5	0/10	0	5	0/10	0	0/20	0
10	0/10	0	10	0/10	0	0/20	0	10	0/10	0	10	0/10	0	0/20	0
15	0/10	0	15	2/10	20	2/20	10	15	0/10	0	15	0/10	10	1/20	5
20	2/10	20	20	4/10	40	6/20	30	20	2/10	20	20	3/10	30	5/20	25
25	4/10	40	25	5/10	50	9/20	45	25	5/10	50	25	3/10	30	8/20	40
30	5/10	50	30	5/10	50	10/20	50	30	5/10	50	30	4/10	40	9/20	45

Cells, Vitamin K-5 (Pre-inoculation); Group V								Cells, Vitamin K-5 (Post-inoculation); Group VI							
Exp. I			Exp. II					Exp. I			Exp. II				
DAI *	No. Dead	% Dead	DAI *	No. Dead	% Dead	Total	%	DAI *	No. Dead	% Dead	DAI*	No. Dead	% Dead	Total	%
5	0/10	0	5	0/10	0	0/20	0	5	0/10	0	5	0/10	0	0/20	0
10	1/10	10	10	0/10	0	1/20	5	10	0/10	0	10	0/10	0	0/20	0
15	7/10	70	15	5/10	50	12/20	60	15	5/10	50	15	3/10	30	8/20	40
20	7/10	70	20	7/10	70	14/20	70	20	7/10	70	20	5/10	50	12/20	60
25	8/10	80	25	9/10	90	17/20	85	25	9/10	90	25	8/10	80	17/20	85
30	9/10	90	30	10/10	100	19/20	95	30	10/10	100	30	10/10	100	20/20	100

* Number of days after inoculation of cells.

Table 23

THE EFFECTS OF VITAMIN K-5 TREATMENT ON MICE INOCULATED WITH H. CAPSULATUM

Cells, Phosphate Buffer; Group III								Cells; Group IV							
Exp. I			Exp. II					Exp. I			Exp. II				
DAI *	No. Dead	% Dead	DAI *	No. Dead	% Dead	Total	%	DAI *	No. Dead	% Dead	DAI *	No. Dead	% Dead	Total	%
5	0/10	0	5	0/10	0	0/20	0	5	0/10	0	5	0/10	0	0/20	0
10	0/10	0	10	0/10	0	0/20	0	10	0/10	0	10	0/10	0	0/20	0
15	0/10	0	15	1/10	10	1/20	5	15	0/10	0	15	0/10	0	0/20	0
20	1/10	10	20	3/10	30	4/20	20	20	1/10	10	20	2/10	20	3/20	15
25	2/10	20	25	3/10	30	5/20	25	25	2/10	20	25	4/10	40	6/20	30
30	3/10	30	30	5/10	50	8/20	40	30	2/10	20	30	4/10	40	6/20	30

Cells, Vitamin K-5 (Pre-inoculation); Group V								Cells, Vitamin K-5 (Post-inoculation); Group VI							
Exp. I			Exp. II					Exp. I			Exp. II				
DAI *	No. Dead	% Dead	DAI *	No. Dead	% Dead	Total	%	DAI *	No. Dead	% Dead	DAI *	No. Dead	% Dead	Total	%
5	0/10	0	5	0/10	0	0/20	0	5	0/10	0	5	0/10	0	0/20	0
10	3/10	30	10	2/10	20	5/20	25	10	1/10	10	10	0/10	0	1/20	5
15	5/10	50	15	5/10	50	10/20	50	15	3/10	30	15	2/10	20	5/20	25
20	7/10	70	20	8/10	80	15/20	75	20	6/10	60	20	6/10	60	12/20	60
25	8/10	80	25	8/10	80	16/20	80	25	8/10	80	25	8/10	80	16/20	80
30	9/10	90	30	9/10	90	18/20	90	30	9/10	90	30	9/10	90	18/20	90

* Number of days after inoculation of cells.

