AN ABSTRACT OF THE THESIS OF

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(Name) (Degree) (Major)

Date thesis is presented May 12, 1966

Title Growth Characteristics and Metabolism of Beggiatoa

Abstract approved [redacted]
(Major professor)

The addition of catalase to culture medium increased the period of viability of Beggiatoa from one week to two months. Addition of catalase also produced a marked increase in cell yield and enzyme activity. The addition of cysteine or hydrogen sulfide to the growth medium or the use of semi-solid medium stimulated growth. Oxygen was found to be required for growth, but carbon dioxide was not produced. Citrate and sulfite were inhibitory to growth whereas malate and acetate stimulated growth. Glucose and thiosulfate were not oxidized, and cytochromes were not detectable by spectrophotometric analysis.

Cells grown on agar surfaces in the absence of catalase exhibited an absorption peak characteristic of peroxides. This absorption peak was removed by addition of catalase during or after growth.

A proposed system which would permit acetate incorporation into four carbon compounds without the presence of key enzymes of the citric acid cycle or glyoxylate bypass was described. In this

system acetyl-CoA is condensed with glyoxylate to form malate which, in turn, is converted to oxaloacetate. Oxaloacetate then reacts with glutamate to produce α-ketoglutarate, which is subsequently converted to isocitrate. Cleavage of isocitrate produces glyoxylate and succinate to complete the cycle. Citrate and fumarate are not involved in the proposed cycle. Fumarase, aconitase, catalase, citratase, pyruvate kinase, enolase, phosphoenopyruvate carboxylase, lactic dehydrogenase, α-ketoglutarate dehydrogenase and condensing enzyme were not detectable in crude extracts of Beggiatoa.

Succinate was oxidized by a soluble enzyme not associated with an electron transport particle.

Sulfite, sulfide and ascorbic acid oxidation appeared to be correlated with peroxide production by flavoproteins during oxidation of organic substrates.

GROWTH CHARACTERISTICS AND METABOLISM OF *BEGGIATOA*

by

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A THESIS

submitted to

OREGON STATE UNIVERSITY

in partial fulfillment of
the requirements for the
degree of

DOCTOR OF PHILOSOPHY

June 1964

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Date thesis is presented May 12, 1964

Typed by Lucinda Nyberg

ACKNOWLEDGMENT

Sincere appreciation is expressed to Dr. Richard Y. Morita for his guidance during this investigation.

I am grateful to Dr. E. G. Pringsheim and Dr. S. Maier for the cultures they sent.

Thanks are expressly due my family for the help and consideration they have given.

This investigation was supported by United States Public Health Grant WP00443.

S. D. B.

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GROWTH CHARACTERISTICS AND METABOLISM OF *BEGGIATOA*

INTRODUCTION

Investigations on *Beggiatoa* have been hindered by the inability to grow sufficient quantities of cells to warrant biochemical studies and by the difficulty in maintaining stock cultures. In nature, however, these organisms are often found in very large masses and appear to be able to withstand adverse conditions for considerable periods of time. An interesting observation led to a possible explanation of these facts. Catalase negative *Beggiatoa*, when mixed with a slow growing catalase positive bacterium, exhibited luxurious growth and remained viable for several months. Growth of *Beggiatoa* in the presence of a catalase positive bacterium occurred at the surface of the culture medium whereas growth of pure cultures remained several mm below the surface.

Acetate has been reported to cause an increase in the growth of *Beggiatoa* (24, 35). Winogradsky (66, 22, p. 54) included a small amount of acetate in some of the media he used even though he considered *Beggiatoa* to be autotrophic.

The classification of *Beggiatoa* into various species according to the diameter of the trichome is obviously not satisfactory. This

method has been used because insufficient information is available to devise a classification based on biochemical characteristics. The description of the family Beggiatoaceae in the seventh edition of Bergey's Manual of Determinative Bacteriology (7) ends with a plea for additional information. The situation is further complicated by the fact that the few pure cultures described in the literature are not available for comparative purposes.

In view of the above information, the investigation of cultural conditions on the growth of Beggiatoa was initiated to determine conditions which would provide good cell yields and increase the length of time of stock culture viability. The investigation of the metabolism of these microorganisms was begun to clarify possible means of acetate utilization. The accumulation of such data should provide a basis for an improved classification.

PREVIOUS INVESTIGATIONS

"Motile, colourless threads, with oil-like contents in a water containing organic matter in solution, may with certainty be referred to as Beggiatoa alba." This quotation from Ellis (22 p. 93) typifies the rather loose definition that has allowed investigators to report the occurrence of Beggiatoa, often based on casual observations, in sulfur springs, sewage, sewage outflows, stagnant ponds, decaying algae, desert waters, warm mineral springs, thermal waters, marine tidal flats and submarine canyons (2, 3, 7, 10, 11, 14, 17, 21, 22, 23, 24, 27, 29, 30, 33, 35, 37, 39, 40, 44, 46, 52, 53, 59, 61, 62, 63, 65, 66, 67). Rarely have pure cultures been obtained and the description of the microorganisms given in sufficient detail to assure the validity of the reports.

It is undoubtedly due to work with impure cultures that has led to the reported highly pleomorphic nature of Beggiatoa (22, 68).

The first well-documented investigations on Beggiatoa were performed by Winogradsky (65, 66, 67, p. 1-149). Although he never attained pure cultures, he was able to observe oxidation of hydrogen sulfide and demonstrated that this was the source of intracellular sulfur granules found in Beggiatoa. He formulated the concept of autotrophy from these observations and later proved this concept with the Nitrobacteraceae (67, p. 150-861). His concept

of the autotrophic nature of Beggiatoa still has considerable influence today.

Kiel (33) provided the most convincing and the last evidence of the autotrophic nature of Beggiatoa. He was able to obtain pure cultures and grow them in the absence of any organic matter. Although his work has not been duplicated by others, it is possible that autotrophic strains occur.

Cataldi (11) developed an enrichment procedure for hetertrophic strains of Beggiatoa. This method has been used to obtain most of the pure cultures isolated since 1940. Her enrichment medium consisted of dried hay that had been treated with hot water to remove most of the soluble organic matter.

Scotten and Stokes (53) obtained pure cultures of Beggiatoa using Cataldi's enrichment procedure and observed reduction of methylene blue by intact trichomes when placed with numerous organic compounds. Formate, lactate and succinate provided the most rapid reduction of methylene blue. Oxygen uptake occurred with lactate, glutamate, α-ketoglutarate, succinate, pyruvate and acetate whereas glucose and glycerol were inactive. Their studies were hampered by uncontrollable lysis and subsequent loss of activity.

Faust and Wolfe (24) isolated several strains of Beggiatoa which were unable to utilize a number of organic compounds for

growth but were stimulated by acetate and low levels of sulfide. Their investigations on the metabolism of Beggiatoa were limited by low cell yields.

Samuel-Maharajah (49) investigated the American Type Culture Collection strains 11028 and 11029 of Beggiatoa alba and reported the occurrence of carbon dioxide uptake in the presence of sulfur and reduced sulfur compounds. He identified radioactive phosphoglyceric acid, fructose-6-phosphate, hexose-diphosphate, glucose-1-phosphate, aspartic acid, serine and alanine after exposure to $C^{14}O_2$ for ten sec.

Johnson and Baker (30) used raw sewage as an enrichment source for Beggiatoa for morphological studies. They were unable to obtain pure cultures or demonstrate stimulation of growth by hydrogen sulfide.

Lackey (35) obtained pure cultures of Beggiatoa and noted that good growth occurred in acetate medium on initial isolation but that growth was poor on subsequent transfers. He concluded that Beggiatoa have an absolute need for hydrogen sulfide and oxygen. He stated that Beggiatoa are not satisfactory indicators of pollution.

Bahr and Schwartz (2) found the presence of hydrogen sulfide and a reducing potential between 250 and 400 millivolts to be important for growth of Beggiatoa in natural conditions.

Pringsheim (46) considers Beggiatoa to be a pigment-free

relative of Oscillatoria and that it can be either autotrophic or heterotrophic.

Maier (37) investigated the cytology of the family Beggiatoaceae and concluded that this family should be placed in the class Schizophyceae rather than in the class Schizomycetes.

Morita and Stave (40) prepared ultrathin sections of Beggiatoa grown in Cataldi's enrichment medium and were able to demonstrate cell membranes and cell walls. No set number of sulfur granules occurred within any of the cells.

Most of the pure cultures of Beggiatoa that have been isolated have been lost and are not available for current work.

MATERIALS AND METHODS

Microbiological Methods

A Beggiatoa strain isolated by Maier (37) from mud dredged from the bottom of Lake Erie was used in these studies. The trichomes, stained gram negative, were not sensitive to lysozme and did not contain cellulose. Motility was due to a gliding motion, and flagella were not detectable. Cell diameter varied with cultural conditions but averaged from 2.0 -2.5 μ. On the basis of trichome diameter Maier (37) considered this strain to be B. leptomitiformis.

The medium for culture maintenance and for cell production was composed of yeast extract (Difco), 2.0 g; calcium chloride, 0.1 g; sodium acetate, 0.5 g; tap water, 1,000 ml; and unless otherwise stated, 2.0 g agar. Catalase was added to make a final concentration of ten Sigma units per ml when noted. For mass production of cells, Fernback flasks containing 250 ml sterile medium were inoculated with 2.5 ml of an actively-growing culture and incubated without agitation.

Smith fermentation tubes filled with liquid media were used to determine the effect of several compounds on growth and evaluate CO_2 production. All cultures were incubated at 28 C.

Oxidation of hydrogen sulfide by intact trichomes was ascertained by flushing 48 hr cultures with sterile hydrogen sulfide followed by

aerobic incubation. The cultures were examined microscopically for sulfur granules.

Compounds tested for their stimulatory action on the growth of Beggiatoa were added to semi-solid medium. Their effects on growth were estimated visually. Heat labile compounds were sterilized by filtration through membrane filters (Millipore, 0.45 mμ pore size). The inocula were prepared from stock cultures never exposed to catalase.

Chemical Assays

Glucose was determined with "Glucostat" (Worthington Enzyme Co.). Thiosulfate was determined using standard idiometric titrations. Protein and nucleic acids were estimated by the method of Warburg and Christian (64).

Preparation of Cell-Free Extracts

Cell-free extracts were prepared by treating washed cells for ten min in a 10-kc Raytheon sonic oscillator. Cell debris was removed by centrifugation at 10,000 x g for ten min.

Photomicrographs

Photomicrographs were taken using a Leitz Ortholux microscope equipped with a Heine phase contrast condensor #74 and Pv Fl 70/1.15

n objective. Photographs were taken using a green filter, Kodak High Contrast Copy film and "Mikas" camera attachment.

Chemicals and Biological Reagents

The following items were obtained from Sigma Chemical Company: catalase, stock # C-3, crude sterile; catalase, stock # C-100, 2X crystallized; lactic dehydrogenase, type II, (free of pyruvate kinase); Coenzyme A, (CoA); acetylphosphate, (lithium salt); thiamin pyrophosphate chloride, (TPP); tris-(hydroxymethyl)-aminomethane, (tris); and Clostridium kluyveri, (dried cells, used as source of phosphotransacetylase).

Crystalline fumarase, dl-isocitrate, (disodium salt); phosphoenolpyruvic acid, (trisodium salt, A grade); nicotinamide adenine dinucleotide, (NAD); reduced nicotinamide adenine dinucleotide, (NADH) and nicotinamide adenine dinucleotide phosphate, (NADP) were obtained from California Corporation for Biochemical Research.

Glyoxylic acid and phenazine methosulfate (PMS) were obtained from Nutritional Biochemicals Corporation. All other reagents and compounds were of reagent grade and used without further purification.

Spectrophotometric Analysis

Absorption spectra were determined in a Cary Model II recording

spectrophotometer. Routine spectrophotometric assays were performed with a Beckman DU spectrophotometer.

Enzyme Assays

The determination of malate synthetase activity was performed by a coupled reaction with phosphotransacetylase as described by Ajl (1). Phosphotranacetylase was prepared from C. kluyveri as described by Novelli (41) and assayed as described by Stadtman (60).

A coupled reaction with malic dehydrogenase was employed to assay citratase. The reaction mixture contained in μmoles: NADH, 0.2; citrate, 1.0; tris-HCl buffer (pH 7.4), 200. Enzyme (containing a minimum of 500 units malic dehydrogenase) and distilled water were added to bring the volume to three ml. The change in absorption was observed at 340 mμ for five min. Malic dehydrogenase activity was determined by following NADH oxidation as described by Ochoa (42), with the exception that the 0.25 M glycylglycine buffer was replaced with 0.2 M tris-HCl (pH 7.4) buffer.

The chemical transfer of acetyl group of acetyl-CoA to hydroxylamine and the colorimetric estimation of acetohydroxyamate was used to determine acetyl thiokinase activity (31). A similar process (48) was used to assay for acetokinase.

