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A STUDY OF THE PHYSIOLOGY OF

HISTOPLASMA CAPSULATUM

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A STUDY OF THE PHYSIOLOGY OF HISTOPLASMA CAPSULATUM

CHAPTER I

INTRODUCTION

Histoplasmosis is a systemic mycosis of man and animals caused by the dimorphic fungus, Histoplasma. It was first recognized by Darling (1), who named the fungus Histoplasma capsulatum suspecting that it was a protozoan. da Rocha-Lima (2) corrected the error concerning the taxonomic position of the organism, and expressed his view that Histoplasma was not a protozoan, but a fungus. DeMonbreun (3) was first to isolate and culture the organism from a natural infection. He found that the organism exhibited thermal dimorphism. It grew as a small unicellular budding, yeast-like form when incubated at 37° C on moist enriched medium or in animal tissue. However, the organism formed cottony mycelium with tuberculated chlamydospores in culture incubated at room temperature.

Emmon (4) was first to isolate the organism from soil. He demonstrated, by direct microscopic examination of saline suspensions

of the soil sample, tuberculated chlamydospores, typical of those developing in cultures of the fungus at room temperature.

The metabolism of the mycelial phase of H. capsulatum and of other systemic mycotic agents has not been thoroughly investigated.

The first study of oxidative metabolism in a systemic fungus was by Bernheim (5) who investigated Blastomyces dermatitidis. He reported that sugars were not fermented by either phase of this organism. Glucose and mannose were oxidized at the same rate, provided that the high endogenous rate was suppressed. Of five sugars tested, arabinose, rhammose, and fructose were not oxidized. Most amino acids increased oxygen consumption of the organism. Levine et al. (6) found that the optimal pH range for endogenous respiration and glucose oxidation by B. dermatitidis was between 6 and 8. In the presence of acetate the effect of the hydrogen ion concentration on oxygen uptake resembles results obtained with other yeasts, with the greatest uptake at pH 7. Nickerson and Edward (7) showed that the mycelial phase of the organism oxidized added substrates exogenously and that the endogenous respiratory rate of the yeast phase was several times that of the mycelial phase.

Robbins and Ma (8), investigated the nutrition of Trichophyton mentagrophytes and found that the organism was unable to use ammonium nitrate, but grows readily with amino acids as the source of nitrogen. They found no evidence for the indispensability of any amino acid,

but no single amino acid promoted growth so well as did a suitable mixtures of these compounds. The authors concluded that amino acids added in their medium were incorporated into the fungus protein more rapidly than those supplied by metabolic transformation of any single amino acid. Nickerson and Chadwick (9) reported that in the respiration of dermatophytes, two points of maximum oxygen uptake occur; the first at pH 3, and the second at pH 8. Between these two degrees of hydrogen ion concentration, the rate of respiration decreases, reaching a minimum at pH 5-5.5.

The study of the nutritional requirements of H. capsulatum, particularly the yeast phase was undertaken by Salvin (10). He reported an excellent yield of yeast cells in a medium devoid of blood, blood derivatives, or other proteins. Salvin (11) also reported that biotin was required by the yeast phase of several strains of H. capsulatum, when cysteine was used as the sole source of nitrogen. When alanine was used as a nitrogen source only one of the six mycelial strains tested showed this requirement. Also 8 yeast and 6 mycelial isolates showed a need for reduced sulfur compounds such as cystine, cysteine, methionine, and thioglycollate.

Pine (12) found that cysteine was necessary for the growth of the yeast phase of several strains of H. capsulatum. Rowley and Pine (13) reported that the conversion of the yeast to the mycelial phase of some isolates was stimulated by the presence of calcium pantothenate

and certain amino acids. Pine (14) reported on the comparison of the vitamin requirements of the mycelial and yeast phases of several strains of H. capsulatum. Although no single vitamin was found to be required by the mycelial phase at 25° C, eight of the yeast phase strains required thiamine for the maximum rate of growth at 37° C. The growth rate of the yeast phase at 25° C was equal to or greater than that at 37° C. Howell (15) and more recently Scheer (16) reported that temperature of 35° C or greater are inhibitory to the mycelial phase of H. capsulatum.

The first attempt to study the respiration of H. capsulatum was made by Cozad et al. (17). They studied the effects of various substances on the respiration of the yeast phase of this organism, and found that glucose, mannose, fructose, xylose, and maltose stimulated respiration, while lactose, sucrose, and arabinose did not show any stimulation. All the L-amino acids tested increased the oxygen uptake, except L-arganine. The results obtained from the tricarboxylic acid cycle intermediates suggest that part of the Krebs cycle is involved in the metabolism of H. capsulatum.

The purpose of this dissertation was to investigate the physiology of the mycelial phase, and to compare the pathway of glucose dissimilation of both the mycelial and the yeast phase of H. capsulatum.

CHAPTER II

MATERIALS AND METHODS

The Ward strain of Histoplasma capsulatum used in all experiments was obtained from U. S. Public Health Service, CDC, Kansas City Field Station, University of Kansas Medical Center, Kansas City, Kansas.

Basal medium. The basal medium used in all experiments contained the following ingredients:

Potassium phosphate (dibasic).....	1.50 g
Magnesium sulfate ($7H_2O$)	0.50 g
Calcium chloride	0.33 g
Sodium molybdate	0.01 mg
Copper chloride	0.01 mg
Zinc chloride	0.07 mg
Ferrous sulfate.....	0.05 mg
Distilled water	1000 ml

Preparation of standard inoculum: A sixteen day old culture of the mycelial phase of H. capsulatum was harvested with sterile saline and transferred to 75 ml vaccine bottle containing 20% by volume glass

beads. The bottle was shaken at maximum speed of 250 strokes per minute on the Eberbach reciprocating shaker for 30 minutes to break the large mycelial mats into fine particles.

Growth Studies on Farm Soil

Preparation of media: The following samples were collected from a farm near Norman: chicken manure from the chicken house, a soil sample taken on the floor of the house, a soil sample taken outside of the house, and a soil sample taken at a distance from the house.

One thousand milliliter of distilled water was added to 100 grams of each sample, stirred for 10 minutes, filtered through 4 layers of gauze, and then centrifuged for 10 minutes at 2000 x g to remove the soil particles (crude extract). Half of each crude extract was filtered through a Seitz filter and a clear brown refined extract was collected.

The original pH of the crude and refined extracts of each sample was determined and found to range from pH 7.5-7.8. Each sample was then divided into three 100 ml portions (a, b, c), and the pH of two portions were adjusted with 0.1N NaOH and HCl as follows: (a) pH 6.5, (c) pH 8.5. Portion (c) was left at the original pH.

Refined extracts a, b, and c were sterilized separately by again being passed through Seitz filters. The extracts were then divided into 50 ml portions and dispensed into 250 ml sterile Erlenmeyer flasks. A duplicate set was solidified by the addition of previously prepared and sterilized agar-agar.

The crude extracts were divided the same way as the refined extract. The liquid and solid media were autoclaved at 120° C, 15 psi pressure, for 15 minutes.

Each flask was inoculated with 1.5 ml of mycelial suspension and incubated at 25° C for 16 days. The liquid media were shaken in a reciprocating shaker at the rate of 95 strokes per minute during this period. All experiments were done in duplicate.

Effect of Nitrogen Source on Growth of the Mycelial Phase of H. capsulatum

The following amino acids and inorganic nitrogen sources were used for this study: L-glycine, L-alanine, DL-serine, L-valine, L-threonine, L-leucine, L-isoleucine, L-cysteine hydrochloride, L-methionine, L-aspartic acid, Dl-histidine, L-proline, ammonium sulfate, and potassium nitrate. Two milliliters of a solution of each of the above nitrogen sources were added to respective flasks containing 45 ml of sterile basal medium. This resulted in a final concentration of organic nitrogen of 0.1 M, and the inorganic nitrogen of 0.015 M.