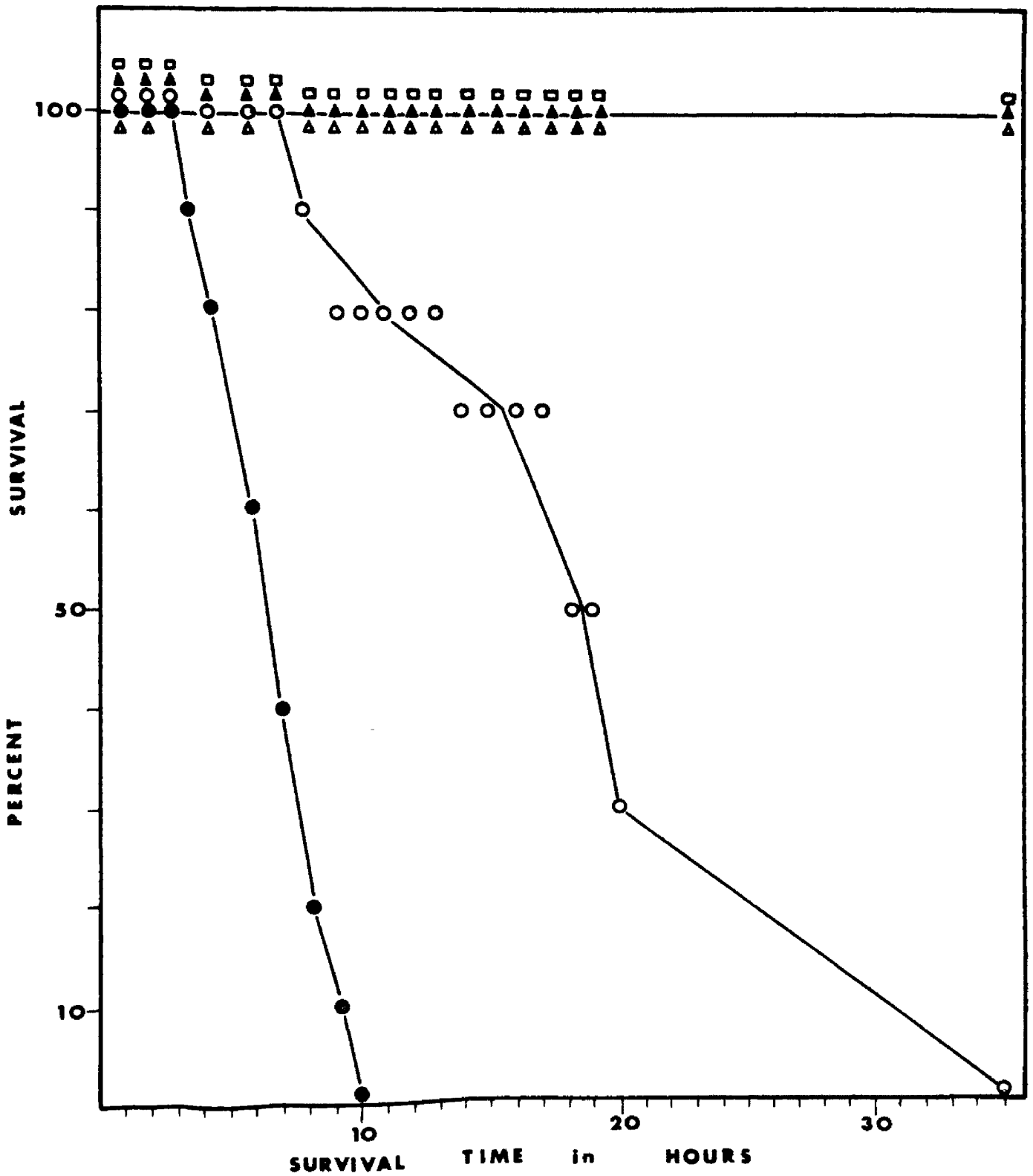


FIG. 12

The Effect of Vitamin K-5 in Mice Injected with Streptococcus pyogenes.

●—● Cells, Vitamin K-5
○—○ Cells, Phosphate Buffer
▲—▲ SPBS, Vitamin K-5
△—△ 2.5% Gastric Mucin, Phosphate Buffer
□—□ 2.5% Gastric Mucin, Vitamin K-5

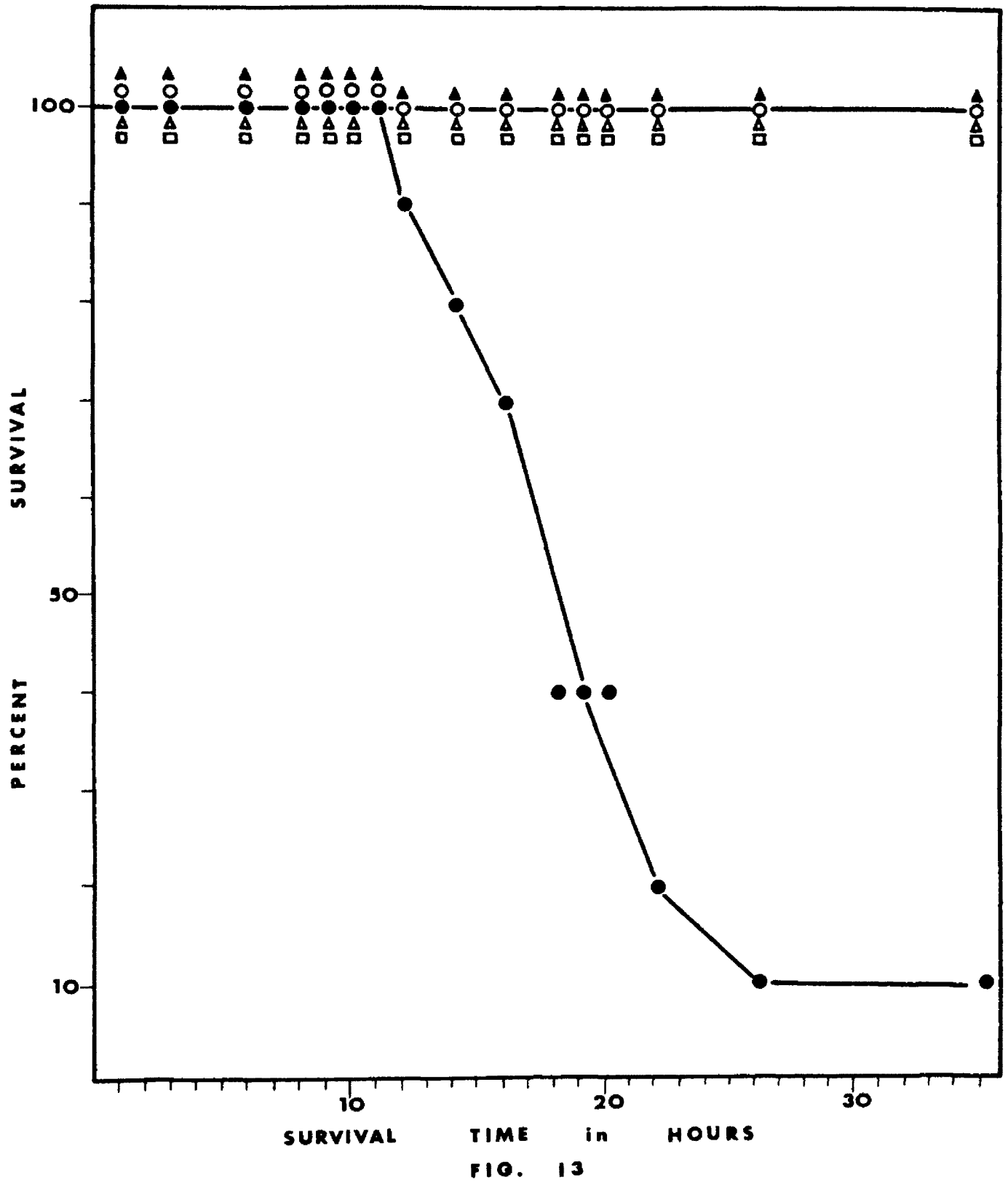


FIG. 13

The Effect of Vitamin K-5 in Mice Injected with Salmonella typhosa.