The reduction of 2, 6-dichlorophenolindophenol (DCIP) in the presence of PMS was used to measure succinate oxidation by extracts

of Beggiatoa. The reaction mixture contained in μmoles: tris-HCl, 200; (pH 8.7); succinate, 150; DCIP, 0.2; sodium cyanide, 10.0. The volume was adjusted to 2.9 ml after addition of enzyme. The reaction was begun by adding 0.1 ml of 0.1% PMS and the change in absorption observed for three min. Controls minus succinate were used to determine non-specific reduction of DCIP.

The change in absorption at 252 mμ in the presence of semicarbazide and isocitrate (43) and the spectrophotometric determination of the 2, 4-dinitrophenylhydrazone of glyoxylate after incubation of extracts with isocitrate (58) were used to determine isocitratase activity.

The spectrophotometric assay described in Sigma Chemical catalog (54, p. 8) was used to determine catalase activity. Aconitase and fumarase were assayed by spectrophotometric procedures (47).

The reduction of α-lipoic acid by NADH (51) was used to determine lipoic dehydrogenase activity. The reaction rate was followed by the decrease in absorption at 340 mμ.

DCIP linked NADH diaporase was determined by following the decrease in absorption at 605 mμ as described by Mahler (36).

A coupled reaction with malic dehydrogenase (3) was used to determine phosphoenolpyruvate carboxylase activity.

Enolase activity was determined as described by Bücher (8). The coupled reaction with lactic dehydrogenase (9) was used to assay

pyruvate kinase. The procedure described by Kornberg (34) was used to assay lactic dehydrogenase activity.

The spectrophotometric determination of DCIP reduction by α-ketoglutarate dehydrogenase (50) and the reduction of NADP by isocitrate dehydrogenase (45) were used to determine these enzymes.

The procedure described by Cohen (13) for glutamic-aspartic transaminase was modified by using malic dehydrogenase to measure oxaloacetate formation.

The non-enzymatic reduction of DCIP by residual sulfide after incubation of the crude extracts with hydrogen sulfide was used to determine sulfide oxidation. The reaction mixture contained in a total of three ml: 0.2 μmole sulfide, 600 μmoles tris-HCl buffer (pH 7.2), and enzyme. At the end of the reaction time 0.1 to 0.3 μmole of DCIP was added, and the maximum decrease in absorption was noted. Residual sulfide was determined by comparison with reduction caused by known amounts of sulfide.

The decrease in absorption at 320 mμ of a NAD-sulfite complex (15) and the decrease in absorption at 320 mμ of a PMS-sulfite complex were employed to determine sulfite oxidation. The former reaction contained in three ml: 1.0 μmole sulfite, 6.0 μmole NAD, 200 μmoles tris-HCl buffer (pH 7.4), and enzyme. The latter reaction contained in three ml: 0.5 μmole sulfite, 0.1 mg PMS, 200 μmoles tris-HCl buffer (pH 7.4), and enzyme. A similar procedure

was used to measure ascorbic acid oxidation. The reaction mixture contained in 3.0 ml: 0.5 μmole ascorbic acid, 0.1 mg PMS, 200 μmoles tris-HCl buffer (pH 7.4) and enzyme. The reaction was followed at 320 mμ.

RESULTS

The American Type Culture Collection strains of Beggiatoa alba, 11028 and 11029 were examined and found to be lacking many of the characteristics that are descriptive of Beggiatoa. Both cultures grew well on nutrient agar, metabolized glucose, exhibited motility similar to that of flagellated bacteria and produced heat resistant spherical bodies. The effect of hydrogen sulfide on these cultures was variable, but oxidation of sulfides was never ascertained.

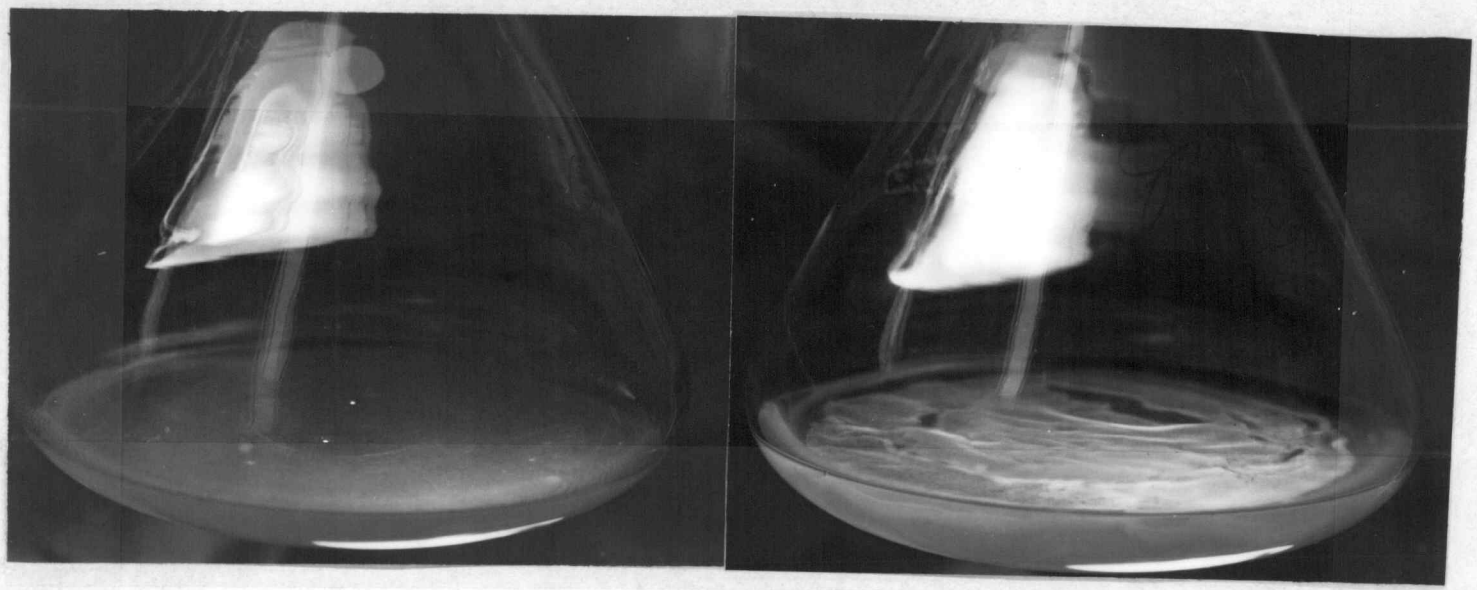
A culture sent by Dr. Pringsheim was badly dehydrated on arrival, and the mold-like culture obtained by subculturing was considered a contaminant picked up in transit.

Several cultures of a Beggiatoa strain isolated from Lake Erie mud were received from Dr. Maier; but the only viable culture also contained a non-motile, catalase positive, pink-pigmented cocci. During the process of isolating the Beggiatoa from this contaminant, it was noticed that the Beggiatoa grew much better in the presence of the cocci. The Beggiatoa grew well on solid agar surfaces near the contaminant but grew much slower away from the cocci. A marked difference in growth also occurred in semi-solid medium. The mixed culture grew rapidly at the surface of the medium whereas the pure culture of Beggiatoa grew best in freshly steamed medium and remained several mm below the surface.

Preliminary experiments with pure cultures of Beggiatoa and sterile catalase (2X crystallized or crude) produced identical beneficial effects as those observed in mixed cultures. The addition of cysteine or hydrogen sulfide to the medium also stimulated growth in the absence of catalase, but 2-mercaptoethanol was inhibitory to growth when added. No growth occurred under strict anaerobic conditions.

The effect on growth of Beggiatoa of adding ten Sigma units per ml of denaturated (heated 20 min at 121 C) or native catalase to semi-solid medium is illustrated in Fig. 1. Heat-denatured catalase did not stimulate growth, and the trichomes were dispersed throughout the medium with the exception of the top two to five mm. In the medium to which active catalase was added the growth was almost exclusively at the surface where it formed a heavy mat.

The effect of catalase on growth of Beggiatoa in semi-solid medium in culture tubes is illustrated in Fig. 2. The Beggiatoa in the medium with heat-denatured catalase grew at first only at the very bottom of the medium and moved at the end of 48 hr to the position shown. In 98 hr the narrow band of growth came to a stop about one cm from the surface. The trichomes in the presence of catalase at first grew throughout the entire medium, then moved to the top as the oxygen became depleted in the lower portion of the medium. The medium in both tubes was in equilibrium with the



A B

Figure 1. Effect of catalase on growth of Beggiatoa in semi-solid medium in Fernback flasks. Catalase was added to both flasks in a final concentration of 10 Sigma units per ml. Flask A was sterilized (at 121 C for 20 min) after addition of catalase. Flask B was sterilized prior to addition of catalast. Photographs were taken 48 hr after addition of one % inoculum.

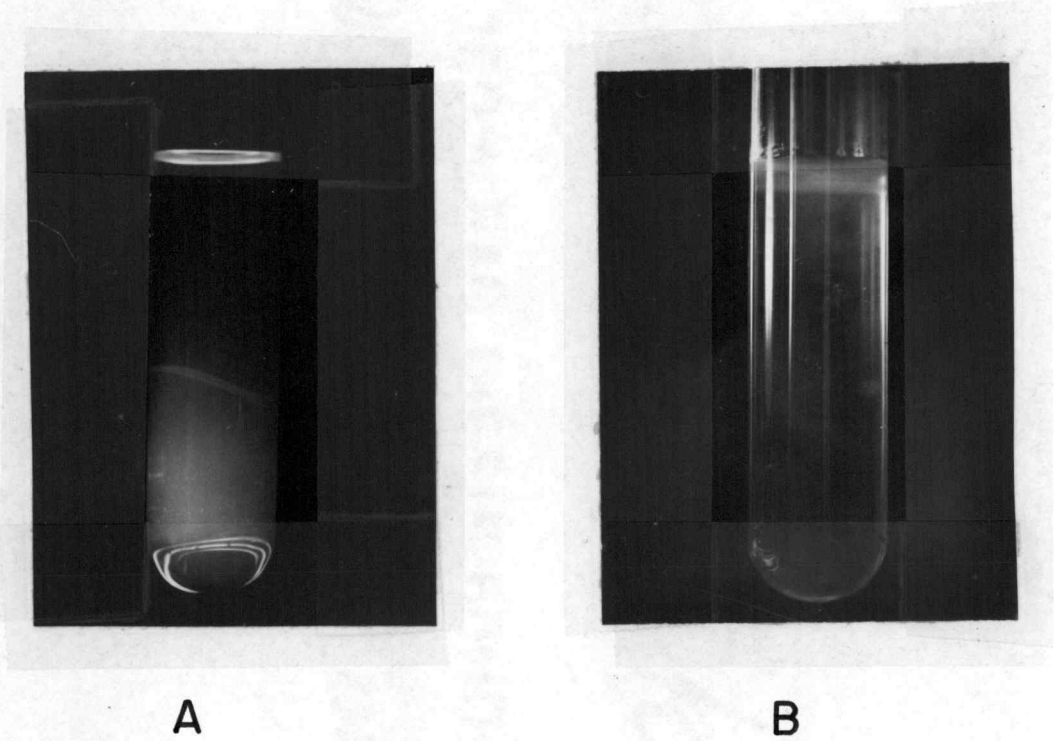


Figure 2. Effect of catalase on growth of Beggiatoa in semi-solid medium in culture tubes. Conditions of catalase addition and incubation are the same as those described in Fig 1.

atmosphere prior to inoculation.

The growth of Beggiatoa on solid agar surfaces with denatured and native catalase added is illustrated in Fig. 3. The agar with denatured catalase had only meager growth whereas the agar with active catalase was covered with profuse growth.

The ultraviolet absorption spectra of extracts obtained from cultures grown on solid agar surfaces in the presence of native and denatured catalase is shown in Fig. 4. In the absence of active catalase a component with an absorption peak characteristic of peroxides occurred (12) and increased with the age of the culture. The component was removed on addition of catalase to the extract.

The oxidized and reduced spectra of Beggiatoa extracts are shown in Figs 5 and 6. No cytochrome peaks were evident in the extracts.

The effects of catalase on growth are summarized in Table 1. The culture grown in liquid medium with catalase had 3.8 times as much protein, 5.2 times as much nucleic acid, 24 times as much wet weight, and 93 times as much malic dehydrogenase activity as the culture grown with denatured catalase. The packed trichomes grown in the presence of catalase were very gelatinous whereas the trichomes grown with denatured catalase were densely packed.