The sterile agar-agar was added to duplicate flasks for solid media. Into two sets, one with solid media, one with liquid media, were added 3 ml of sterile glucose solution to yield the final glucose concentration of 1%. No glucose was added to the duplicate set.

Each flask was inoculated with 1.5 ml of mycelial suspension and incubated at 20° C for 16 days. The liquid culture media were

shaken on a reciprocating shaker during the incubation period. At the end of 16 days the flasks were autoclaved at 121° C, 15 psi, for 15 minutes and each mycelial mat was collected on filter paper using Buchner funnel, washed, dried in 110° oven for 2 hours and its weight was determined. All experiments were done in duplicate.

The Effect of Carbohydrates and Fatty Acids on the Growth of the Mycelial Phase of H. capsulatum

Basal medium with 0.2% ammonium sulfate, was used in this experiment. Each of the following carbohydrates and fatty acids were added separately to the respective basal medium to give a final concentration of 1%: dextrose, mannose, fructose, dulcitol, mannitol, xylose, arabinose, glycerol, sodium pyruvate, lactose, maltose, sucrose, sodium acetate, sodium, propionate, butyric acid, sodium lactate, and caproic acid. The same procedure was employed for the preparation of media as described in the amino acid studies. All experiments were done in duplicate.

Manometric Studies of H. capsulatum, Mycelial Phase The Effects of Various Substances on the Respiration

One hundred fifty milliliter of basal medium containing 2% sodium citrate and bacto-dextrose was dispensed into a 500 ml Erlenmeyer flask, and then autoclaved at 12° C, 15 psi pressure for 15 minutes. The medium was inoculated with 1.5 ml mycelial suspension, shaken on a reciprocating shaker for 16 days at 25° C. At this time

numerous uniform pellets were formed. These pellets were centrifuged, washed three times aseptically with distilled water, then suspended in 150 ml distilled water and subjected to constant shaking for 48 hours in an attempt to exhaust the stored metabolites. The pellets were then washed and centrifuged for 10 minutes at 1800 x g and the supernatant was discarded.

Two hundred fifty mg of wet pellets were weighed on an analytical balance and added to each Warburg flask containing 1.2 ml of phosphate buffer, pH 6.5. The center well contained 0.2 ml of 20% KOH, 0.5 ml of 0.1 M substrate was added to the sidearm. Manometric measurements were done at 37° C, and 0_2 uptake was measured by the method of Umbreit et al. (18). All experiments were done in duplicate.

Detection of Enzymes in Cell Free Extract of Both Mycelial and Yeast Phase of H. capsulatum Pathway of Glucose Dissimilation

Preparation of cell free extract:

Yeast phase. The yeast phase of H. capsulatum was grown on blood agar for four days at 37° C. The cells were removed from the blood agar slants with cold 0.03 M phosphate buffer, pH 7, centrifuged and washed three times with the same buffer at 1800 x g. The enzymes were extracted in buffer by exposure to Mickle's disintegration for fifteen minutes in the cold room. The extract was then centrifuged at 13,800 x g for 30 minutes in a Lourdes refrigerated centrifuge. The

cell-free supernatant was then dialyzed against 0.003 M phosphate buffer, pH 7, at 4° C for 12 hours and was used for enzyme studies.

Mycelial phase. The same procedure was used for mycelial phase, except that the pellets were prepared by growing the organism in Sabouraud's broth for 16 days on a reciprocating shaker at 25° C. The pellets were ground in a pre-cooled mortar containing small glass (Ballotini) beads for enzyme extraction.

Pathway of Glucose Dissimilation of the Mycelial and Yeast Phases of H. capsulatum

Protein determination. The protein content of cell-free extract was determined according to Biuret method (Garnell, Burdawill and David), (19). The yeast and mycelial phase of cell-free extracts contained, respectively 4.69 and 1.87 mg of protein per ml.

Determination of enzyme activities. Hexokinase of cell-free extract was determined by the spectrophotometric method described by Slein, Cori, and Cori (20). Phosphoglucose isomerase was detected by the resorcinol method of Roe (21) as used by Slein (22). Aldolase activity was determined by colorimetric method of Sibley and Lebninger (23), and 6-phosphofructokinase by method of Kuo-Huang Ling, Birne, and Lardy (24). The DeMoss (25) method was used to detect the presence of glucose-6-phosphate and 6-phosphogluconic dehydrogenase activities. The method of Dische and Borenfreund (26) as modified by Axelrod and Jang (27) was used for testing pentose phosphate isomerase.

The formation of sedoheptulose, the product of transketolase activity, was shown by the method of Dische, Shettles, and Osnos (28) as modified by Axelrod et al. (29). The method of Walter Christian (30) was employed for detection of D-glyceraldehyde-3-phosphate dehydrogenase.

A Beckman DU spectrophotometer, using 3 ml quartz curvettes, light path 1 cm was employed in all experiments.

CHAPTER III

RESULTS AND DISCUSSION

Growth on various extracts. Table 1 shows that the organism grew moderately in all the extracts regardless of pH. Microscopic examination showed that the liquid cultures, particularly chicken manure extract, produced many tuberculated spores, but the solid media stimulated mycelial growth.

According to Ajello (31), soils from chicken house area were found to be more acid than soils negative for the presence of H. capsulatum, and had significantly higher organic carbon content and moisture-holding capacity than soils from other areas. It is true that environmental conditions may play an important part for growth of this organism in soil, but this study indicated that pH, within the limit of this experiment, had no limiting influence on the growth in extracts obtained from soils in or around the vicinity of chicken house. Although the organism can grow in some soils, it is more often found on those having a high organic content, as shown by Larsh et al. (32), and Furcolow et al. (33). It is reasonable to assume that the organism can grow better in organic matter, since such an environment provides nitrogen, carbon, and other

Table 1. Growth of the mycelial phase of Histoplasma capsulatum on various extracts

Sources	pH	Substrate			
		Crude Extract		Refined Extract	
		Liquid	Solid	Liquid	Solid
		Growth	Growth	Growth	Growth
Chicken Manure	6.5	+	+	+	+
	7.5	++	++	++	++
	8.5	++	++	++	++
Soil Sample Chicken House Floor	6.5	++	++	++	++
	7.8	++	++	++	++
	8.5	++	++	++	++
Soil Sample Outside of the Chicken House	6.5	++	+++	++	++
	7.6	++	+++	++	++
	8.5	++	+++	++	++
Soil Sample Near the Vicinity of the Chicken House	6.5	++	++	++	++
	7.5	++	++	++	++
	8.5	++	++	++	++

\+ Scant growth
++ Moderate growth
+++ Good growth

growth stimulating factors which may not be abundant in other soils.

Growth of H. capsulatum on various amino acids and inorganic nitrogen. Table 2 shows the amount of growth, based on dry weight, made on various amino acids and inorganic compounds as a sole source of nitrogen. The organism grew much better on alanine, serine, valine, leucine, isoleucine, and aspartic acid in glucose basal medium. Phenylglycine and cysteine hydrochloride supported scanty growth, while proline, histidine, and methionine showed little or no effect on the growth of H. capsulatum.

The maximum amount of growth was obtained when ammonium sulfate was used as a nitrogen source, but the organism failed to grow in the medium to which potassium nitrate was added. This would suggest that the organism could use only NH_3-N, but not NO_3-N as a sole source of nitrogen.