●—● Cells, Vitamin K-5
○—○ Cells, Phosphate Buffer
▲—▲ SPBS, Vitamin K-5
△—△ 2.5% Gastric Mucin, Phosphate Buffer
□—□ 2.5% Gastric Mucin, Vitamin K-5

CHAPTER V

DISCUSSION

The two strains of B. dermatitidis, S. schenckii and H. capsulatum were found to vary in sensitivity to the inhibitors employed. Most noticeable was the difference between B. dermatitidis strain LBa and B. dermatitidis strain 6046. The former strain exhibited tolerance levels half that of the latter with 6-azauracil and puromycin, while sensitivity to 8-azaguanine was the same for both strains. Growth of all the organisms was obtained at the indicated inhibitor concentrations; however, in each case fresh inocula from stock cultures were used at the start of each experiment to prevent possible adaptation or attenuation of the organisms which might occur following long term maintenance on media containing the respective inhibitors. It was also felt that high concentrations of the inhibitors should be used to obtain the greatest possible effect, whatever it might be. For this reason, the concentration which proved to be one which was near maximum in tolerance for the organism was employed.

The effect of the inhibitors on the total nucleic acid content of the cells was found to vary with the specific inhibitor tested. Puromycin did not appear to inhibit or alter the total RNA or DNA content of the organisms following incubation in the presence of the antibiotic for 5 days. Similarly, the total DNA content of the organisms following growth in the presence of 8-azaguanine or 6-azauracil did not change significantly. For the most part, these results are consistent with

previously published reports. Early work suggested that puromycin might block nucleic acid synthesis resulting in a decrease in RNA and DNA formation. However, in studies with the synthesis of purine compounds by Trypanosoma cruzi, Fernandes and Castellani (1958) showed that preformed bases were used to build up the purine nucleotides and polynucleotides and that incubation with the antibiotic did not inhibit this utilization. Asanuma (1959) reported that puromycin inhibited the synthesis of protein in Pseudomonas fluorescens but did not alter the synthesis of RNA. Similarly, Takeda et al. (1960), working with the same organism noted that P^{32}-orthophosphate was incorporated into cellular RNA during exposure to the antibiotic but that protein synthesis was completely inhibitied. In studying the effect of puromycin upon certain tissues from rats, including liver, heart, kidney and thymus, Gorski et al. (1961), found reduced protein synthesis in all instances. Inhibition of RNA synthesis, however, was found to occur only in the thymus. Mueller et al. (1962), studying HeLa cells discovered that the antibiotic at a concentration of 2×10^{-5} M inhibited the incorporation of C^{14}-leucine by 90% and at 5×10^{-5} M blocked all incorporation. They also found that this inhibition could be reversed by transferring to a puromycin-free medium. Furthermore they noted that the concentration of puromycin which inhibited protein synthesis prevented the acceleration of DNA synthesis without inhibiting the established rate of DNA synthesis. In this investigation total DNA and RNA contents were determined following a five-day period; therefore, the data presented represent the net differences at the time of sampling and do not measure the changes in rates of synthesis. Since the effect of

puromycin is more closely associated with translation rather than transcription it is reasonable to assume that available RNA would not be affected. The inhibition of protein and enxyme syntheses have been demonstrated by other investigators and could have an effect on RNA synthesis. In this respect it might be possible that the availability of RNA polymerase is not affected and that only synthesis of the enzyme is repressed. Nucleic acids may be derived from the pool with little or no need for increased nucleic acid synthesis until depletion warrants synthesis or degradation of available nucleic acids.

In contrast to the effect of puromycin, 6-azauracil reduced the total RNA content of the organisms studied, while the total DNA content remained unchanged. Although the decrease of the RNA does not represent a marked reduction, results obtained were consistently lower than normally-grown cells. Studies with E. coli have shown that 1 µg/ml of 6-azauracil caused complete inhibition of growth (Otsuji and Takagi, 1958). They also reported that the short term effects of the uracil analog resulted in slight increases in total RNA content; however, following longer periods, near normal concentrations were noted. In addition, Habermann (1960) stated that in intact biological systems, 6-azauracil is transformed to 6-azauracil-riboside-5'-phosphate which inhibits orotidylic acid decarboxylase, so that the synthesis of pyrimidine precursors of nucleic acids is inhibited. In spite of this transformation, the analog does not seem to be incorporated into nucleic acid. The former authors suggested that interference with polymerization enzymes might have taken place. In this respect the noted decrease in RNA content may have resulted directly from an inhibition of nucleic acid polymerization

into RNA mocecules. On the other hand, an indirect inhibition is also feasible; that is, if orotidylic decarboxylase is inhibited by the accumulation of 6-azauridine-5-phosphate the inhibition of uridylic acid synthesis could be rate-limiting in terms of nucleic acid precursors. This could in turn result in a depletion of the nucleic acid pool with a subsequent reduction in total RNA content. In addition, inhibition of enzyme synthesis, due to reduced RNA availability, may also have played an independent or additive role in yielding the data presented.