The activity of Beggiatoa on several compounds is shown in Table 2. In no instance was there any growth in the anaerobic side of the flask or was CO_2 detected. Glucose and thiosulfate were not

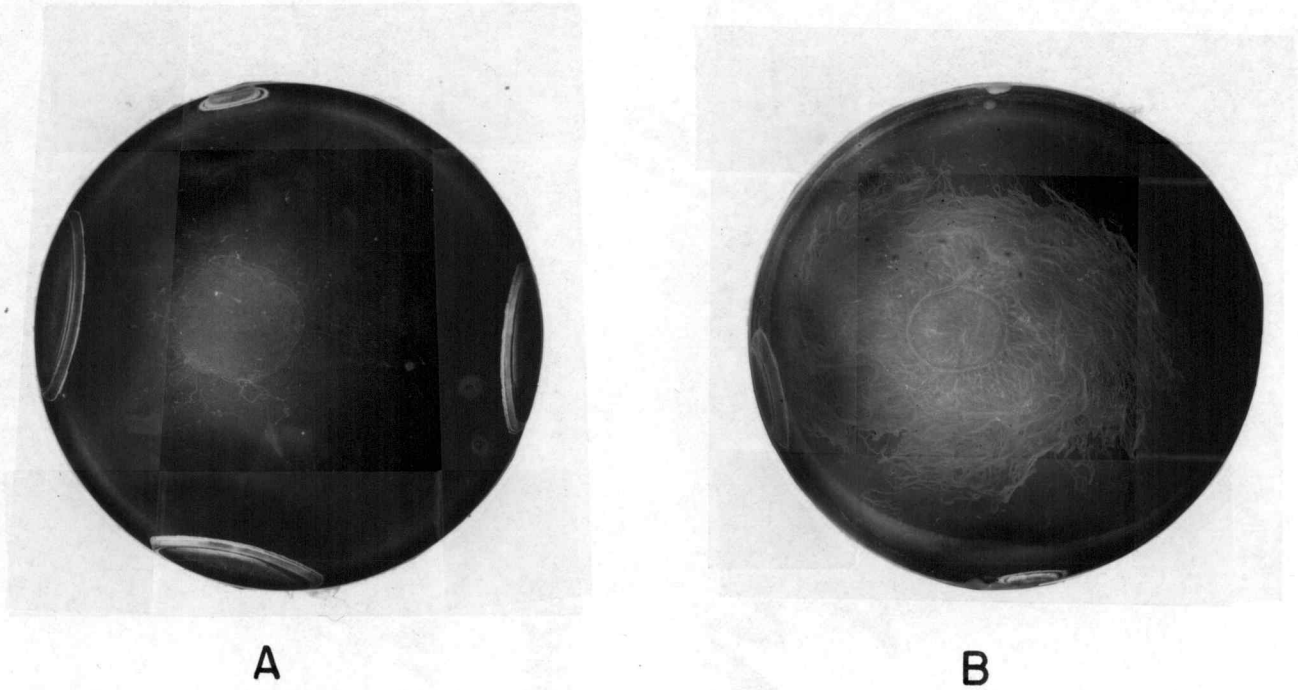


Figure 3. Effect of catalase on growth of Beggiatoa on solid agar surfaces. Conditions of catalase addition and incubation are identical to those described in Fig 1.

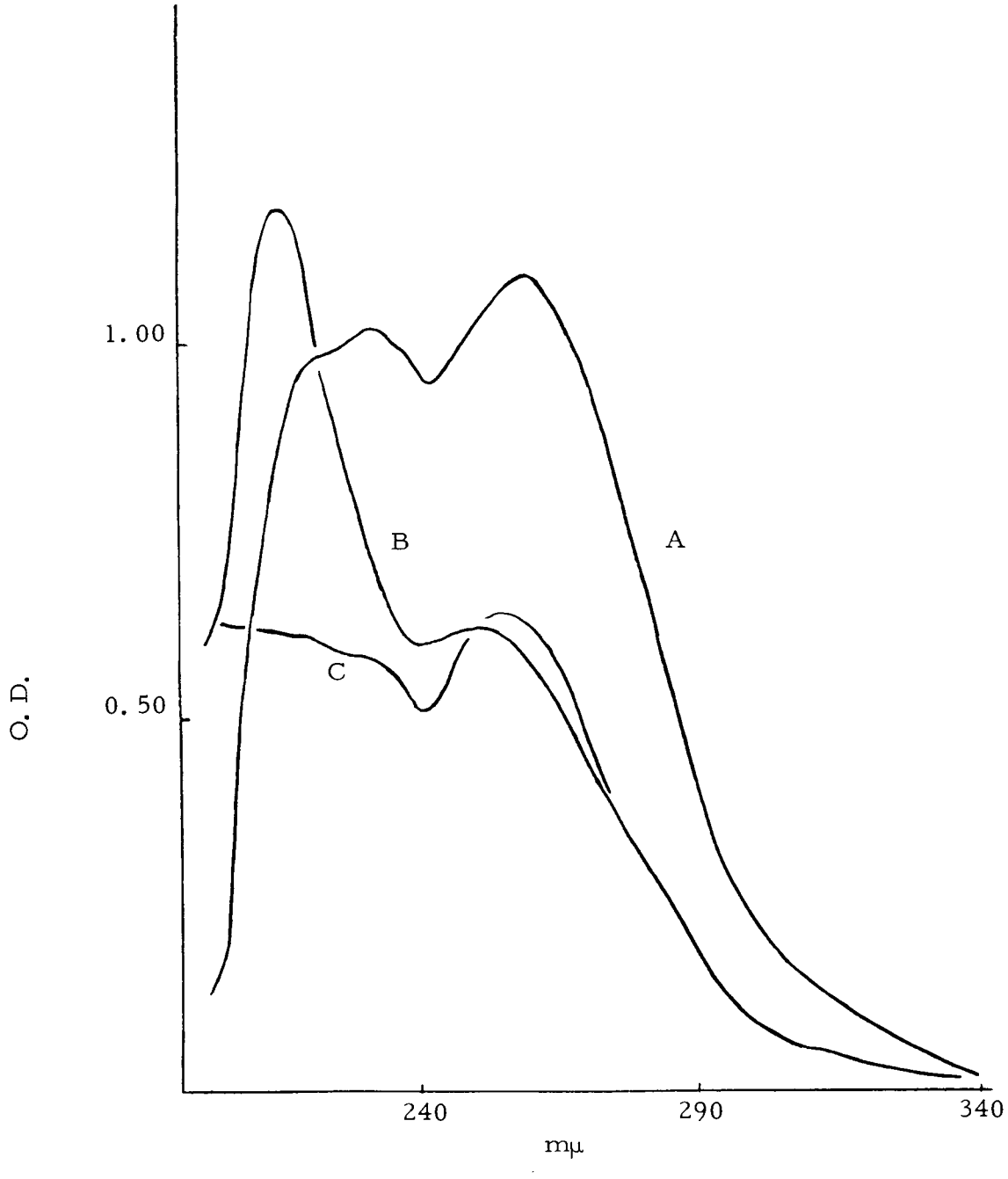


Figure 4. Effect of addition of catalase to growth medium on ultraviolet absorption spectra of cell-free extracts of Beggiatoa. Curve A, extract obtained from culture grown in medium to which catalase was added after sterilization. Curve B, extract obtained from culture grown in medium to which catalase was added prior to sterilization. Curve C, same extract as curve B, but 30 min after addition of 300 Sigma units of catalase, (catalase also added to reference cell).

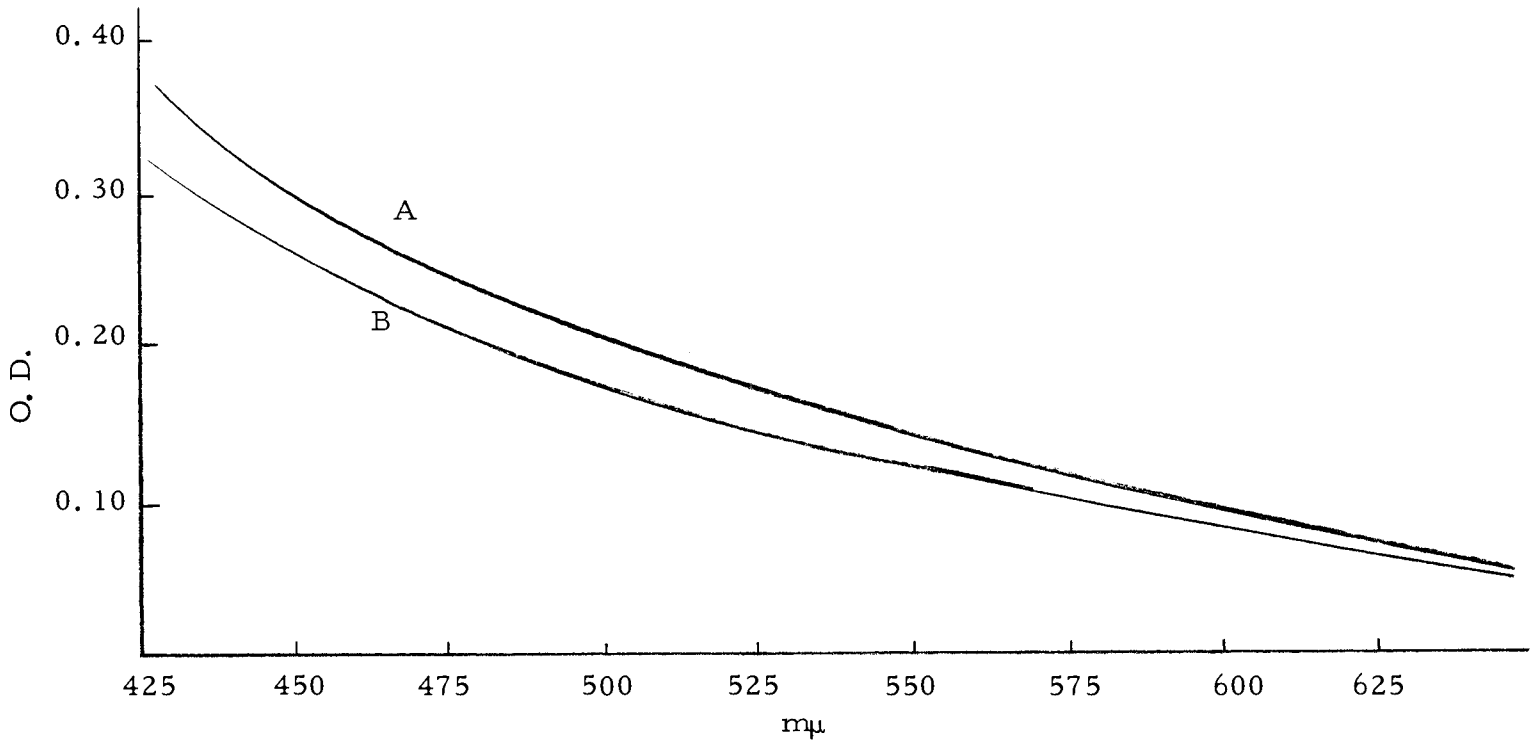


Figure 5. Oxidized and reduced visible absorption spectra of Beggiatoa extracts. Cells washed three times with 0.01% $CaCl_2$ solution before preparation of cell-free extracts. Sodium desoxycholate (0.01%) added after treatment of washed cells for 10 min in a 10-kc Raytheon sonic oscillator. Cell debris allowed to settle out prior to determination of spectra. Solid sodium hydrosulfite added to curve B.