The excellent growth of this organism in salt medium containing ammonia as a nitrogen source in the presence of glucose indicates that the organism synthesizes all the amino acids necessary for protein synthesis through a reaction between ammonia and alpha-keto acids (pyruvic, oxalacetic, etc.) formed in carbohydrate dissimilation. Therefore, this fungus, like many other filamentous fungi, has no absolute requirement for a specific amino acid.

It was shown that various amino acids (Table 2) permit satisfactory growth in the presence of glucose. It seems plausible that the

Table 2. Effect of nitrogen source on the growth of mycelial phase of H. capsulatum with or without 2% glucose

Nitrogen Source	Glucose		Without Glucose	
	Dry wt. mg.	Final pH	Dry wt. mg.	Final pH
Ammonium sulfate..	58.9	3	0	6.9
Potassium nitrate ..	0	6.9	0	6.9
L-Glycine	6.9	6.4	0.7	6.9
L-Alanine	26.3	6.4	4.0	8.2
DL-Serine	24.5	6.5	7.5	7.8
L-Valine	24.1	5.6	8.0	7.2
L-Leucine	24.0	6.1	4.3	7.4
L-Isoleucine	26.7	6.1	5.2	7.4
L-Cysteine-HCl	7.8	6.5	2.4	7.1
L-Methionine	0	6.9	0	6.9
L-Aspartic acid	20.7	7.7	3.3	8.4
DL-Histidine.....	1.9	6.7	1.3	7.5
L-Proline	2.0	7.1	1.2	7.0

amino acids were first deaminated and the organism then utilized ammonia released to synthesize other amino acids necessary for protein synthesis. This postulated deamination may be easily demonstrated using medium containing an amino acid as a sole source of carbon. The change of pH of the media from acidic to alkaline may indicate the formation of ammonia in excess of the amount required for protein synthesis. Britten (34) showed a similar situation when Eschericia coli was grown in C^{14} glucose salt medium with the addition of glutamic acid. He found that a considerable amount of radio active glutamic was formed. Therefore, he concluded that most glutamic acid in the medium was used as a sole source of nitrogen.

Effect of carbon compounds on growth. The results of carbon utilization are presented in Table 3. The organism grew increasingly well in glucose, mannitol, and mannose. Scanty growth was obtained on maltose, sucrose, and glycerol media. No growth was observed on lactose, dulcitol, arabinose, xylose, pyruvate, lactate, acetate, propionate, butyric acid and caproic acid.

Microscopic examination of positive cultures showed that the organism produces many microconidia and tuberculated chlamydospores in the presence of sucrose and mannitol, but only few tuberculated chlamydospores in the presence of mannose and glucose. Glycerol stimulated the production of many microconidia, while few microconidia and tuberculated chlamydospores were seen in medium containing

Table 3. Effect of various carbon compounds on growth of the mycelial phase of H. capsulatum

*Carbon Sources	7 Days		14 Days		20 Days	
	Solid	Liquid	Solid	Liquid	Solid	Liquid
	Growth	Growth	Growth	Growth	Growth	Growth
Glucose	+	+	++	+++	+++	+++
Mannose	+	+	+++	+++	++++	++++
Fructose	±	±	+	+	+	+
Lactose	±	±	±	±	±	±
Maltose	+	±	+	+	+	+
Sucrose	+	+	+	+	+	+
Xylose	–	–	–	–	–	–
Arabinose	–	–	–	–	–	–
Glycerol	+	+	+	+	+	+
Sodium pyruvate	–	–	–	–	–	–
Dulcitol	–	–	–	–	–	–
Mannitol	+	+	++	++	+++	+++
Sodium lactate	–	–	–	–	–	–
Sodium acetate	–	–	–	–	–	–
Sodium propionate	–	–	–	–	–	–
Butyric acid	–	–	–	–	–	–
Caproic acid	–	–	–	–	–	–
Sabouraud control	+	+	+++	+++	++++	++++

*Each compound added separately into the basal medium.

+ Scant growth
± Doubtful growth
– No growth
++ Moderate growth
+++ Good growth
++++ Abundant growth

fructose or maltose.

It would seem that the organism was non-exacting as to its carbon sources and had no apparent growth factor requirements. It is probable that certain carbohydrates stimulate luxurious mycelium production, whereas others stimulate production of microconidia and tuberculated chlamydospores.

Manometric Studies

Effect of carbon sources. The intact cells of M-phase of H. capsulatum were capable of utilizing all sugars tested except lactose, xylose, and arabinose. Table 4 shows that the cells consume O_2 more rapidly in the presence of mannose, than did any other sugars tested, and that they oxidize maltose and sucrose slowly. Xylose, arabinose, and lactose does not stimulate O_2 consumption in sufficient amount to be considered utilization. Fructose stimulates the O_2 uptake more than did glucose. Bernheim (5) reported that in B. dermatitidis, fructose did not have any effect on its respiration. Mannitol stimulated the O_2 uptake, but not as much as did mannose, although the two are structurally related. Dulcitol had no effect on oxygen consumption.

The data presented in Figure 1 show the rate of oxygen uptake of cells (M-phase) of H. capsulatum on glucose, mannose, and fructose. There was no lag in the initial rate of oxygen uptake, as seen in some fungi and bacteria. It is reasonable to assume that H. capsulatum has constitutive enzymes for these sugars.

Lower fatty acids did not stimulate the oxygen uptake. Butyric and caproic acids depressed O_2 uptake. Apparently these two compounds

Table 4. Rates of oxidation of various carbohydrates, fatty acids, organic acids and alcohols by mycelial phase of H. capsulatum

Substrate	QO_2*
Dextrose	4.76
Mannose	9.86
Fructose	7.44
Xylose	0.98
Arabinose	0.06
Lactose	0
Maltose	3.88
Sucrose	1.02
Mannitol	2.21
Dulcitol	0.56
Sodium lactate	1.80
Sodium acetate	0.58
Sodium propionate	0
Butyric acid	0
Caproic acid	0

* Oxygen uptake per mg. dry weight per hr. corrected for endogenous respiration.

have a toxic effect on the respiration of H. capsulatum.

All of the compounds used in this experiment gave no measurable change in the oxygen uptake, when freshly harvested unstarved pellets were used; however, when starved pellets were used, the oxygen uptake increased. The same effect was noticed by Bernheim (5) in the mycelial of Blastomyces dermatitidis and by Gale (35) in Rhizopus oryzae.

It would seem that the rate of growth of this organism in synthetic medium containing a single carbon source showed some correspondence to QO_2 values. Rate of oxidation, however, is by no means the only factor that decides the growth-promoting quality of a nutrient. For example, H. capsulatum grew more rapidly on glucose than on fructose when supplied as single carbon sources, although the QO_2 values of fructose was greater than glucose.

Effect of various amino acids. All the amino acids tested increased oxygen uptake, except phenylglycine, alanine, and aspartic acids. Table 5 shows that cystiene hydrochloride, tyrosine, leucine, isolucine, glutamic acid, proline, valine, and histidine were utilized more rapidly than were serine, cystine, methionine, tryptophan, and threonine. Bernheim (5) showed similar results on B. dermatitidis except that aspartic acid had little or no effect on oxygen uptake. He reported that an increased O_2 uptake did not indicate complete oxidation of any amino acids added. The additional oxygen consumed was sufficient only to permit an oxidative deamination, but such occurrence did not seem likely, since the amount of ammonia produced was too low to verify oxidative deamination. He concluded that amino acids in-

Table 5. Rates of oxidation of various amino acids by mycelial phase of H. capsulatum

Substrate	QO_2*
Glycine	0.07
L-Alanine	0.95
L-Valine	2.85
L-Leucine	3.20
L-Isoleucine	3.16
DL-Serine	1.24
L-Threonine	2.45
L-Tyrosine	3.71
L-Tryptophan	1.33
L-Cysteine-HCl	4.72
L-Cystine	1.75
L-Methionine	1.78
L-Glutamic acid	3.20
L-Aspartic acid	0.50
DL-Histidine	2.58
L-Proline	3.00

* Oxygen uptake per mg. dry weight per hr. corrected for endogenous respiration.

creased the O_2 uptake, but were not deaminated in the process, however, they were able to stimulate the oxidation of some unknown substances in the cell. In many respects, the M-phase of H. capsulatum and B. dermatitidis are metabolically similar, but it seems that Bernheim's interpretation is rather inconclusive, since he did not describe those unknown substances in the cell.