The most noticeable effect of an analog on the nucleic acid content was obtained with 8-azaguanine-grown cells. Organisms grown in the presence of this purine analog exhibited marked increases in total RNA content, while the total DNA content remained unchanged. Although all the cells showed increases in total RNA, the degree of increase varied with different organisms. In contrast to puromycin and 6-azauracil, 8-azaguanine has been shown to be incorporated into RNA replacing a portion of the guanine residues. Since it has been experimentally demonstrated that 8-azaguanine is incorporated into RNA, the implication that the analog will serve as a substrate with a specificity similar to guanine appears reasonable. In this context it may be possible that the analog not only serves as a substrate but in addition it could induce the RNA synthetic pathway, hence, resulting in increased RNA synthesis. The synthesized RNA may be nonsense codes; however, this was not studied in this investigation. On the other hand, 8-azaguanine was not detected in the DNA (Smith and Matthews, 1956). Otaka (1960) discovered that the synthesized RNA which contains the guanine analog is accumulated in the ribosome fraction. In spite of this incorporation into RNA, however,

the analog increases the rate of RNA formation while DNA formation is not changed appreciably (Chantrenne and Devreux, 1959).

Sensitivity to ultraviolet irradiation varied somewhat among the organisms studied. Normally-grown S. schenckii appeared to be the least sensitive while again a strain difference could be detected between B. dermatitidis strains LBa and 6046. B. dermatitidis strain LBa consistently exhibited lower sensitivity to irradiation. H. capsulatum appeared to resemble more closely the strains of B. dermatitidis than S. schenckii. Culturing and preparation of the cells prior to and following irradiation were identical in all cases. It must be assumed, therefore that the physiological states of the organisms within each experimental group were the same, as were the conditions in which the organisms were irradiated. Plate counting of the systemic fungi poses additional problems over that of bacteria in that colonial morphology of the yeasts often prevent accurate counts. For this reason, preliminary experiments had to be performed to arrive at optimum cell concentrations and incubation times. The optimum, starting concentration was found to be 1×10^4 cells/ml while the best incubation period was 7 days.

Cells grown in the presence of 6-azauracil did not differ appreciably from their normally-grown counterparts nor did recovery on SAB plus uracil medium significantly alter recovery rates. In contrast, 8-azaguanine-grown cells consistently exhibited greater sensitivity to irradiation. Recovery of the organisms on the SAB-guanine medium appeared to be greater than on SAB alone, particularly following 35-60 minutes of irradiation. The significance of the observed difference

between recovery on the two media may be questionable, principally in the 45-60 minute periods as the number of colonies counted were, in some cases, below the minimum (30) considered to be significant in standard plate count methods.

Puromycin-grown cells definitely exhibited the greatest sensitivity to ultraviolet irradiation. Recovery rates of the puromycin-grown cells were repeatedly low and resulted in values of approximately one-half that of the normally-grown organisms. It has been observed that irradiation of cells with ultraviolet results in injury, which in the case of yeast cells, is manifested by mutation, interference with budding or cell death in order of increasing dose. Furthermore, Witkins (1946) stated that the genetic make-up of the cells could, in part, contribute to some variation, and other factors previously mentioned have been suggested by Dimond and Dugger (1941). Thus it was to be expected that the organisms used in this investigation would differ in sensitivity to ultraviolet irradiation although the effect of growth in the presence of inhibitors could not be anticipated.

In pursuing other parameters, respiration and fermentation do not appear to be as sensitive to irradiation as cell division (Rothstein, 1959). In addition, Giese and Swanson (1947) found that activity such as storage of carbohydrates is much more readily affected than the total respiration of fermentation in S. cerevisiae. Interesting studies by several workers have shown that the enzyme content of a cell may play an important role in sensitivity to ultraviolet irradiation (Novelli, 1961; Stapleton and Engle, 1960). The concentration of amino acids, phosphate esters, organic acids, proteins and other substances which can

react with free radicals have also been shown to effect sensitivity. Rothstein (1959) stated that the effect on any particular substance in a cell appears to be a function of its concentration. If a substance is present in surplus of its requirements, its inactivation will not be reflected in a change of that function. If, on the other hand, the substance is a rate-limiting factor, then its inactivation will result in diminished function. The differences in sensitivity to irradiation of the normally-grown fungi as opposed to inhibitor-grown cells could be, in part, due to an alteration of the normal biochemical make-up of the cell resulting from the effects of the inhibitors. Thus the increased sensitivity to irradiation of puromycin-grown cells may be a function of reduced protein and enzyme formation and activity which has been found to occur following incubation with the antibiotic. More specifically, Creaser (1955) reported that puromycin-induced inhibition resulted in the complete absence of β-galatosidase in S. aureus while, Sypherd and Strauss (1963) found that the antibiotic interfered with the synthesis of the enzyme in E. coli. Although this enzyme is not found in the yeast cells, it might be assumed that other enzymes may be similarly affected by the antibiotic. General inhibition of protein synthesis has also been demonstrated by several workers. Similarly, it has been suggested that 8-azaguanine also interferes with protein synthesis (Hollinshead, 1963) and that several enzymes of Aerobacter aerogenes are repressed completely by this analog (Levine and Magasnik, 1959; Dewey and Kidder, 1960).

The effect of 6-azauracil on protein synthesis does nto appear to be as great as is the case with the other inhibitors studied. Although, Habermann (1960) demonstrated that the pyrimidine analog caused a decrease

in protein synthesis and activity of some enzymes, other workers suggest that this is not the primary site of inhibition. It is possible then that the differences noted in ultraviolet sensitivity of yeasts following incubation in the presence of the three inhibitors could be a function of a decrease in ultraviolet absorbing substances in the cells with a concomitant reduction in enzyme acitvity. In the case of 8-azaguanine-grown cells, an increase in total RNA content was noted. However, in terms of ultraviolet sensitivity, the effect of this increase may have been masked by a possible marked decrease in other ultraviolet absorbing materials. Although, results obtained from this investigation do not show marked differences between recovery on SAB and SAB plus the natural purine or pyrimidine Lodmell (1963) found that recovery of B. dermatitidis was variable on different media. He further stated that media having the highest ratio of nitrogen to carbon was better for growth and that SAB medium was one of the better media for recovery of irradiated yeast. It should also be pointed out that addition of the natural purine or pyrimidine to the recovery medium does not necessarily predispose utilization of that substance. The multiple effect of growth in the presence of an inhibitor plus that of ultraviolet irradiation may have reduced the metabolic and enxymatic activity of the cell to the state that normal utilization of guanine or uracil was negated. This inhibition of protein and enzyme synthesis may also be of significance in the variation noted in the oxygen uptake of normal and inhibitor-grown organisms.