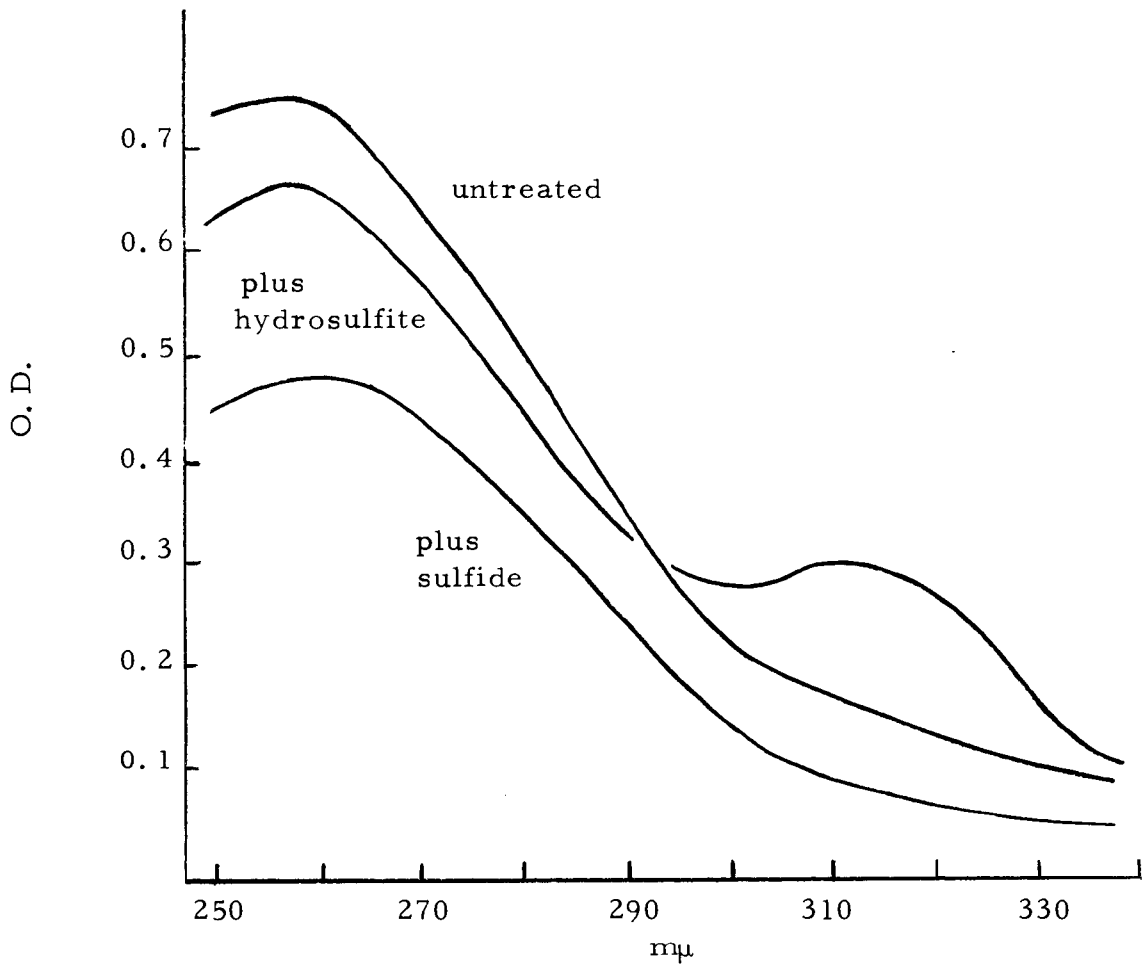


Figure 6. Oxidized and reduced ultraviolet absorption spectra of Beggiatoa extracts. The extract described in Fig 5 diluted 1/10 and 0.1 ml of 0.1% solutions of sodium hydrosulfite and sodium sulfide added to both reference cell and sample cell as noted.

Table 1. Effect of Catalase on Growth of Beggiatoa.

		Components Measured		
	Wet weight	Soluble protein*	Nucleic acid	Malic dehydrogenase
Type catalase added**	Total gm	Total mg	Total mg	Total units
Native	1.20	54	13	14,400
Heat denatured	0.05	14	2.5	156

Trichomes from 1 liter of medium were harvested after 48 hr growth at 28 C and washed once with 0.01% $CaCl_2$ solution. Volume brought to 10 ml prior to treatment in 10-kc Raytheon sonic oscillator.

* Non-sedimented at 10,000 x g for ten min after treatment in 10-kc Raytheon sonic oscillator for ten min.

** Ten Sigma units per ml activity prior to heat denaturation (20 min at 121 C).

Table 2. Effect of Various Substrates on the Growth of Beggiatoa.

Additions to basal medium	Final concentration %	Amount of growth	Gas Production	Residual substrate %
Acetate	0.05	+++++	-	*
Malate	0.05	++++	-	*
Thiosulfate	0.02	++	-	0.02
Thiosulfate and Acetate	0.02 0.05	++	-	0.02 *
Sulfite	0.02	-	-	*
Sulfite and Acetate	0.02 0.05	-	-	*
Glucose	0.01	+++	-	0.01
Citrate	0.02	-	-	*
None		+++	-	*

Basal medium composed of 2.0 gm yeast extract, 0.1 gm $CaCl_2$ and 1,000 ml tap water. Culture incubated 96 hr in Smith fermentation tubes.

* Not determined.

The following notations are used to indicate growth:

- = negative, $\bar{+}$ = questionable, + = very poor, ++ = poor, +++ = fair, ++++ = good, +++++ = best.

metabolized during growth. Citrate and sulfite inhibited growth.

The effect of acetate and malate concentration on growth is presented in Table 3. Sodium malate became toxic at considerable lower concentrations than did sodium acetate.

Cultures flushed with hydrogen sulfide produced sulfur granules extractible with carbon disulfide. Beggiatoa filaments without sulfur are shown in Fig. 7 and filaments with sulfur are shown in Fig. 8.

The components required for acetyl thiokinase activity are listed in Table 4. Sodium salts were inhibitory to the reaction while CoA and ATP were required.

The assay system employing semicarbazide to determine isocitratase exhibited activity with both dialized and undialized extracts. When glyoxylate was measured after the enzyme reaction, activity was demonstrable only with dialized extracts. Undialized extracts removed added glyoxylate. The absorption spectra of the products formed in the 2, 4-dinitrophenylhydrazine test for keto acids after enzymatic action on isocitrate are shown in Fig. 9.

The absorption spectra of pyruvate, α-ketoglutarate and glyoxylate dinitrophenylhydrazones are given in Fig. 10. The pH for maximum isocitratase activity was between 7.8 and 8.2 (Fig. 11).

The oxidation of succinate by whole cells and crude extracts is shown in Figs. 12 and 13. The components required for maximum

Table 3. Effect of Acetate and Malate Concentration on Growth of Beggiatoa.

Final Concentration %	Acetate	Malate
	Growth	
0.00	+++	+++
0.02	++++	++++
0.05	+++++	++++
0.10	+++	+
0.20	++	+
0.50	-	-
1.00	-	-

- = negative
± = questionable
+ = very poor
++ = poor
+++ = fair
++++ = good
+++++ = best

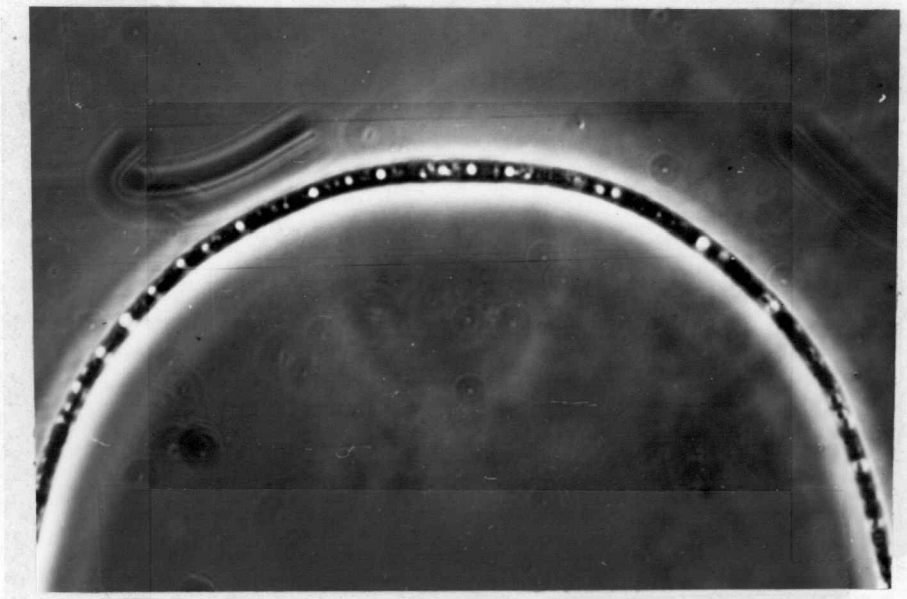


Figure 7. Beggiatoa trichomes prior to exposure to hydrogen sulfide. Culture grown 48 hr in presence of catalase.

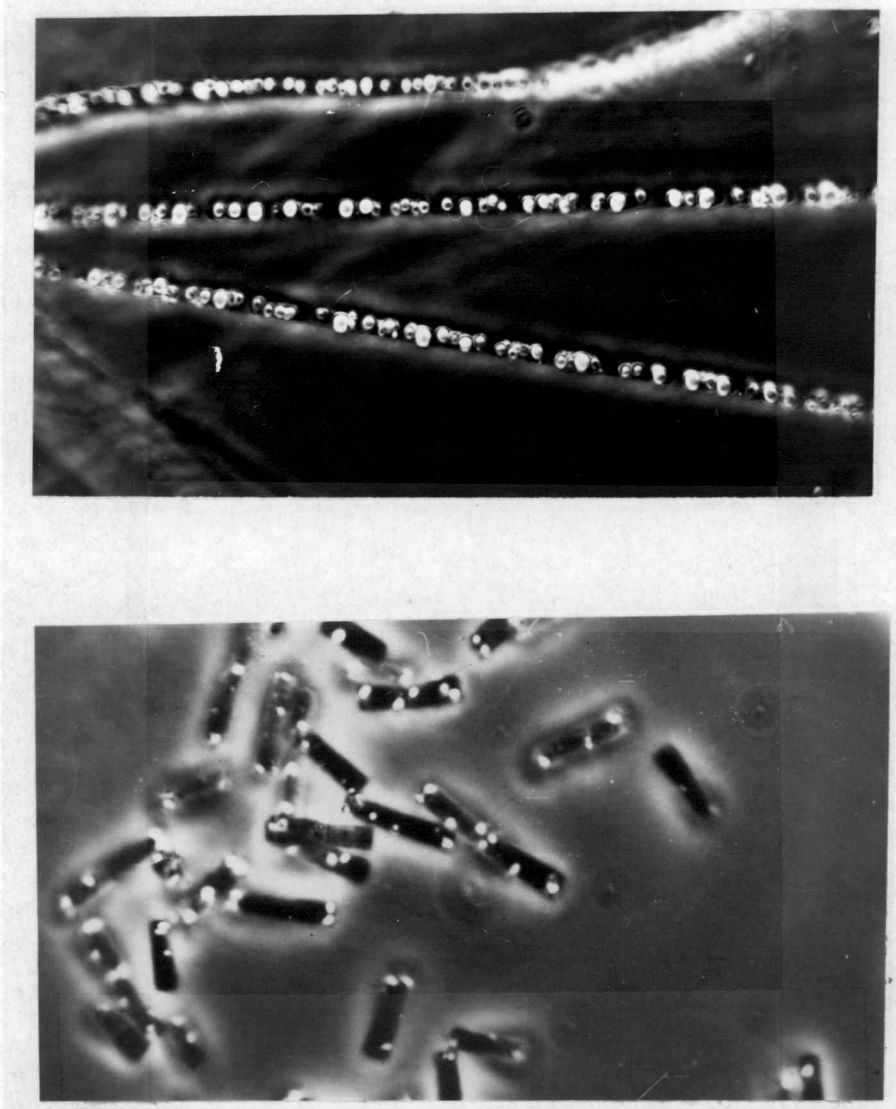


Figure 8. Beggiatoa trichomes after exposure to hydrogen sulfide. A 48 hr culture was exposed to sterile H_2S for one min then incubated at room temperature. Photographs taken 12 hr after exposure to H_2S. The trichomes in the bottom photograph are beginning to break down to individual cells.

Table 4. Acetyl Thiokinase Activity of Beggiatoa Extracts.

Assay System	Units Enzyme Activity
Complete system *	0.70
- Enzyme	0.00
- CoA	0.20
- ATP	0.00
+ EDTA (1 μmole)	0.60
+ NaCl (200 μmoles)	0.00
- KF	0.70

*Complete system contained in one ml: 25 units CoA, ten μmoles ATP, 20 μmoles potassium acetate, 100 μmoles tris-HCl buffer (pH 8.2), 200 μmoles hydroxylamine, 50 μmoles KF, ten μmoles $MgCl_2$, ten μmoles glutathione and 0.2 mg enzyme.

1 unit enzyme = 0.4 μmoles acetylhydroxamic acid produced per 20 min.