Effect of tricarboxylic acid intermediates. The oxidative activity of the whole cells is shown in Table 6. Most of the Krebs cycle intermediates, except cis-aconitic acid were oxidized at a rate greater than that of endogenous respiration. The greatest amount of stimulation occurred with pyruvic and succinic acids, while fumaric, malic and alpha-ketoglutaric acids were oxidized much more slowly. The results of this study suggest a possibility that such a cycle may exist in H. capsulatum, but proof of its existence depends upon additional studies using radio-active carbon C^{14} tracers and the isolation and identification of the enzymes in this cycle.

Pathway of Glucose Dissimilation in Both the Mycelial and Yeast Phase of H. capsulatum

The presence of hexokinase activity of cell-free extracts were measured by noting TPN reduction (Fig. 2) the low enzymatic activity might be due to destruction of enzyme during the preparation of cell-free extracts. No change in optical density observed when DPN was substituted for TPN.

The presence of G-6-P and 6-phosphogluconic acid dehydrogenase was shown by the reduction of TPN (Fig. 3). DPN was ineffec-

Table 6. Rates of oxidation of various Kreb cycle intermediates by mycelial phase of H. capsulatum

Substrate	QO_2*
Pyruvic acid	4.80
Citric acid	1.40
Cis-aconitic acid	0.00
L-Isocitric acid	1.34
Alpha-Ketoglutaric acid	0.80
Succinic acid	4.77
Fumaric acid	0.54
Malic acid	0.38

* Oxygen uptake per mg. dry weight per hr. corrected for endogenous respiration.

tive. Both reactions appeared to be specific for the mycelial and yeast phase of H. capsulatum.

The cell-free extracts had phosphofructokinase activity (Fig.4). The reduction of TPN was very rapid as compared with DPN in the yeast phase. In the mycelial phase the enzyme activity was lower, but the reduction of TPN was higher than DPN.

D-glyceraldehyde-3-phosphate dehydrogenase activity of cell-free extracts were measured by observing the TPN and DPN reduction (Fig. 5). The yeast phase reduced TPN very rapidly as compared with DPN, but the mycelial phase reduced only TPN.

Both the yeast and mycelial phase of cell-free extracts showed aldolase activity (Fig. 6).

Pentose phosphate isomerase activity of cell-free extracts were detected by the formation of ribulose-5-phosphate, and the keto sugar was measured by cysteine-carozole reaction after 1/2 hour incubation period and the change of optical density against blank was read at 540 mu. The change of optical density for the yeast and mycelial phase were 0.75 and 0.25 respectively, indicating the presence of this enzyme in cell-free extracts.

The detection of transketolase and transaldolase activities were based on the increase in absorption spectra with the products formed as a result on the enzymatic activity on pentose-5-phosphate (Fig. 7). The maximum absorption shown at 415 and 520 mu corres-

ponds to hexose phosphate and sedoheptulose respectively. An incubation period of 3 hours or longer was necessary to show the peak corresponding to by-products.

From evidence presented here, it appears that both phases of organism are capable of oxidizing glucose through Embden-Myerhof - and hexose monophosphate shunt pathways. The study is not sufficient, however, to prove conclusively that these are the only two pathways of glucose dissimilation in both phases. The same pathways were shown by Clark and Wallace (36), McDonald et al. (37) in Pullulara pullulans and Claviceps purpurea respectively.

Apparently, most of the dehydrogenases in both phases are TPN specific. Clark and Wallace (36) showed the same specificity in P. pullulans. Roa et al. (38) recently showed the same specificity in cell-free extract of Candida albicans. They concluded that the almost absolute requirement of the enzyme for TPN may be associated with the highly aerobic nature of the organism.

In conclusion, it is reasonable to postulate that there is no significant difference enzymatically, within the limit of this experiment, between the yeast and mycelial phases of H. capsulatum. The lower enzyme activity of the M-phase was due to lower protein content of the cell-free extract.

The following abbreviations are used in this report: DPN, diphosphopyridine nucleotide; TPN, tri-phosphopyridine nucleotide;

ATP, adenosine triphosphate; G-6-P, glucose-6-phosphate; F-6-P, fructose-6-phosphate; F-1, 6-P, fructose -1, 6-diphosphate; P-5-P, pentose-5-phosphate; 6-PG, 6-phosphogluconate.

CHAPTER IV

SUMMARY

Growth of H. capsulatum in extracts from chicken manure and different soil samples at varying pH's was determined. It was found that the organism grew in these extracts regardless of pH of the media used in this experiment.

Of 13 compounds tested as a suitable single source of nitrogen for H. capsulatum, ammonium sulfate, alanine, serine, valine, leucine, isoleucine, and aspartic acid were found to be satisfactory in the glucose medium. The organism did not grow when nitrate nitrogen or methionine were used as a single source of nitrogen. The organism grew much better when ammonia nitrogen was used as a nitrogen source. It was concluded that the amino acid was used as a nitrogen source for the synthesis of all nitrogenous cellular constituents rather than a direct incorporation of amino acid into protein structure. It was found that the organism used most amino acids as a source of carbon and nitrogen when glucose was absent in the synthetic medium.

Results of carbohydrate utilization by H. capsulatum showed that the organism grew very well in mannose, glucose, and mannitol

but poorly in maltose, sucrose, and glycerol. Lactose, dulcitol, arabinose, lactate, pyruvate, and lower fatty acids did not stimulate growth. Microscopic examination showed that carbohydrates which support scanty growth of the organism usually stimulate production of microconidia and tuberculated chlamydospores.

The effects of various substances on the respiration of the M-phase were determined. It appears that the organism has constitutive enzymes for the utilization of mannose, fructose, glucose, maltose, and mannitol, but not for lactose, arabinose, xylose, dulcitol, and sucrose. The organism oxidizes mannose and fructose more rapidly than glucose, maltose, and mannitol. No oxygen uptake was observed with some of the lower fatty acids. Butyric and caproic gave a negative oxygen uptake, therefore, it was concluded that these two compounds may have a toxic effect on the respiration. Whether this toxic effect is endogenous or exogenous has not been established.

The organism oxidized most of the amino acids tested, except phenylglycine, alanine, and aspartic acid. Serine, isoleucine, cysteine hydrochloride, valine, glutamic acid, tyrosine, proline, and histidine, were utilized more rapidly than were serine, cystine, methionine, tryptophan, and threonine.

All of the tricarboxylic acid cycle intermediates stimulated oxygen uptake, except cis-aconitic acid, indicating a possibility of the Krebs cycle in this organism.

The enzyme studies were done on the mycelial and yeast phases of H. capsulatum. The results showed that the two phases possess enzymes of the Embden-Mayerhof and hexose-monophosphate shunt pathways of carbohydrate metabolism.