The effects of the three inhibitors on respiration were studied and all were found to inhibit the rate of oxygen uptake. Although variation

was noted with respect to the organisms and substrates tested, all rates were consistently lower than that of the normally-grown organisms. Normally-grown cells were found to utilize glucose, fructose, xylose and monosodium glutamate. Previous work by Levine and Novak (1950) demonstrated that fructose and ribose did not stimulate oxygen uptake in B. dermatitidis. In this investigation, however, fructose was found to stimulate marked oxygen uptake in both strains of B. dermatitidis, S. schenckii and H. capsulatum. In contrast, ribose stimulated only slight uptake in B. dermatitidis strain LBa and H. capsulatum and none in S. schenckii or B. dermatitidis strain 6046. Following growth in the presence of any of the inhibitors, B. dermatitidis strain LBa no longer demonstrated any utilization of ribose. H. capsulatum showed reduced, though consistently positive rates of respiration.

The rates of oxygen uptake of 6-azauracil-grown cells were somewhat lower than that of the normally-grown organisms; however, the rates were always higher than both 8-azaguanine and puromycin-grown cells. In addition, the influence of the substrate on oxygen uptake was noted with other systems. The 6-azauracil-grown cells showed the least amount of uptake when glutamate was employed as the substrate. Although 6-azauracil has been shown to cause a decrease in protein synthesis and repression of some enzymes (Habermann, 1960), these manifestations are probably an indirect result of the action of the uracil analog on other sites. It has been suggested by Shigura (1962) that the analog is converted to the active form, 6-azauridine-5'-phosphate by one of the phosphorylating enzymes. Earlier, Handschumacher (1960) demonstrated that 6-azauridine-5'-phosphate was an inhibitor of orotidylic decarboxylase

in rat liver cells and yeasts. This could result in a blockage of the pathway which results in the decarboxylation of orotidylic acid to form uridylic acid. Skoda and Sorm (1959) reported that the inhibitory concentrations of 6-azauracil resulted in accumulation of orotic acid and ortidylic acid in cultures of E. coli. In addition, Pasternak and Hands (1955) noted marked inhibition of oretic acid incorporation into the nucleic acids of various mouse tissues by 6-azauracil and suggested that interference with the metabolism of oretic acid might be the primary site of action. In this same context, a point of indirect importance may also involve the formation of cytidine nucleotides. It is apparent that the formation of cytidine nucleotide is dependent on the phosphorylation of uridylic acid to form uridine triphosphate and the subsequent amination of this triphosphate to yield the corresponding cytidine triphosphate. Thus the primary site of action of 6-azauracil may result in indirectly producing the observations of several workers that the analog inhibits the synthesis of proteins and enzymes. The results of this study suggest that enzymes associated with oxidative respiration and repair of irradiation injury may be among those inhibited.

Cells grown in the presence of 8-azaguanine exhibited definite decreases in oxygen uptake with all the substrates tested. Warburg studies indicate that with the exception of ribose, xylose was the least actively utilized substrate. In B. subtilis and other bacteria 8-azaguanine has been shown to be a strong protein inhibitor (Otaka, 1961). It has also been demonstrated, with this same organism, that as much as 40 percent of the RNA guanine is replaced by 8-azaguanine following incubation in the presence of the analog. It appears that the amount of analog incorporated

into RNA reaches a peak then declines to a relatively stable level. This is followed by an accumulation of "abnormal" RNA with a subsequent decrease in cellular protein (Chantrenne and Devreux, 1960). Smith and Matthews (1957) suggest that the effect of 8-azaguanine is not due to competitive inhibition of enzymes engaged in purine metabolism, but rather is more satisfactorily explained by the failure of at least some of the analog containing RNA to function normally. In the case of the tobacco mosaic virus, Matthews (1955) found that the decreased infectivity of preparations containing 8-azaguanine (RNA) in the virus RNA suggests that infectivity is impaired when the analog is incorporated into its nucleic acid. The accumulation of "abnormal" RNA lends support to the results of this investigation if we assume that the RNA anomalies are altered only at the guanine site. In such an instance nonsense or missense coding could result with a subsequent production and accumulation of nonfunctional proteins and enzymes. In such a case a decrease in enzyme concentration might result with a concomitant decrease in metabolic activity. Since enzyme analyses were not performed in this study, it is difficult to hypothesize whether the inhibition is specific for certain enzyme systems or whether a general nonspecific reduction in enzyme activity occurred. In any event, a decrease in oxygen uptake involves a number of interrelated pathways which if inhibited singly or in combination could account for the results obtained.

Growth in puromycin produced the greatest effect on the oxygen uptake of the organisms tested. In all instances puromycin-grown cells had the lowest rate of respiration. Glucose and fructose appeared to stimulate

the greatest amount of uptake, and xylose the least. The rates of uptake of puromycin-grown cells were more similar to that of 8-azaguanine-grown cells than organisms incubated in the presence of 6-azauracil. More specifically, when glucose was used as the substrate, puromycin-grown B. dermatitidis strain 6046, S. schenckii and H. capsulatum all exhibited respiration rates closely allied to the 8-azaguanine-grown organisms on the same substrate. Although species and strain variation did occur, the trends in oxygen uptake of these organisms were similar. It is interesting to note that both of the agents known to be strong protein inhibitors studied in this investigation, produced the greatest change from the normal controls. In 1954 Agosin and Brand reported that puromycin inhibited the overall carbohydrate metabolism of Trypanosoma equiperdum and that the degree of inhibition was proportional to the concentration of the antibiotic. It appears that concurrent with a general inhibition of protein synthesis, puromycin also interferes with the formation and activity of many enzymes resulting in reduced metabolic activity.