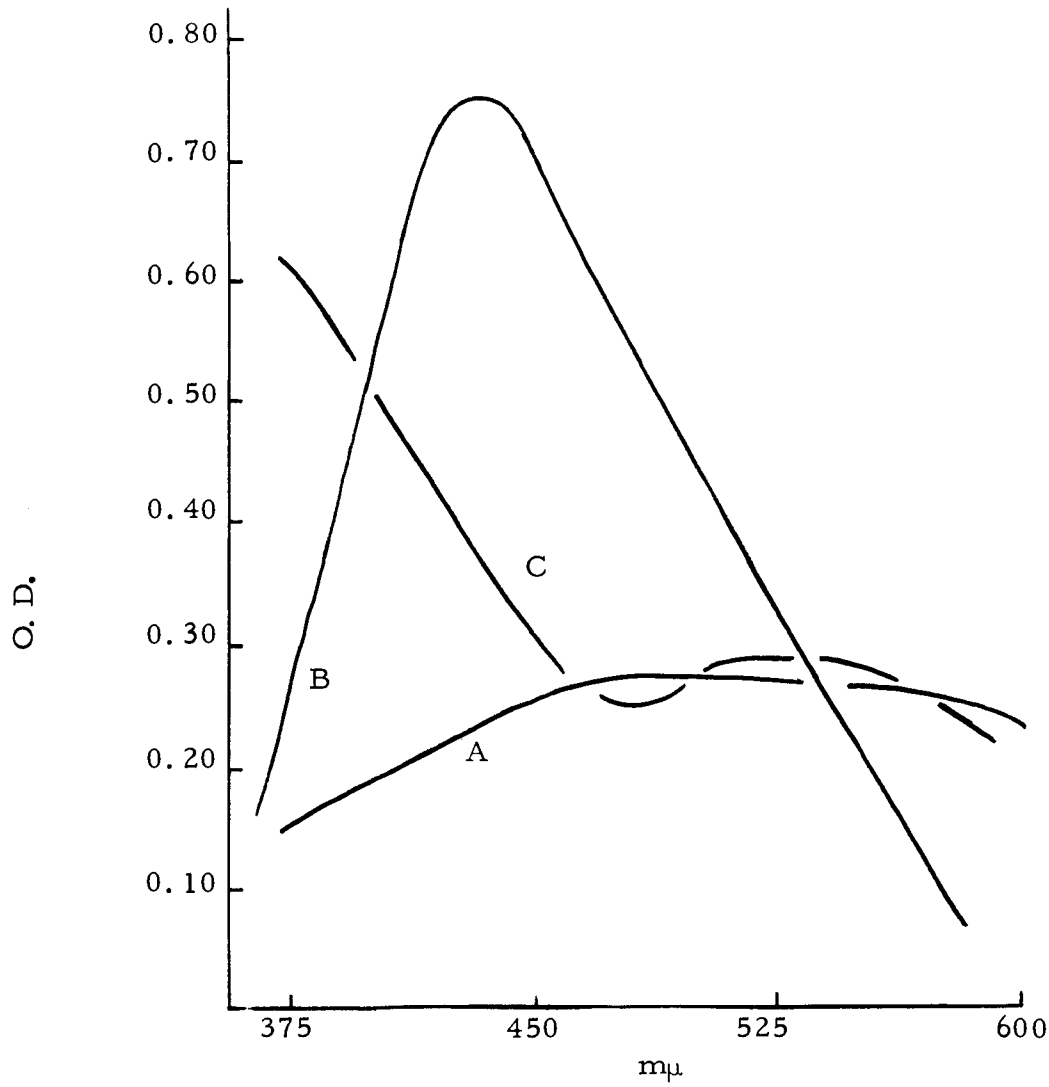


Figure 9. Absorption spectra of the end products obtained from the action of cell-free extracts of Beggiatoa on isocitrate during various incubation procedures. All reaction mixtures contained in 1.5 ml: 20 μmoles dl-isocitrate, 100 μmoles tris-HCl (pH 8.0), 3 μmoles $MgCl_2$ and 2 μmoles cysteine. The following additions were made: A, 0.2 mg undialized extract; B, 0.2 mg dialized extract; and C, 0.2 mg dialized extract plus 0.2 μmoles NADP. One ml samples were assayed for keto acids as described in Fig 10.

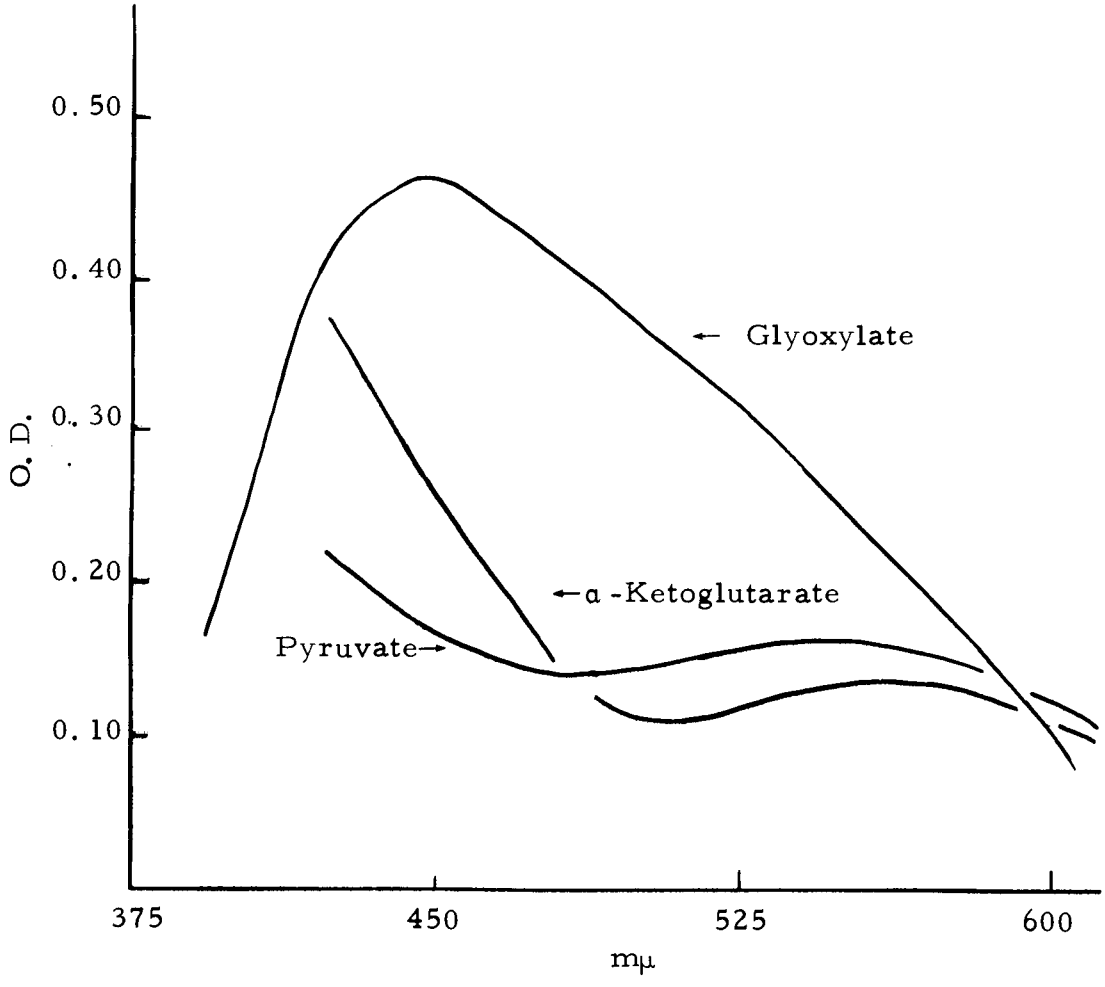


Figure 10. Absorption spectra of glyoxylate, pyruvate and α-ketoglutarate phenylydrazones. Commercial samples (0.1 μmole in 1 ml water) of each keto acid treated with 1 ml 2, 4-dinitrophenylhydrazine (0.1% in 2N HCl) for five min after which 2.0 ml 95% ethyl alcohol, 1.0 ml H_2O and 5.0 ml 1.5 N NaOH were added. Spectra determined three min after addition of NaOH.

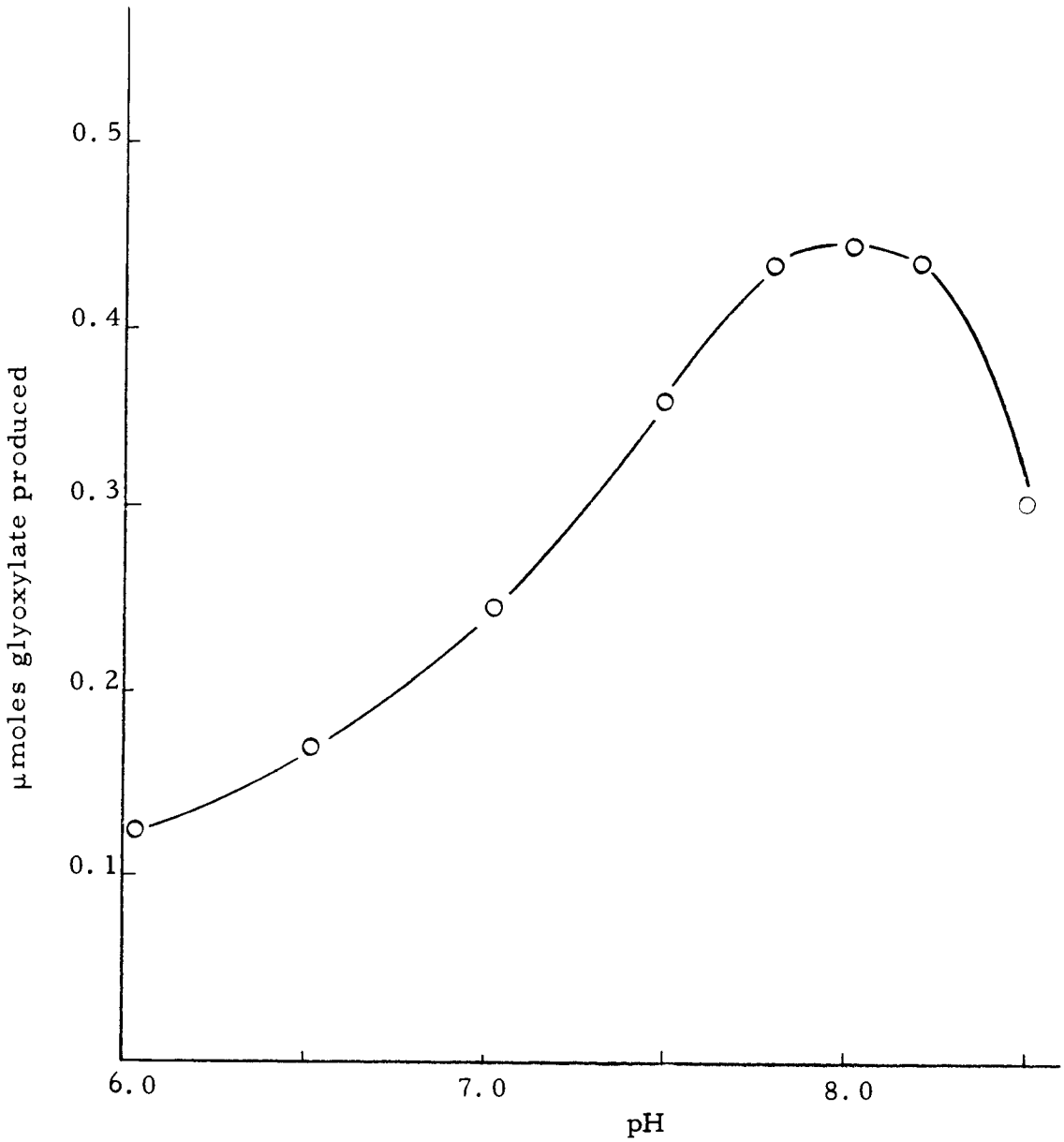


Figure 11. Effect of pH on isocitratase activity of cell-free extracts of Beggiatoa. Reaction mixture contained in 1.5 ml: 100 µmoles tris-HCl (at stated pH), 3 µmoles $MgCl_2$, 2 µmoles cysteine, 5 µmoles dl-isocitrate and 0.1 mg protein. Glyoxylate determined after 10 min incubation at 30 C.

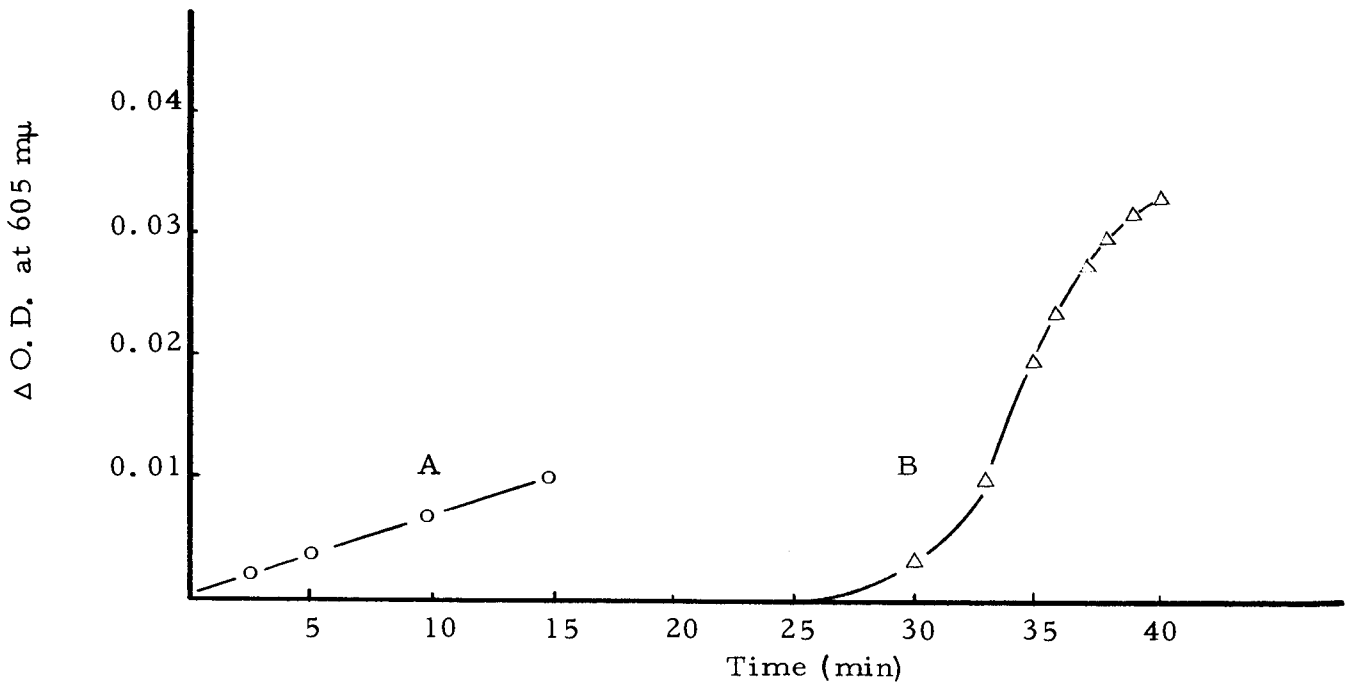


Figure 12. Oxidation of succinate by whole cells and crude extracts of Beggiatoa. Reaction mixture contained in 3. 0 ml: 150 μmoles succinate, 200 μmoles tris-HCl buffer (pH 7. 4) and 0. 2 μmoles DCIP. The following additions were made: A, 0. 5 ml whole cells (O. D. 0. 5 at 625 mμ); B, crude extract (0. 5 mg protein). Temperature maintained at 30 C.