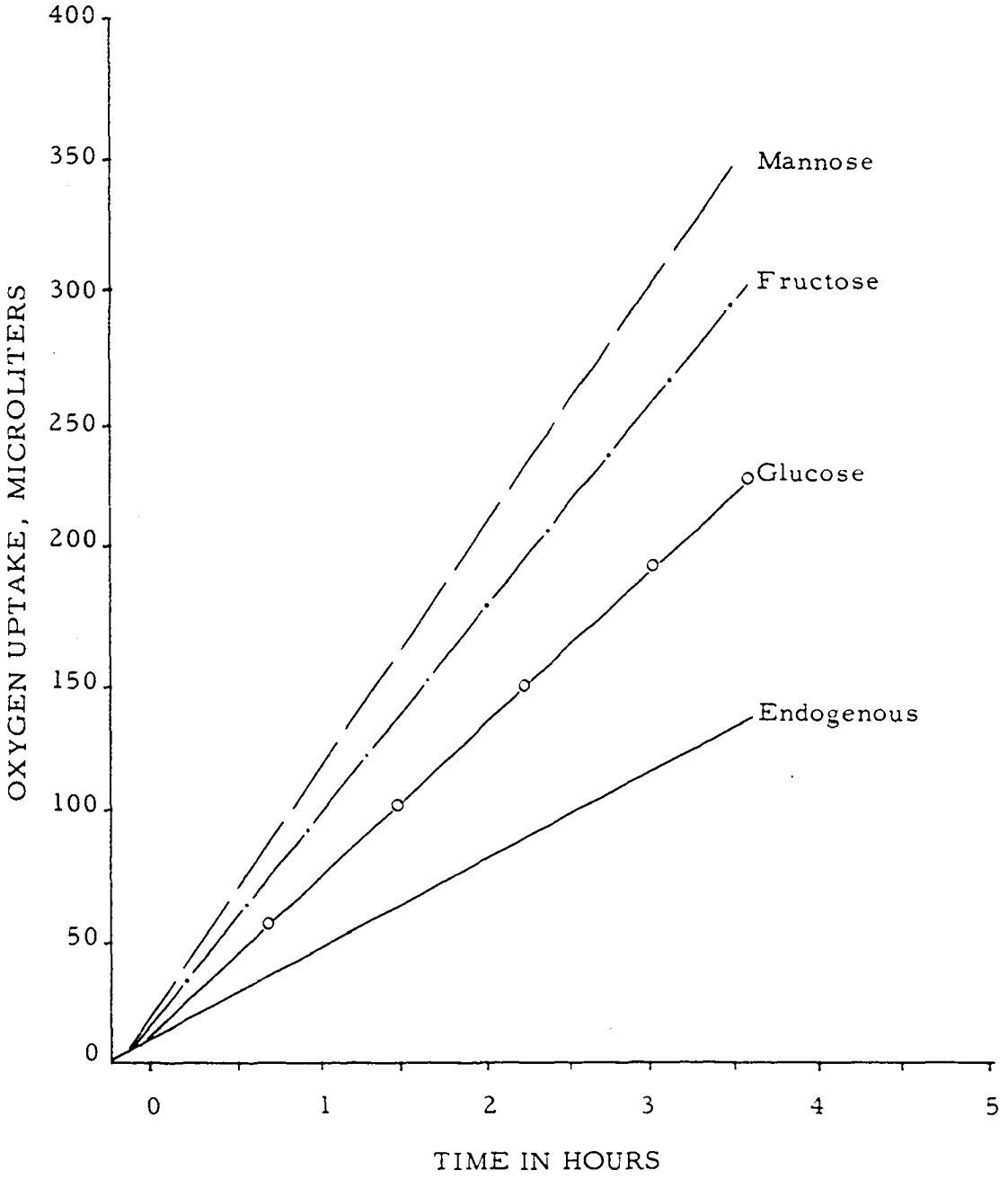

Figure 1. The Effect of Mannose, Fructose, and Glucose on Oxygen Uptake of Mycelial Phase of Histoplasma capsulatum.

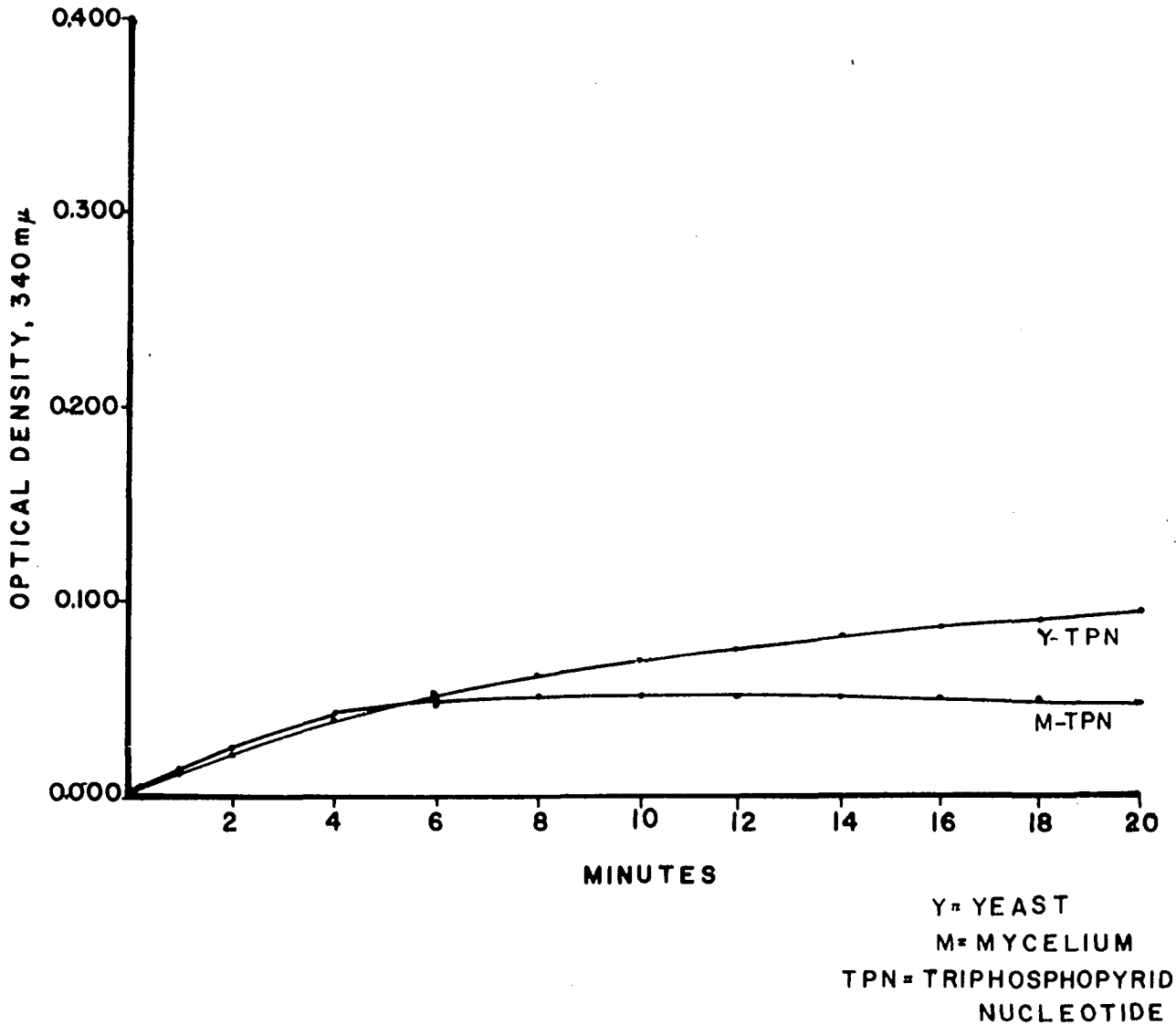

Figure 2. Hexokinase Activity of Cell-free Extracts of the Mycelial and Yeast Phases of H. capsulatum.