The early work of Creaser (1955) on the inhibitory effect of puromycin upon induced β-galactosidase synthesis in bacteria was confirmed by Sypherd et al. (1963). They also reported that when the antibiotic was added at a concentration causing a 40-50 percent reduction in growth rate, a 30 percent decrease in enzyme synthesis occurred. In studies conducted using rat liver, Goldstein (1962) reported that the antibiotic prevented or interfered with the continuing formation of the active enzymes, tyrosine- α- ketoglutarate transaminase and tryptophane pyrrolase. Using cells of the same type, McGeachin and

Potter (1963) demonstrated that puromycin in a concentration of 1.4 X 10^{-4} M prevented amylase production and that the incorporation of C^{14}-leucine into proteins was completely inhibited. Similarly, Garren and Howell (1963) found that with mammalian liver cells, a rapid loss in the activity of the enzymes tryptophane pyrrolase and tyrosine transaminase resulted following incubation with puromycin. It therefore appears that the inhibitory effect of puromycin on the organisms studied in this investigation could result from a reduction in growth due to the inhibition of protein synthesis and also, a related interference with enzyme formation and activity resulting in a reduction of cellular metabolism. No attempt was made in this study to identify the particular enzymes affected.

Vitamin K-5 was found to reduce markedly the oxygen uptake rates of normal yeast cells. Inhibition was noted with all the substrates employed and trends appeared to be similar for all the organisms studied. Following the addition of the vitamin analog into the main compartment of the Warburg flask, a period averaging 1 hour elapsed before inhibition became noticeable. The slopes of graphs for cells receiving the vitamin, in some cases, are plotted as negative rates. Although rigid controls were employed on the preparation and manipulation of the vitamin, no justifiable correlation could be made betweeen the nature of the slope and the substrate employed. In this context, a possible explanation for the negative slope may be that a gas (possibly hydrogen) was evolved following addition of Vitamin K-5 or that the endogenous respiration rate of the cells increased when they were exposed to the vitamin analog. The data obtained tends to support the latter possibility as there was

a slight but consistently higher rate of endogenous oxygen uptake with cells exposed to Vitamin K-5.

It was proposed by Armstrong et al. (1943), that the antibacterial properties of Vitamin K-5 might be due to its rapid oxidation to the corresponding naphthoquinone. Further evidence reported by Schwartzman (1948), Colwell and McCall (1946) and Gonzalez (1945) support the proposal that oxidation of Vitamin K-5 plays an important role in its activity. In independent studies, these workers have demonstrated that reduction of naphthoquinones by -SH compounds, cysteine, ethyl mercaptan and sodium bisulfite suppresses antibacterial and antifungal properties of the naphthoquinones. This is supported by the findings of Colwell and McCall (1946) who reported that the antibacterial and antifungal properties of 2-methyl-1, 4-napthoquinone was suppressed by addition of various reducing agents. Schwartzman (1948) working with Vitamin K-5 found that following solution of the vitamin analog in water, antibacterial activity was strongly evident. However, if 2 mg of sodium bisulfite was added to the solution there resulted an immediate 40 percent loss of activity which was completely restored on exposure to air at 4 C for 4 days

Certain quinones have been shown to play a fundamental role in cellular metabolism and appear to be associated with the electron transport chain and oxidative phosphorylation (Brodie, 1959). Depletion of the bound quinone results in a loss of ability of the cell to conduct electron transport and generate ATP, while restoration of both these activities can be accomplished by the addition of quinones. However, as is the case with bacterial systems, restoration of both

oxidation and phosphorylation requires the addition of the natural bacterial quinone or a closely related homolog (Brodie, 1960a). Naphthoquinones of the Vitamin K-1 and K-2 types, in addition to benzoquinones, are widely distributed in microorganisms (Kashet et al., 1960), and have been shown to be involved in the oxidative phosphorylation system of the cells (Brodie, 1957, 1958, 1960b). In addition, Russell and Brodie (1961) have demonstrated that Mycobacterium phlei can be adapted to be dependent upon Vitamin K-1 for restoration of oxidative phosphorylation, and have been used to study two reduced naphoquinones which were formed during reduction of the vitamin. The two reduced naphthoquinone intermediates were found to accumulate when extracts capable of oxidative phosphorylation were incubated anaerobically with substrate amounts of Vitamin K-1 and either NADPH or malate as electron donors. One of the compounds was found to be identical to synthetic Vitamin K-1 diacetate, while the other which was formed enzymatically, was similar to synthetic monoacetate of the naphthochroman of Vitamin K-1. When analogs of Vitamin K-1 were tested, the naphthochromans were not synthesized and oxidative phosphorylation was not restored. Examination of the structure of Vitamin K-5 reveals that an amino group is substituted at the 4'-position which would make cyclization to the naphthochroman impossible. In addition Kilmer (1950) stated that the antibacterial properties of Vitamin K-5 were due in part to the free amino group contained in the analog. This latter theory cannot be discounted nor has it been shown conclusively to occur.

Limited literature dealing specifically with vitamin K-5 makes conclusive statements impossible; however, a possible mechanism, in view of

work accomplished with the naphthoquinones, is that the 2-methyl-4 amino 1-hydroxynaphthalene (Vitamin K-5) could be oxidized to the quinone, as previous workers have suggested, and the oxidized compound in turn might serve as an inhibitor of oxidative phosphorylation.