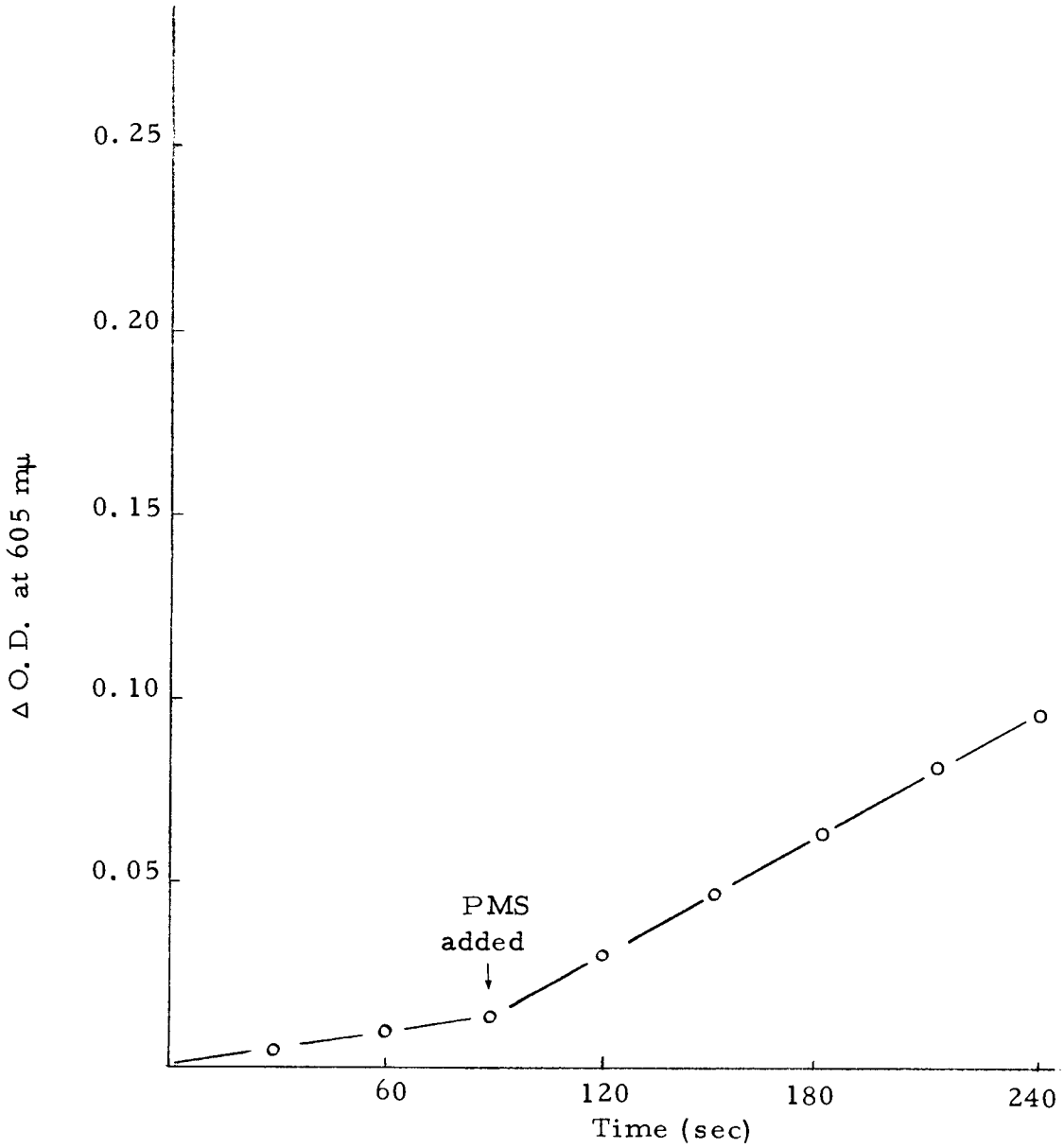


Figure 13. Effect of PMS on succinate oxidation by cell-free extracts of Beggiatoa. Assay mixture contained in 3.00 ml: 200 μmoles tris-HCl buffer (pH 7.4), 150 μmoles succinate, 0.2 μmole DCIP, 10 μmoles sodium cyanide, 0.2 mg protein and 0.1 ml of 0.1% PMS (added at 90 sec). Values corrected for non-specific reduction of DCIP in absence of succinate.

activity and the inhibitory action of malonate are given in Table 5. The optimum pH for maximum activity was 8.7 (Fig. 14). Preliminary attempts to assay succinate oxidation using methylene blue or ferricyanide as hydrogen acceptors were unsuccessful. Both dialized and undialized extracts reduced the DCIP-PMS mixture with isocitrate as substrate. Glyoxylate, pyruvate and α-ketoglutarate did not reduce DCIP or the DCIP-PMS mixture. The addition of lipoic acid, NAD, NADP, TPP or CoA failed to activate any of the above substrates. Fumarase, citratase, aconitase, catalase, pyruvate kinase, enolase, phosphoenolpyruvate carboxylase, and NAD-linked lactic dehydrogenase were also absent from crude extracts.

The activity of the NADP-linked isocitrate dehydrogenase is illustrated in Fig. 15. NAD could not replace NADP.

The activity of malic dehydrogenase was greater than the activity of the other enzymes found in Beggiatoa extracts. The enzymatic reduction of oxaloacetate by NADH is shown in Fig. 16.

The glutamic-oxaloacetate transaminase reaction coupled with malic dehydrogenase is shown in Fig. 17. The reaction was begun by adding α-ketoglutarate.

The condensation of acetyl-CoA and glyoxylate by malate synthetase is demonstrated in Table 6. Phosphotransacetylase was used to produce acetyl-CoA from acetylphosphate and the reaction rate determined by following acetylphosphate disappearance.

Table 5. Oxidation of Succinate by Beggiatoa Extracts.

Assay System	Units Enzyme Activity
Complete system*	23
- CN	0
- Succinate	2
- PMS	2
- Enzyme	0
+ Malonate (150 μmoles)	4
+ Ferricyanide (1 μmole)	0
+ EDTA (2 μmoles)	23

*Complete system contained in μmoles: tris-HCl buffer (pH 8.7), 200; succinate, 150; DCIP, 0.2; sodium cyanide, ten. Water and enzyme (0.2 mg) was added to bring volume to 3.0 ml. Reaction begun by adding 0.1 ml of 0.1% PMS. Enzyme rate followed by observing the reduction in absorption at 605 mμ.

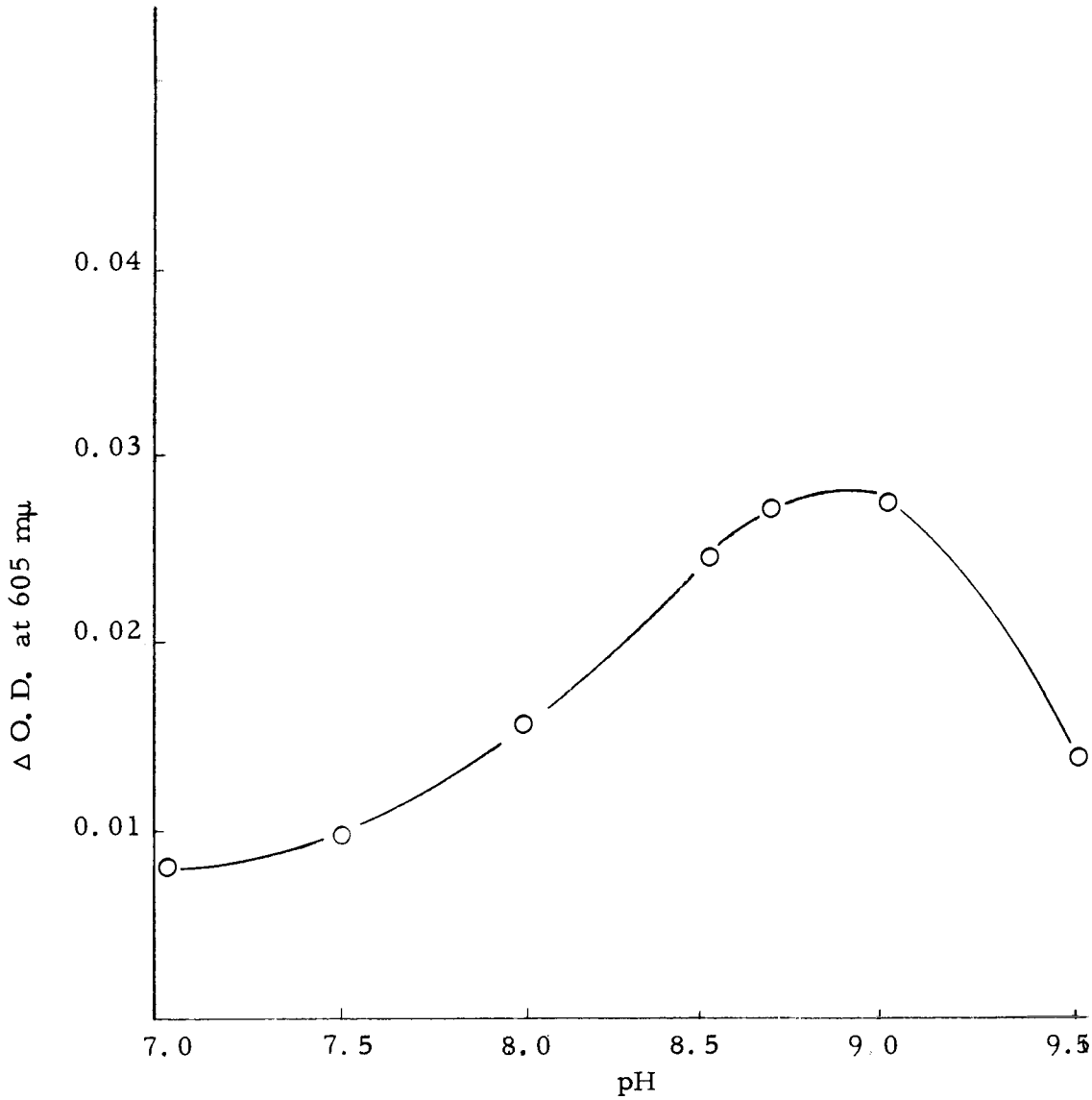


Figure 14. Effect of pH on succinate oxidation by Beggiatoa extracts. Assay mixture contained in 3.0 ml: 400 μmoles tris-HCl buffer (at pH stated), 150 μmoles succinate, 10 μmoles cyanide, 0.2 mg protein and 0.1 ml of 0.1% PMS.