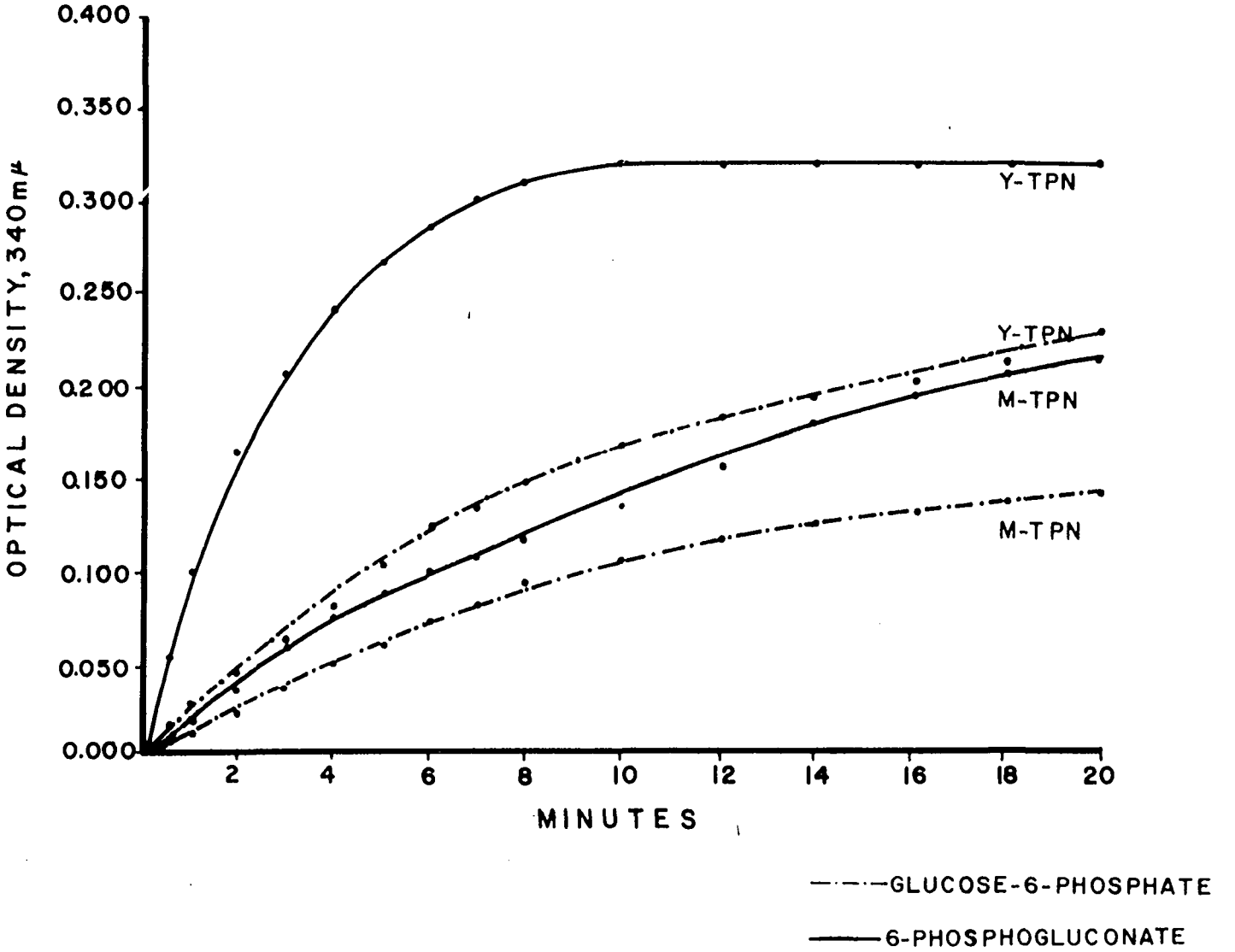

Figure 3. G-6-P and 6-GP Acid Dehydrogenase Activity of the Cell-free Extracts of the M and Y Phases of H. capsulatum.

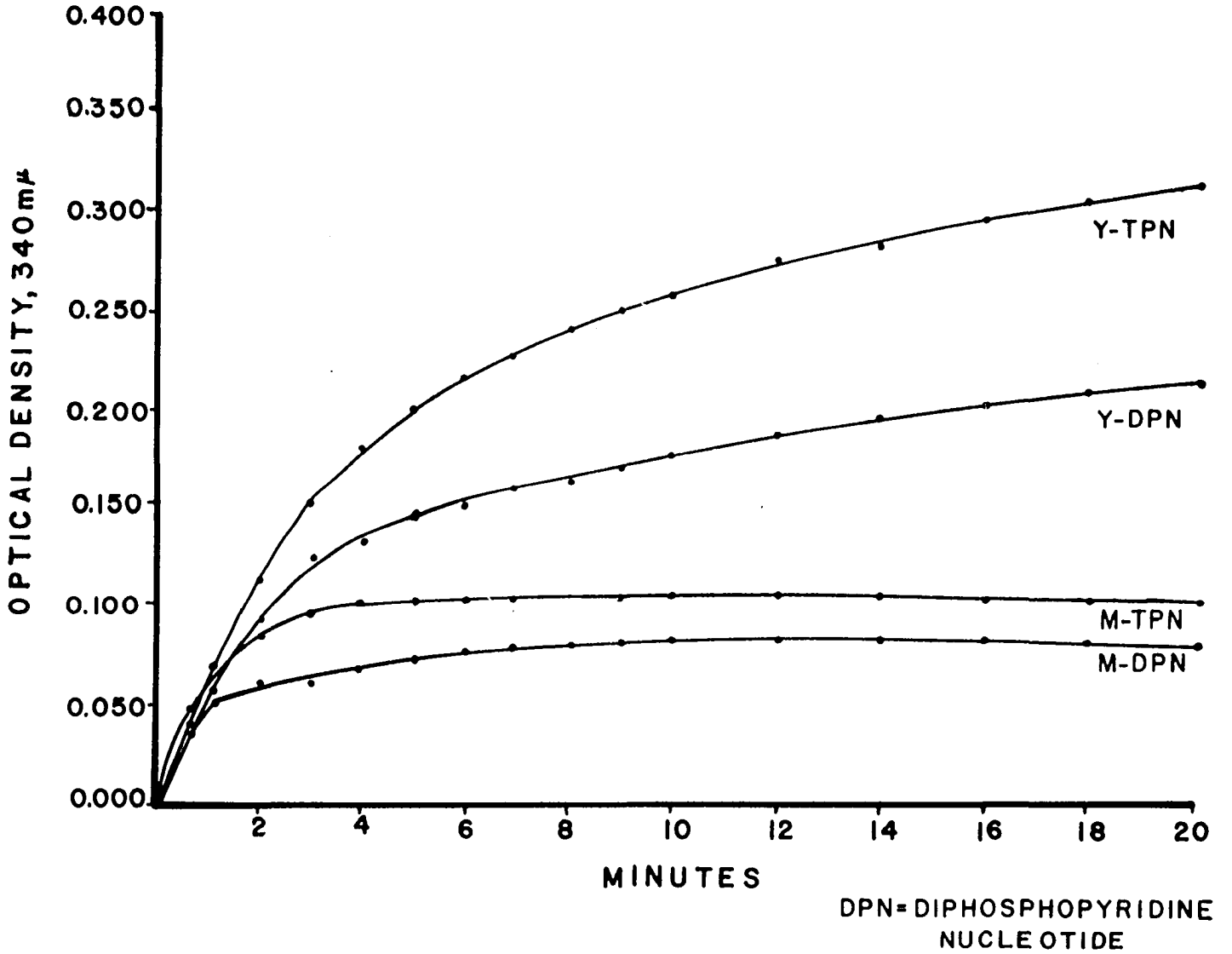

Figure 4. 6-Phosphofructokinase Activity of Cell-free Extracts of the M and Y Phases of H. capsulatum.