All of the organisms studied in this investigation were able to infect and produce death in female white mice of the CFW strain. High infectivity rates were probably aided by the use, in all experiments, of 2.5 percent gastric mucin. The adjuvant was employed in view of previous work done by Howell and Kepkie (1950), Campbell and Saslow (1950), Salvin (1954) and when it was demonstrated that mucin produced a larger percent of deaths, higher number of lesions and higher rate of recoverable organisms following autopsy. Inhibitor-grown cells produced results generally in keeping with other studies conducted for this investigation, that is 6-azauracil-grown cells showed the least amount of variation, in virulence, from that of normally-grown organisms while puromycin-grown yeasts exhibited the greatest difference.

Normally-grown cells of B. dermatitidis strains LBa and 6046 were found to result in different mortality rates following I.P. injection into mice. B. dermatitidis strain LBa was consistently the more pathogenic of the two strains. Previous work in this laboratory confirms this result. As was the case with all the organisms tested, cells were consistently recovered from autopsied mice following 10 or more days of infection. At the time of death, lesions were visible in approximately 50 percent of the mice injected with S. schenckii, 50 percent for B. dermatitidis strain LBa, 30 percent for B. dermatitidis strain 6046 and 30 percent for H. capsulatum. The total mortality rates obtained from

6-azauracil-grown cells did not differ appreciably from the normally-grown cells. This may be correlated to the previously mentioned observations that the pyrimidine analog produced the least amount of change from normally-grown organisms. Organisms were recovered from approximately 90 percent of the mice and lesions were found in 40 percent of the mice injected with S. schenckii, 40 percent with B. dermatitidis strain LBa, 30 percent with B. dermatitidis strain 6046 and 40 percent with H. capsulatum.

Yeasts grown in the presence of 8-azaguanine exhibited a distinct reduction in the mortality rates of mice. In addition, cell recovery on SAB agar media was positive as follows: 80, 75, 65, and 65 percent for S. schenckii, B. dermatitidis strain LBa, B. dermatitidis strain 6046 and H. capsulatum, respectively. The percent of mice, injected with the same respective organisms, exhibiting lesions were 40, 30, 30 and 30 percent.

Puromycin-grown cells produced the lowest mortality rates in the mice. Autopsied mice exhibited lesions on the liver, lungs and spleen only after approximately 28 days following injection. Percent of the mice showing lesions were as follows: 30, 30, 15 and 20 percent for S. schenckii, B. dermatitidis strain LBa, B. dermatitidis strain 6046 and H. capsulatum. The prolonged incubation time here, and the inability of inhibitor-grown cells to recover when transferred to media enriched with normal purine or pyrimidine, suggest that several generations may be required to produce cells of normal metabolic activity and, consequently, normal infectivity. In this series of experiments, the size of the inocula remained constant for comparison between the different organisms.

Criteria on which infectivity are based could vary depending on the nature of the experiment and may include: the production of lesions in test animals, number of recovered organisms, or the death rate in the experimental animals. In this investigation, mortality rates in mice were used as the criterion and subsequent comparative results were based on this parameter. Following I.P. injection, S. schenckii appeared to be the most virulent of the organisms studied. It was followed, in order of decreasing virulence by B. dermatitidis strain LBa, B. dermatitidis strain 6046 and H. capsulatum. Following inoculation with H. capsulatum, the infection is characterized by an intracellular parasitic existence in the cells of the reticuloendothelial system. B. dermatitidis and S. schenckii, however, are not characteristically parasites of phagocytes (Howard and Herndon, 1960). Stained slides of infected tissue, obtained in this investigation, show large granulomatous lesions of the spleen, liver and lungs. However, possibly due to the smaller size of H. capsulatum and the difficulty to observe them in tissue, no slides demonstrating the presence of H. capsulatum were obtained.

Studies in this investigation have shown that the inhibitors can have possible detremental effects on protein synthesis, enzyme formation and activity, oxygen uptake, ultraviolet sensitivity, purine and pyrimidine metabolism and nucleic acid synthesis. It is not unreasonable, therefore, to postulate that the observed alteration of virulence could be due to one or more of these effects. The previously mentioned review of the literature revealed that the principal effect of the inhibitors were on protein, enzyme and nucleic acid synthesis and activity. This tends to support the possibility that viability of the cells is reduced

and that this may be a manifestation of the formation of incomplete proteins and nucleic acid anomalies. The comparative differences noted in virulence may or may not be a direct manifestation of the effects the inhibitors have on the organism; however, a direct correlation between the magnitude of change imposed by the inhibitors on respiration, ultraviolet sensitivity and virulence may be drawn. Whether this alteration of pathogenicity is a reflection of increased susceptibility of the inhibitor-grown cells to humoral and cellular defense mechanisms of the mice or a decrease in the yeasts ability to survive, extend and initiate an infection cannot be accurately stated. In addition, there may be implications relating these observations to the fact that most infections are subclinical in nature and an alteration of the cells may further reduce its infectivity.

Vitamin K-5 has been used as a nontoxic fungistatic agent for the preservation of pharmaceuticals for some time. When this investigation indicated that Vitamin K-5 was a strong inhibitor of cellular respiration of pathogenic funci, experiments were designed to obesrve the action of the vitamin analog *in vivo*. It was initially thought that this marked inhibition of pathogenic fungi might also manifest a reduction or alteration of the course of the infection in experimentally infected mice. Repeated results, however, showed that instead of providing protection by inhibiting the yeasts just the opposite occurred with a definitely higher mortality rate being exhibited in mice receiving Vitamin K-5. Controls were designed to indicate the effect of gastric mucin in combination with the vitamin, gastric mucin alone (phosphate buffer used in lieu of mucin), cellular inoculation with phosphate buffer

(in lieu of Vitamin K-5) and cellular inoculation only. All control results indicate that mucin, buffer and the vitamin singly or in combination with scheduled doses of phosphate buffer did not differ appreciably. Cells in combination with Vitamin K-5, however, exhibited higher mortality rates regardless of the pathogen injected.