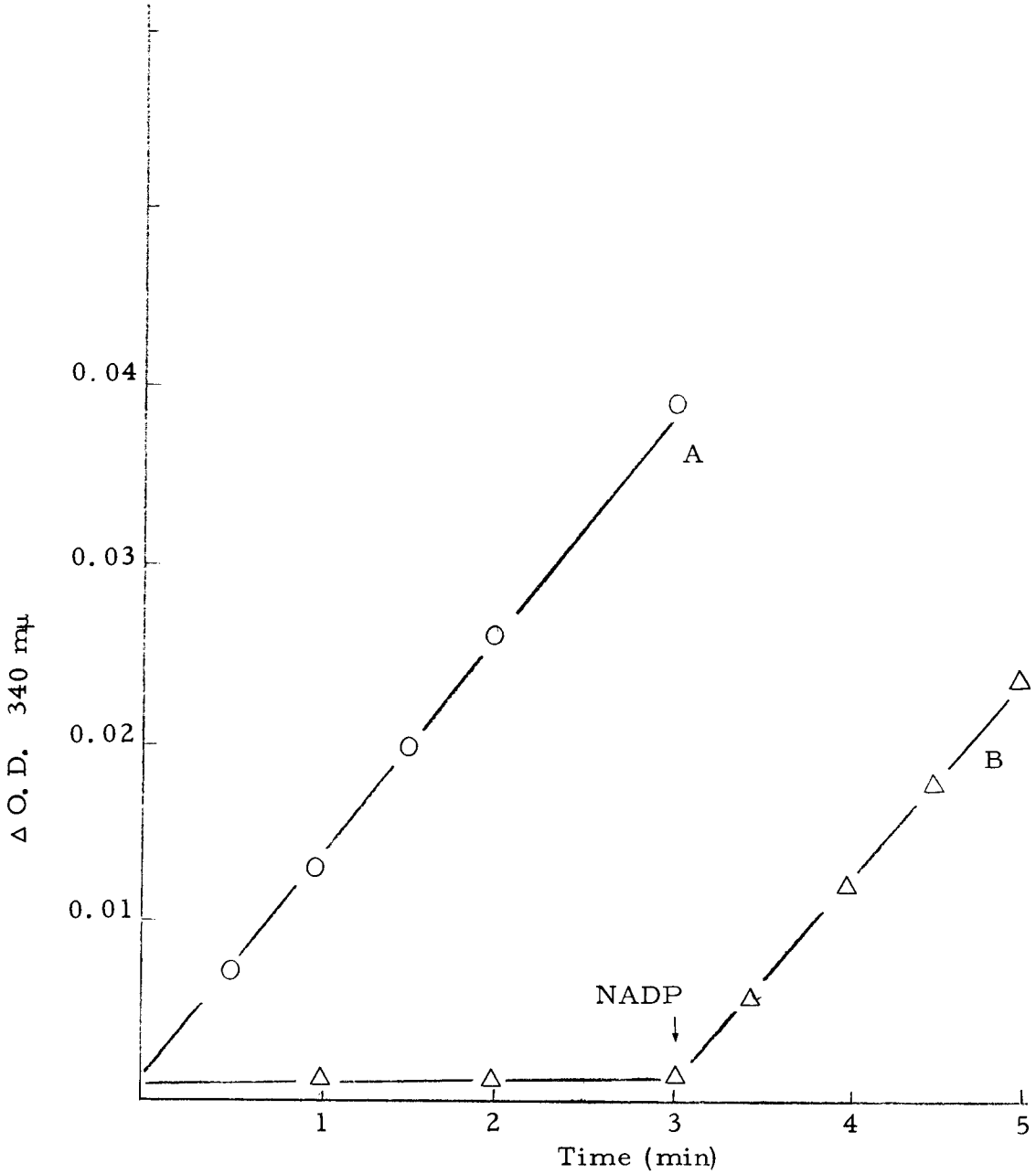


Figure 15. Isocitrate dehydrogenase activity of Beggiatoa extracts. Assay mixture contained in 3.0 ml: 200 μmoles tris-HCl buffer (pH 7.4), 0.3 μmoles ethylenediaminetetra-acetate, 2.0 μmoles $MnSO_4$, 0.2 mg protein and 80 μmoles dl-isocitrate. Curve A had 0.2 μmole NADP added at zero time. Curve B had 0.2 μmole NAD added at zero time and 0.2 μmole NADP added at 3 min.

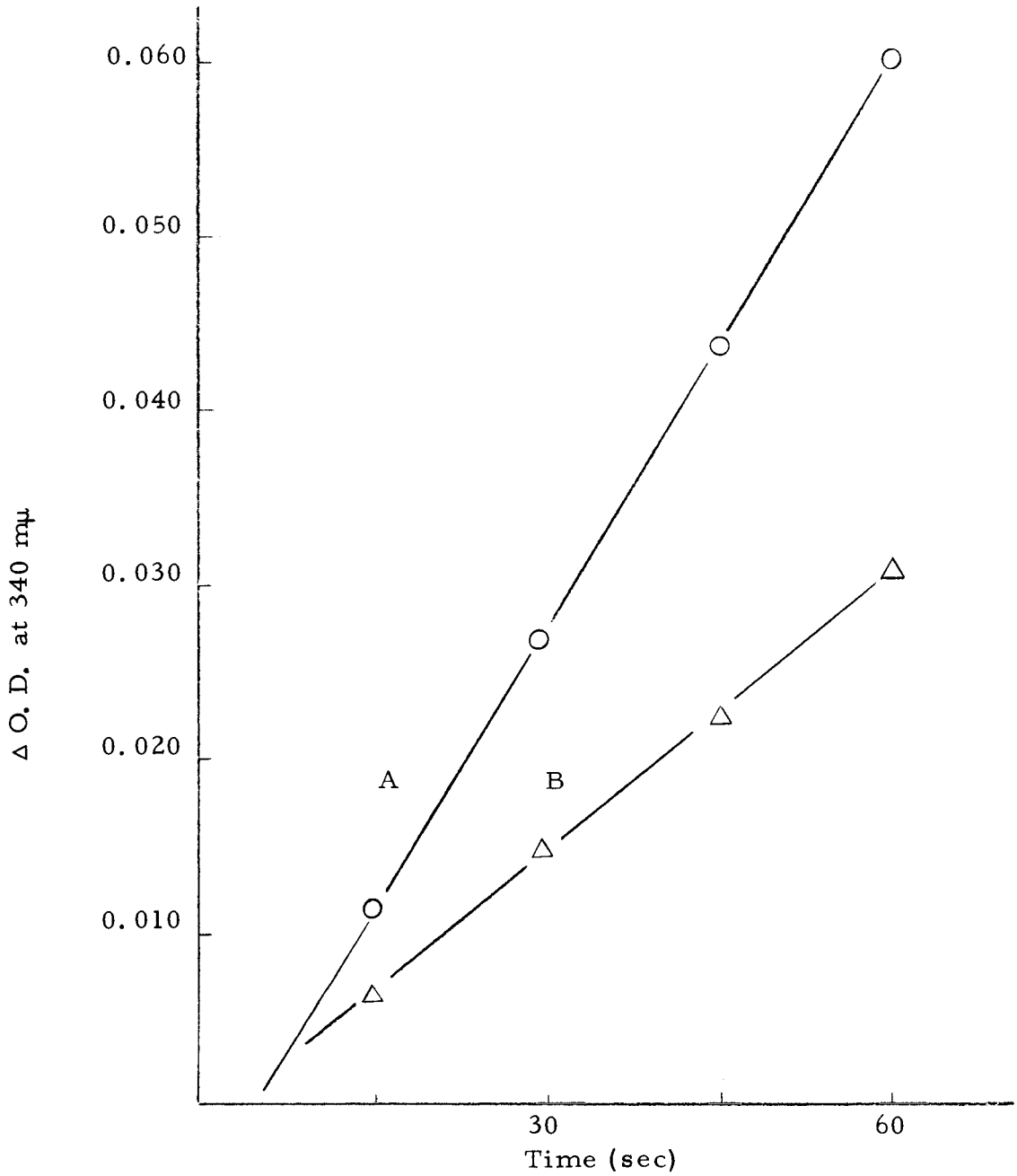


Figure 16. Malic dehydrogenase activity of cell-free extracts of Beggiatoa. Reaction mixture contained in 3.0 ml: 0.2 μmole NADH, 200 μmoles tris-HCl buffer (pH 7.4) and 0.2 μmole oxaloacetate. In addition curve A contained 0.27 mg protein and curve B, 0.135 mg protein. Temperature maintained at 25 C.

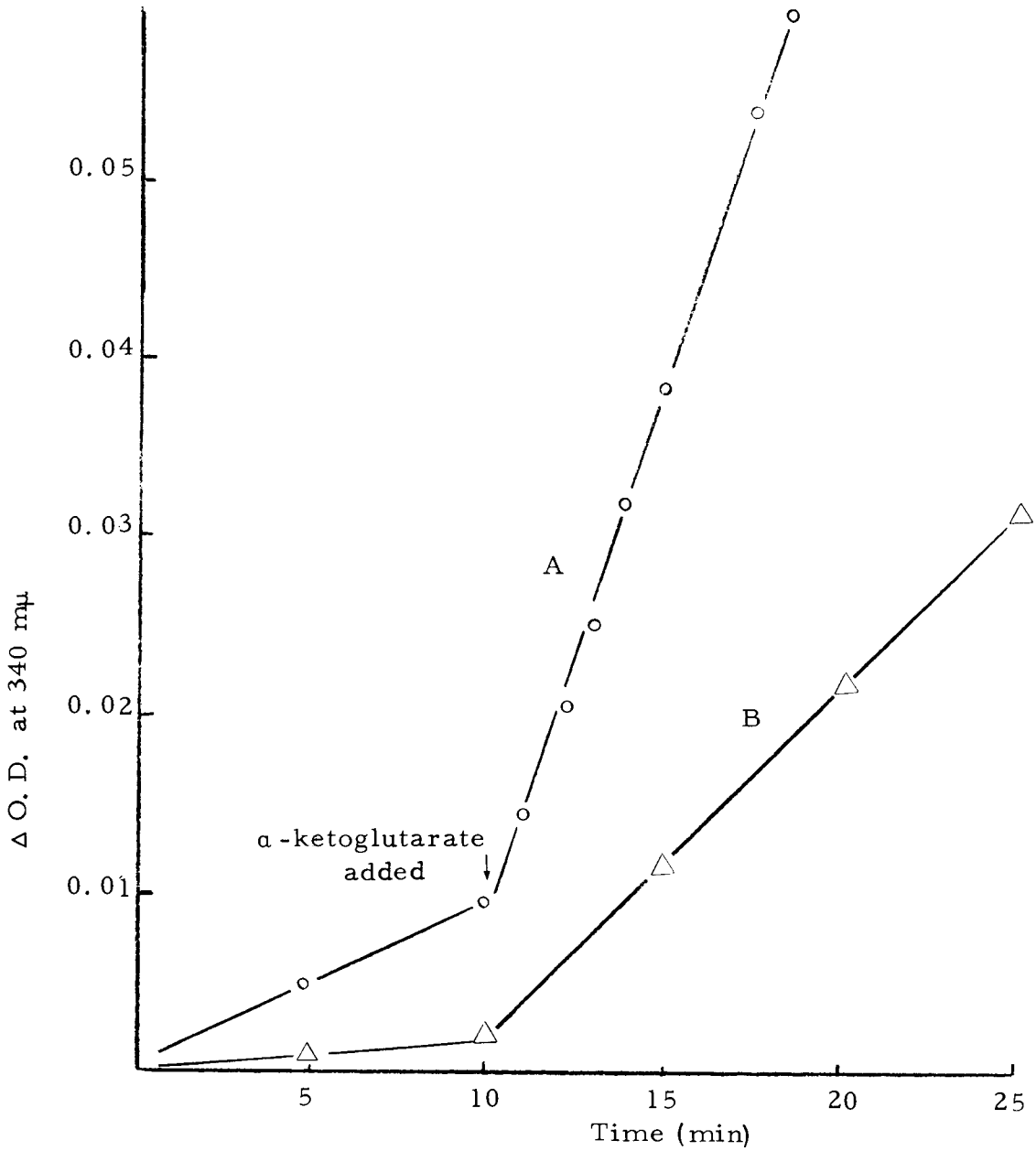


Figure 17. Glutamic-oxaloacetate transaminase activity of cell-free extracts of Beggiatoa. Reaction mixture contained in 3.0: 200 μmoles tris-HCl buffer (pH 7.4), 0.2 μmoles NADH and 20 μmoles α-ketoglutarate (added at 10 min). Curve A contained 0.54 mg protein and curve B 0.27 mg protein. Temperature maintained at 25 C.

Table 6. Malate Synthetase Activity of Beggiatoa Extracts.

Assay System	Units Enzyme Activity
Complete System*	0.40
2X Beggiatoa extract	0.75
2X Beggiatoa extract oxaloacetate replacing glyoxylate	0.10
Minus Beggiatoa extract	0.00
" Phosphotransacetylase	0.00
" Acetylphosphate	0.00
" Glyoxylate	0.00

*Complete system contained in 3.0 ml: ten μmoles acetylphosphate, two μmoles cysteine, ten μmoles $MgCl_2$, 15 μg CoA, 25 μmoles sodium glyoxylate, 50 units phosphotransacetylase, 50 μmoles KCl, 30 μmoles tris-HCl buffer (pH 8.0), and 0.2 mg enzyme.

The activity of lipoic dehydrogenase was reduced by one half by centrifugation (Fig. 18). A DCIP-linked NADH diaphorase was also present and was stimulated by PMS.