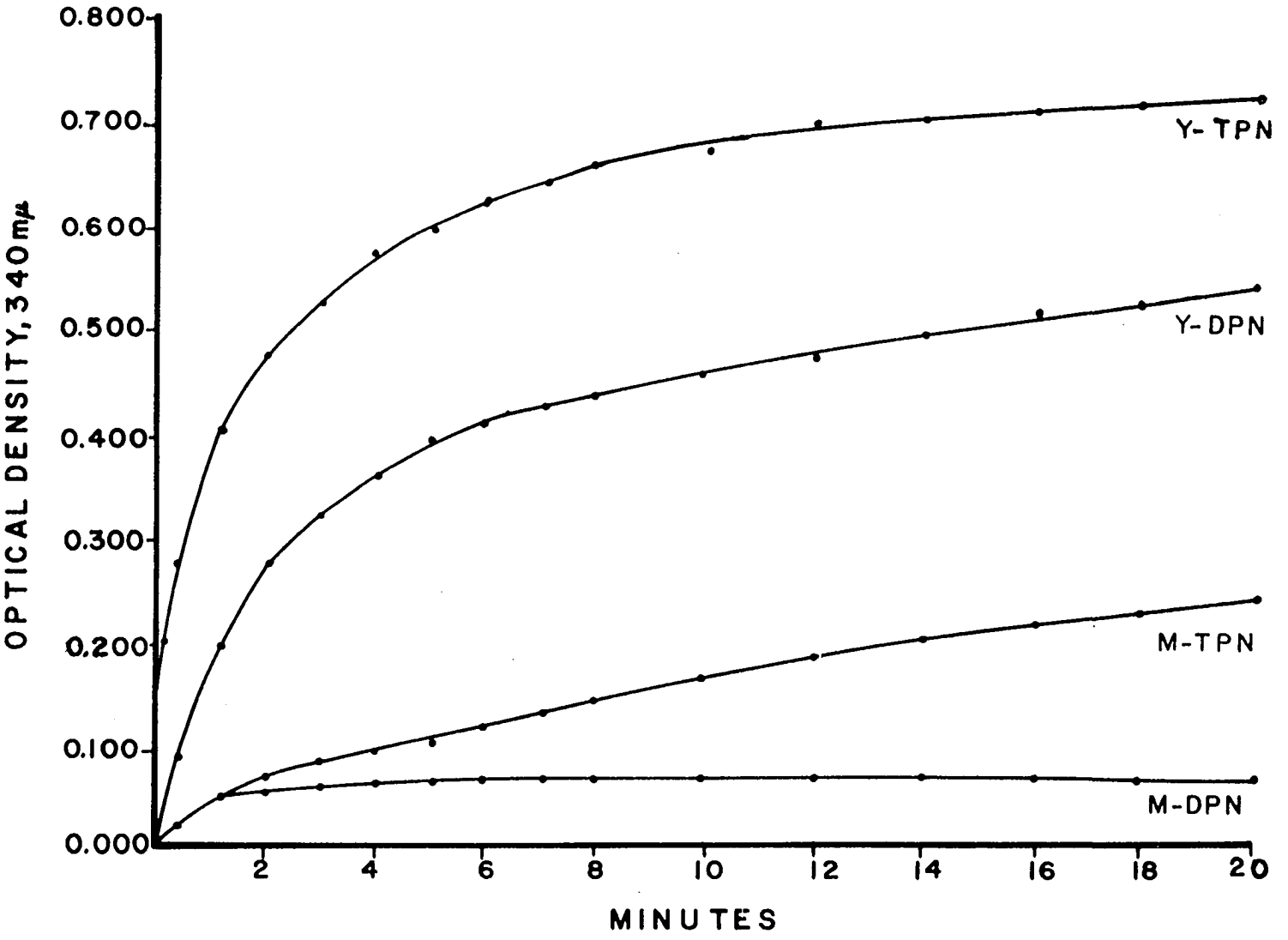

Figure 5. D-Glyceraldehyde-3-Phosphate Dehydrogenase Activity of Cell-free Extracts of the M and Y Phases of H. capsulatum.

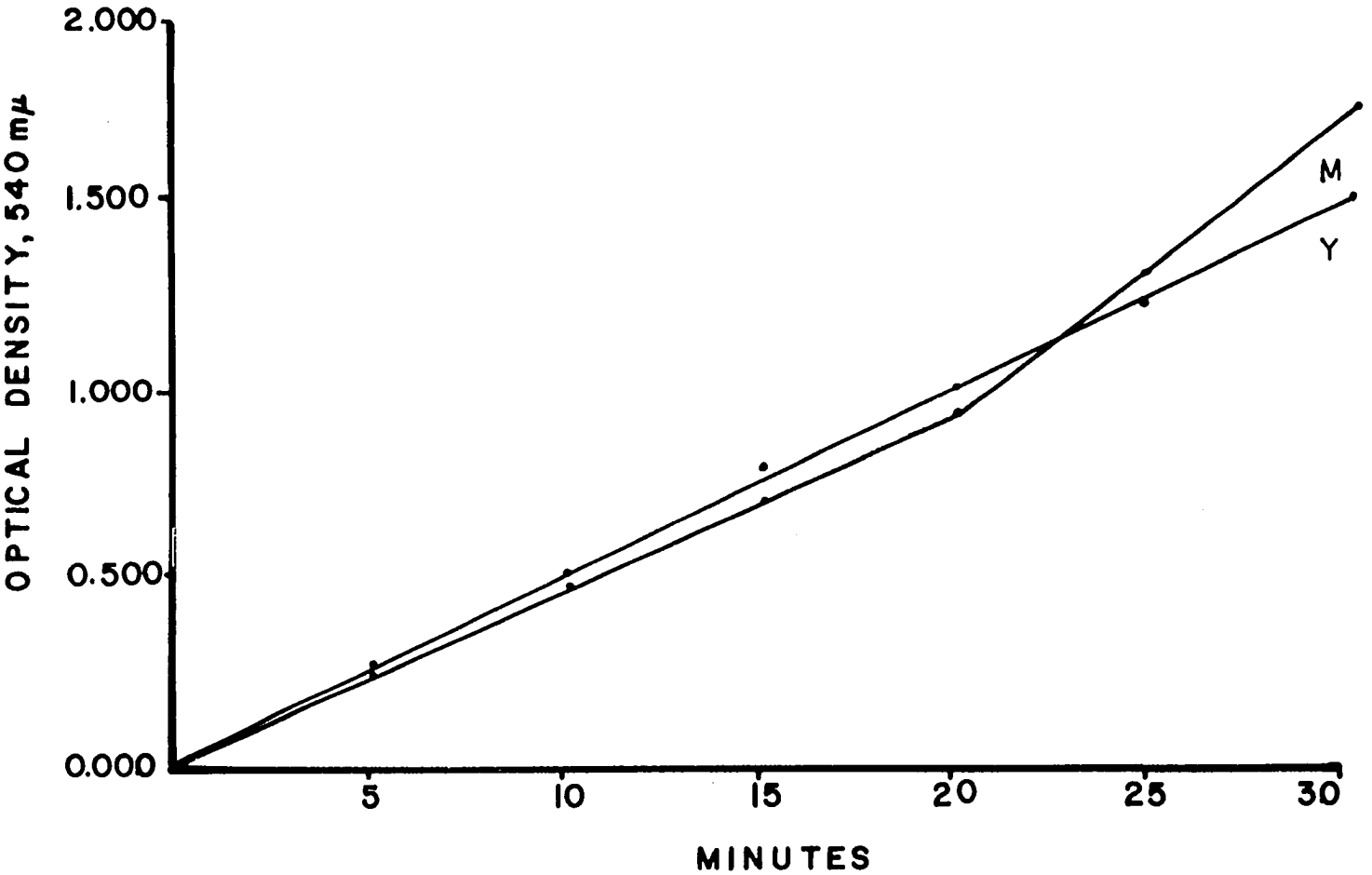

Figure 6. Aldolase Activity of Cell-free Extracts of the M and Y Phases of H. capsulatum