Although no toxic effect could be observed in the mice following Vitamin K-5 treatment, some physiological changes have been noted by previous workers to occur in a variety of animals. Kubovar (1955) found that 2-4 mg/day doses of the vitamin analog exerted a thermo-analgetic effect in mice with concomitant sedation. In newborn infants, Meyer and Angus (1956), Allison (1955) and Laurance (1955) using dose levels varying from mg to 30 mg/day, reported that Vitamin K-5 caused kernicterus, hemolytic anemia and hyperbilirubinemia. Similarly, Moor and Sharmon (1955) found that 10 mg/100 g body weight doses of Vitamin K analogs given intramuscularly in rats caused an intense hemoglobinuria. Finally, Ansbacher (1942) stated that in dogs, cats, rats, rabbits and monkeys, Menadione and Menadiol (Vitamin K analogs) was of low toxicity when given in small doses; however, higher concentrations could result in a fall in blood pressure and anemia. The hematopoietic system, however, did not seem to be affected (Beamish and Storrie, 1956). The above mentioned physiological changes are not necessarily responsible, either directly or indirectly, for the phenomenon noted in this series of experiments. Certainly, the role of kernicterus, blood pressure and anemia in terms of the increased mortality rates are questionable. On the other hand, damage to the liver, kidneys or other reticuloendothelial system organs could be of significance since the organisms studied have a

predilection for these organs. No gross damage to these organs was ever detected, however.

The marked effect, in mice, caused by the concomitant use of several pathogenic bacteria and yeasts and Vitamin K-5 cannot be conclusively explained at this time. It is evident form the controls that this is a result of the two being used in combination and may be due to a multiple effect imposed in the test animals. In addition, it is probably related to the basic immune mechanism of the mice. To determine if viable cells alone could express this phenomenon, one other control system should have been used. Heat-killed cells in combination with the vitamin analog should have been injected to see if particles (cells) caused any variation. This control might help to establish whether the observed result was induced or supported by a toxic effect.

The time interval between inoculation and first recorded deaths with B. dermatitidis strain 6046 was as short as 5 days. Although organisms were recovered, no lesions were visible on any of the mice prior to 18-22 days. The mice injected with the other pathogens, similarly, showed more rapid death rates than the mice not receiving Vitamin K-5 treatment. The first of these deaths were noted between 10-15 days following injection. Again, organisms were recovered upon autopsy; however, lesions were not visible until approximately 18-20 days. The mice that survived through 20-30 days exhibited lesions throughout the abdominal cavity (liver, spleen, intestine) and lungs. Although infection was quite noticeable in these mice, the mice that exhibited early deaths (5-7 days) may suggest a possible toxic effect. Mice which were not injected with Vitamin K-5 showed much slower death rates;

however, increased mortality rates were noted following 40-45 days. For comparative reasons, however, the experiments were terminated after 30 days.

Future investigation in this area should concentrate some effort in describing the pharmacological changes taking place in the Vitamin K-5-treated mice. Once these are determined, experimentally induced changes could be produced using agents other than Vitamin K-5 to see if subsequent injection of pathogenic microorganisms results in the same phenomenon. In addition, experiments should be repeated using not only other Vitamin K analogs, but also, the natural vitamins, K-1 and K-2.

CHAPTER VI

SUMMARY

1. The maximum concentrations of 6-azauracil, 8-azaguanine and puromycin permitting detectable growth of *B. dermatitidis* strain LBa, *B. dermatitidis* strain 6046, *S. schenckii* and *H. capsulatum* were determined.
    A. The fungi exhibited variation in sensitivity. *S. schenckii* showed the least sensitivity while, *H. capsulatum* the greatest.
    B. *B. dermatitidis* strain 6046 could grow in 2-fold higher concentrations of 6-azauracil and puromycin than *B. dermatitidis* strain Lba.
2. Mice were found to show no noticeable signs of toxicity when receiving 0.5 mg/ml doses of Vitamin K-5 every 48 hours, for 15 days.
3. The total DNA and RNA concentrations of normally- and inhibitor-grown organisms were determined.
    A. The DNA content was not affected by any of the inhibitor studied.
    B. Puromycin did not alter the total RNA content of the cells. Azauracil-grown cells exhibited decreases in RNA (to about 10.2% of normal) while, 8-azaguanine-grown organisms showed an increase (to about 19.1%).
4. The effect of the inhibitors on ultraviolet sensitivity was studied.
    A. Cells grown in the presence of 6-azauracil did not differ appreciably from that of normally-grown organisms.

B. Organisms incubated in the presence of 8-azaguanine exhibited slight but consistently greater sensitivity to irradiation. Puromycin-grown cells were found to be extremely sensitive to ultraviolet light.

C. The addition of the natural purine or pyrimidine to the plate count medium did not enhance recovery.

5. Glucose, fructose, monosodium glutamate and xylose were found to stimulate the oxygen uptake of all the organisms tested.

A. Ribose stimulated uptake in B. dermatitidis strain LBa and H. capsulatum, however, no respiration could be detected in B. dermatitidis strain 6046 or S. schenckii with this same substrate.

B. Organisms cultured in the presence of 6-azauracil, 8-azaguanine and puromycin exhibited reduced respiratory activity on all the substrates tested. Puromycin was found to be the strongest inhibitor of oxygen uptake.

C. When normally-grown fungi were exposed to Vitamin K-5, a marked inhibition of oxygen uptake was noted.

6. The relative pathogenicity of normally- and inhibitor-grown cells were studied.

A. Organisms grown in the presence of 6-azauracil caused mortality rates in mice which were similar to their normally-grown counterparts.

B. Distinct reductions in mortality rates, of mice were noted when injected with 8-azaguanine- or puromycin-grown cells. Puromycin-grown organisms were found to be the least virulent.

7. Mice which underwent Vitamin K-5 treatment showed considerably higher mortality rates, when subsequently injected IP with pathogenic bacteria and fungi than untreated mice or mice receiving a placebo.

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