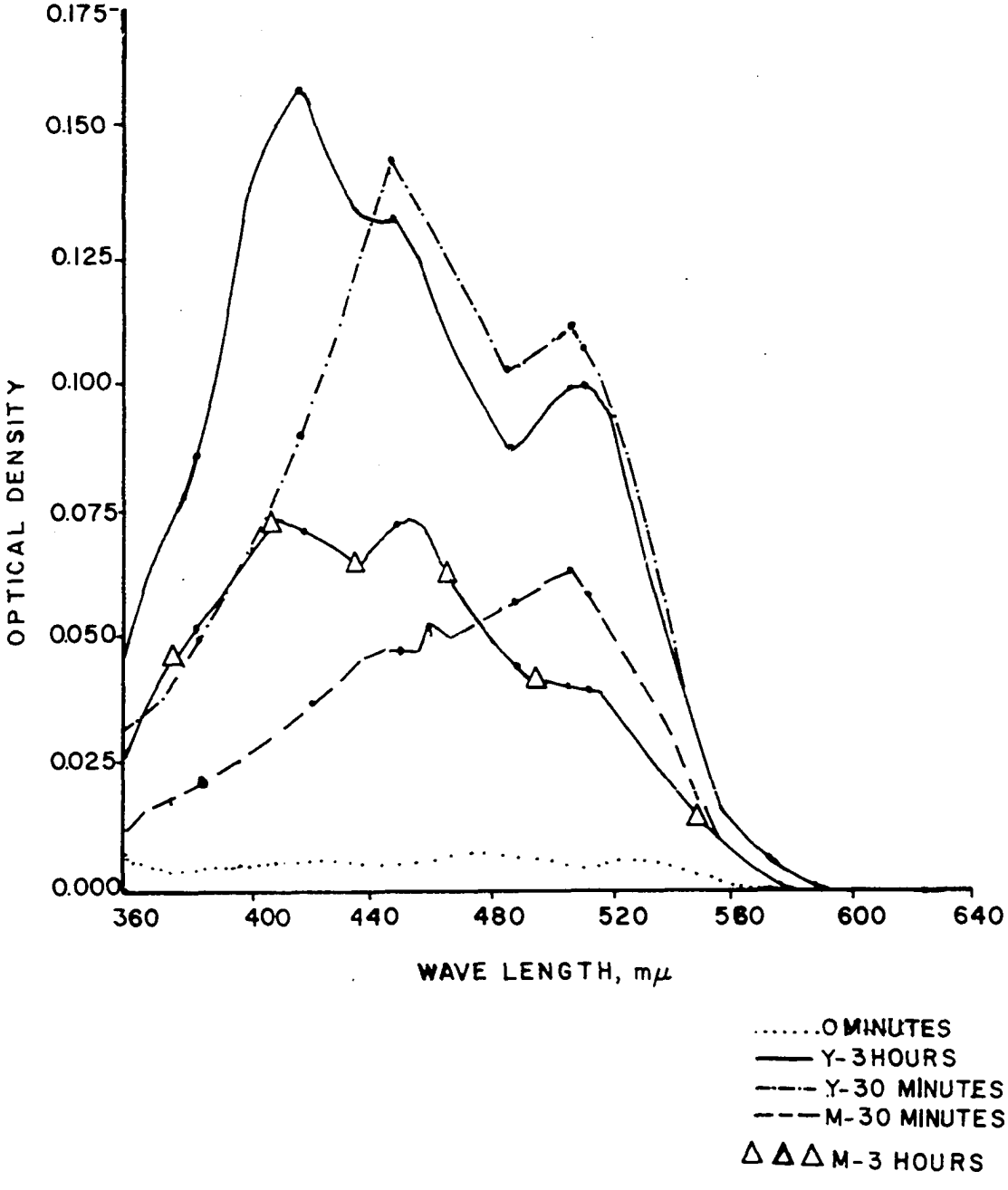

Figure 7. Absorption Spectra in Dische Test Obtained with the Products of Cell-free Extracts of the M and Y Phases of H. capsulatum on Pentose-5-Phosphate.

APPENDIX

Figure 1. The effect of mannose, fructose, and glucose on oxygen uptake of mycelial phase of Histoplasma capsulatum.

Figure 2. Hexokinase activity. Additions: glucose (0.5 M), 0.2 ml; $MgSO_4 \cdot 7\ H_2O$ (0.1 M), 0.15 ml; ATP (0.02 M), 0.5 ml; tris (hydroxymethyl)-aminomethane buffer (0.05 M), pH 7.9, 0.8 ml; TPN (1.0 mg per ml), 0.2 ml, cell-free extract 0.3 ml (Y) or 0.5 (M). Volume adjusted to 3.0 ml with water.

Figure 3. Glucose-6-phosphate and 6-phosphogluconic dehydrogenase activity. Additions: $MgSO_4 \cdot 7\ H_2O$ (0.1 M), 0.2 ml; tris buffer (0.05 M) pH 7.9, 0.9 ml; cell-free extract 0.3 ml (Y) and (M) 0.5 ml; TPN (1.0 mg per ml), 0.2 ml; G-6-P or 6-GP (0.25 M), 0.4 ml. Volume adjusted to 3.0 ml with water.

Figure 4. 6-Phosphofructokinase activity. Additions: fructose-6-phosphate (0.025 M), 0.2 ml; $MgSO_4 \cdot 7\ H_2O$ (0.1 M), 0.1 ml; cysteine HCl (0.2 M), 0.2 ml; sodium arsenate (0.4) molar, 0.2 ml; ATP (0.02 M), 0.3 ml; tris buffer (0.05 M) pH 7.9, 0.9 ml; cell-free extract, 0.3 ml (Y) or 0.5 ml (M); TPN or DPN (1.0 mg per ml), 0.2 ml. Volume adjusted to 3.0 ml with water.

Figure 5. D-glyceraldehyde-3-phosphate dehydrogenase activity. Additions: fructose-1, 6-diphosphate (0.1 M), pH 7.6, 0.3 ml; glycine (0.27 M), 0.3 ml; sodium arsenate (0.17 M), 0.2 ml; TPN or DPN (1.0 mg per ml), 0.2 ml; cell-free extract, 0.3 ml (Y) or 0.5 ml (M). Volume adjusted to 3.0 ml with water.

Figure 6. Aldolase activity of cell-free extracts of the mycelial and yeast phases of H. capsulatum.

Figure 7. Absorption spectra in Dische test obtained with the products of the M and Y phases on pentose-5-phosphate. Additions: P-5-P (0.025 M), 0.3 ml; tris buffer (0.1 M) pH 7, 0.2 ml; thiamine HCl (0.15 M) pH 7.5, 0.05 ml; cell-free extract, 0.3 ml (Y) or 0.5 ml (M). Reaction temperature, 38° C. Reaction stopped by adding 10 volumes of 5 per cent TCA. 1 ml of a 1:2 dilution of this mixture was employed in the Dische test.

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