The oxidation of sulfite as measured by the decrease at 320 mμ of the NAD-sulfite complex is shown in Fig. 19. PMS stimulated this reaction but also reacted non-enzymatically with sulfite. The absorption spectra of PMS before and after treatment with sulfite are given in Fig. 20. The ability of crude extracts to decrease the absorption at 320 mμ (after the non-enzymatic reaction had nearly ceased) is illustrated in Fig. 21. The pH optimum for this reaction was 7.4 (Fig. 22).

The non-enzymatic action of ascorbic acid on PMS was different but was also modified by crude extracts. Ascorbic acid caused continuous non-enzymatic reduction of the absorption of PMS at 320 mμ. The presence of crude extracts decreased the rate at which the absorption was lowered. (Fig. 23).

The oxidation of hydrogen sulfide as measured by the ability of this compound to non-enzymatically reduce DCIP is presented in Table 7. This oxidation varied markedly from preparation to preparation. When the trichomes were not thoroughly washed prior to extraction of the enzyme, crude extracts contained catalase activity; and oxidation of hydrogen sulfide was erratic. Dialized extracts lost their ability to oxidize sulfide whereas incubation of crude

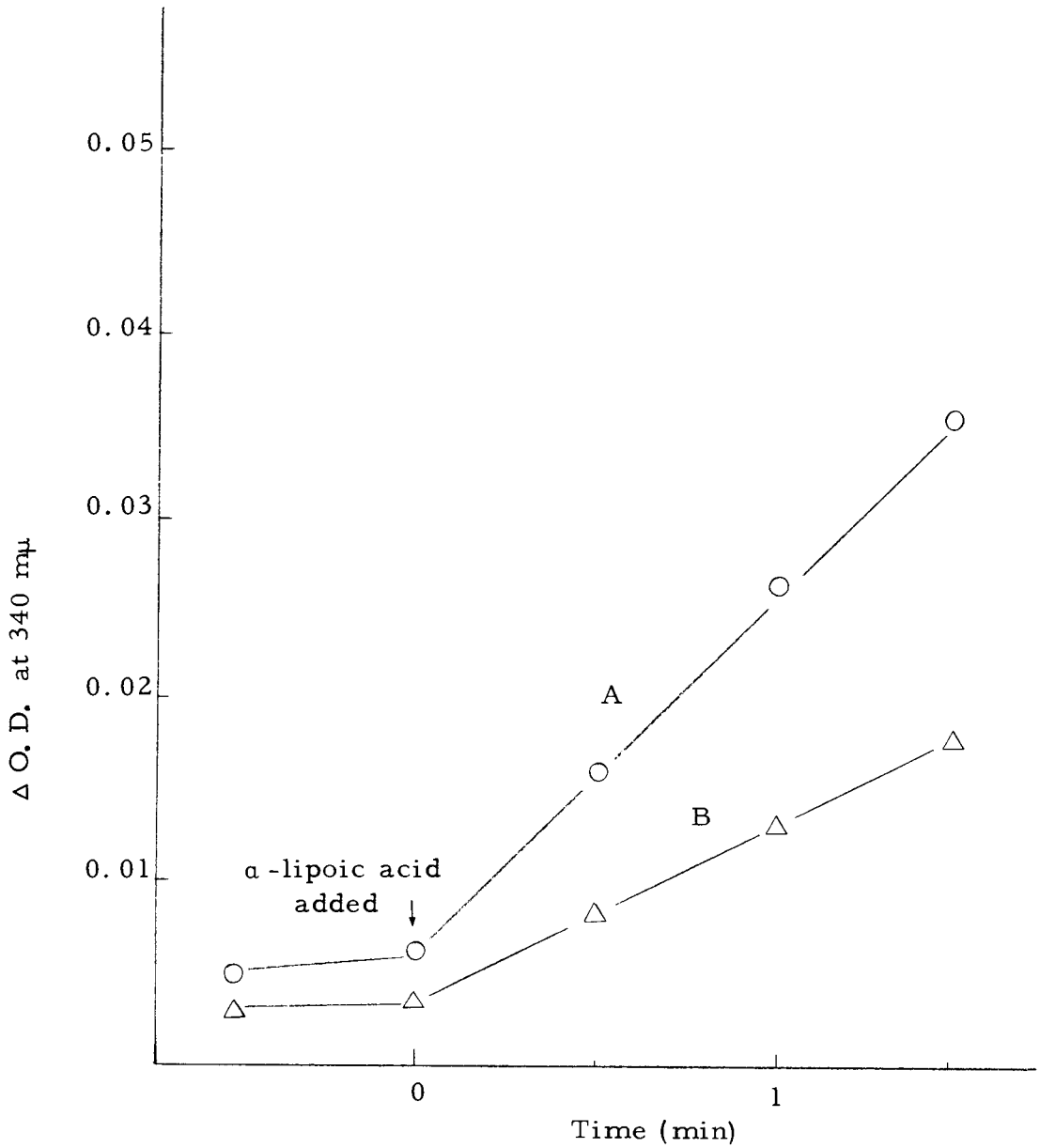


Figure 18. Lipoic dehydrogenase activity of cell-free extracts of Beggiatoa. Reaction mixtures contained in 3.0 ml: 300 μmoles tris-HCl buffer, (pH 7.4), 0.2 μmoles NADH, 2.5 μmoles α-lipoic acid and enzyme (0.54 mg soluble protein). A crude extract (before centrifugation at 10,000 x g) was used for curve A, and the supernant fluid (after centrifugation at 10,000 x g) used for curve B.

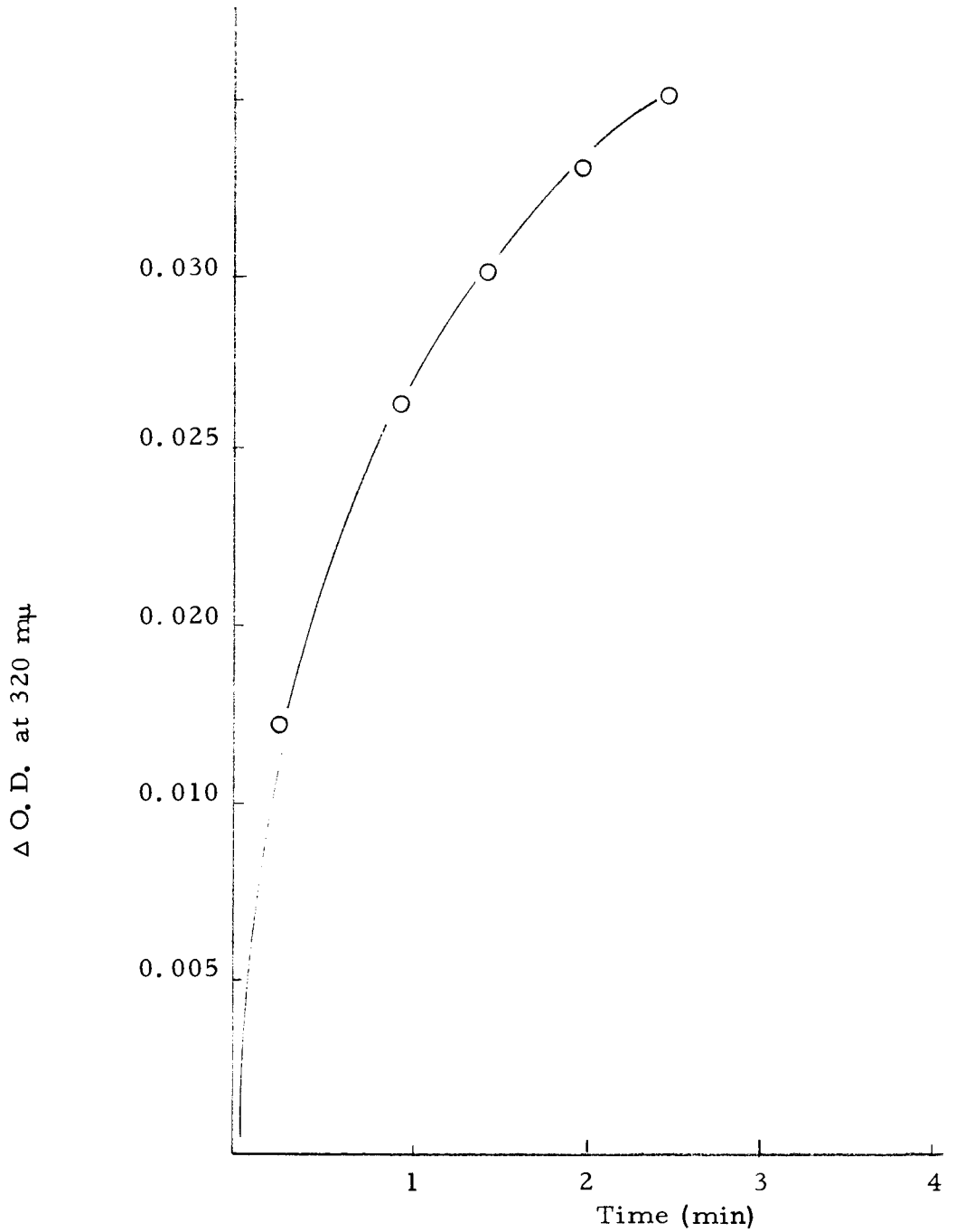


Figure 19. Oxidation of NAD-sulfite complex by cell-free extracts of Beggiatoa. Reaction mixture contained in 3 ml: 1.0 μmole sulfite, 6.0 μmole NAD, 200 μmoles tris-HCl buffer (pH 7.4) and enzyme (0.1 mg protein).

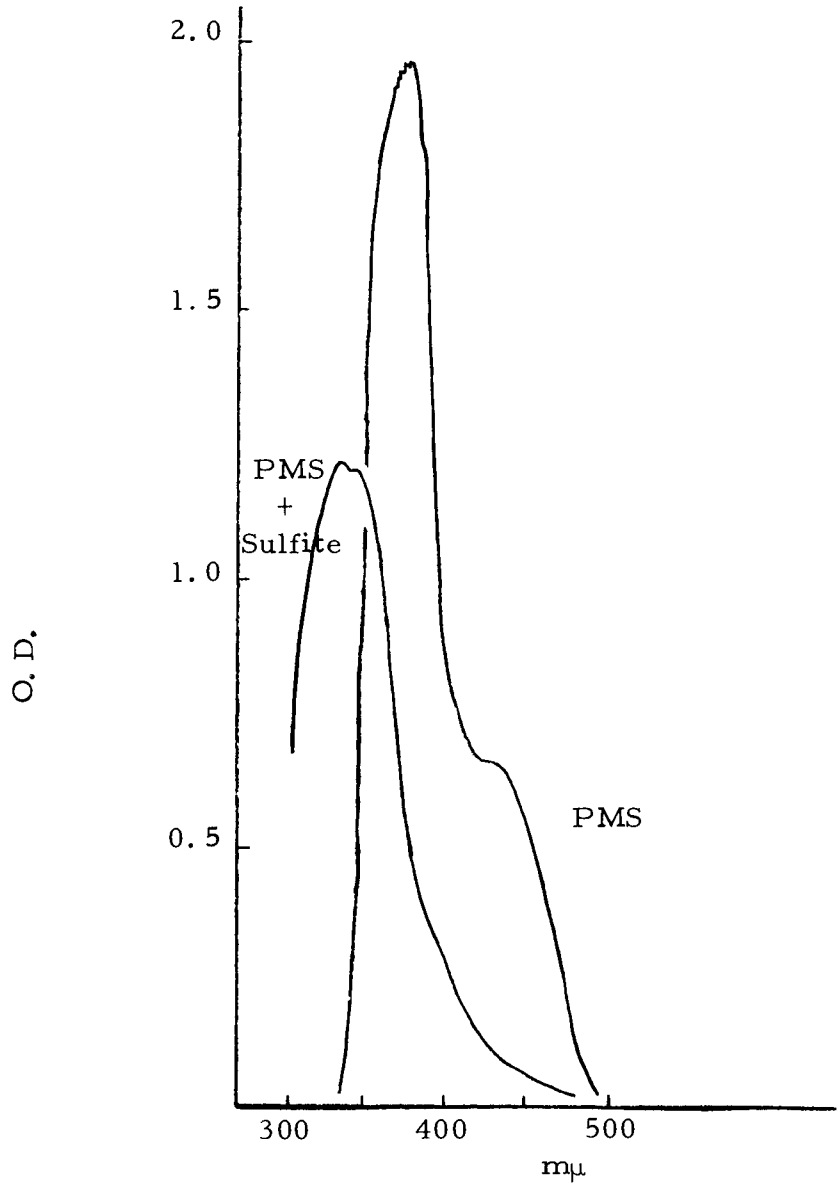


Figure 20. Absorption spectra of PMS and PMS-sulfite complex. PMS (0.01%) prepared in tris-HCl buffer (0.2M, pH 7.2). The spectrum of the PMS-sulfite complex determined 1 min after addition of 0.5 μmole sulfite.

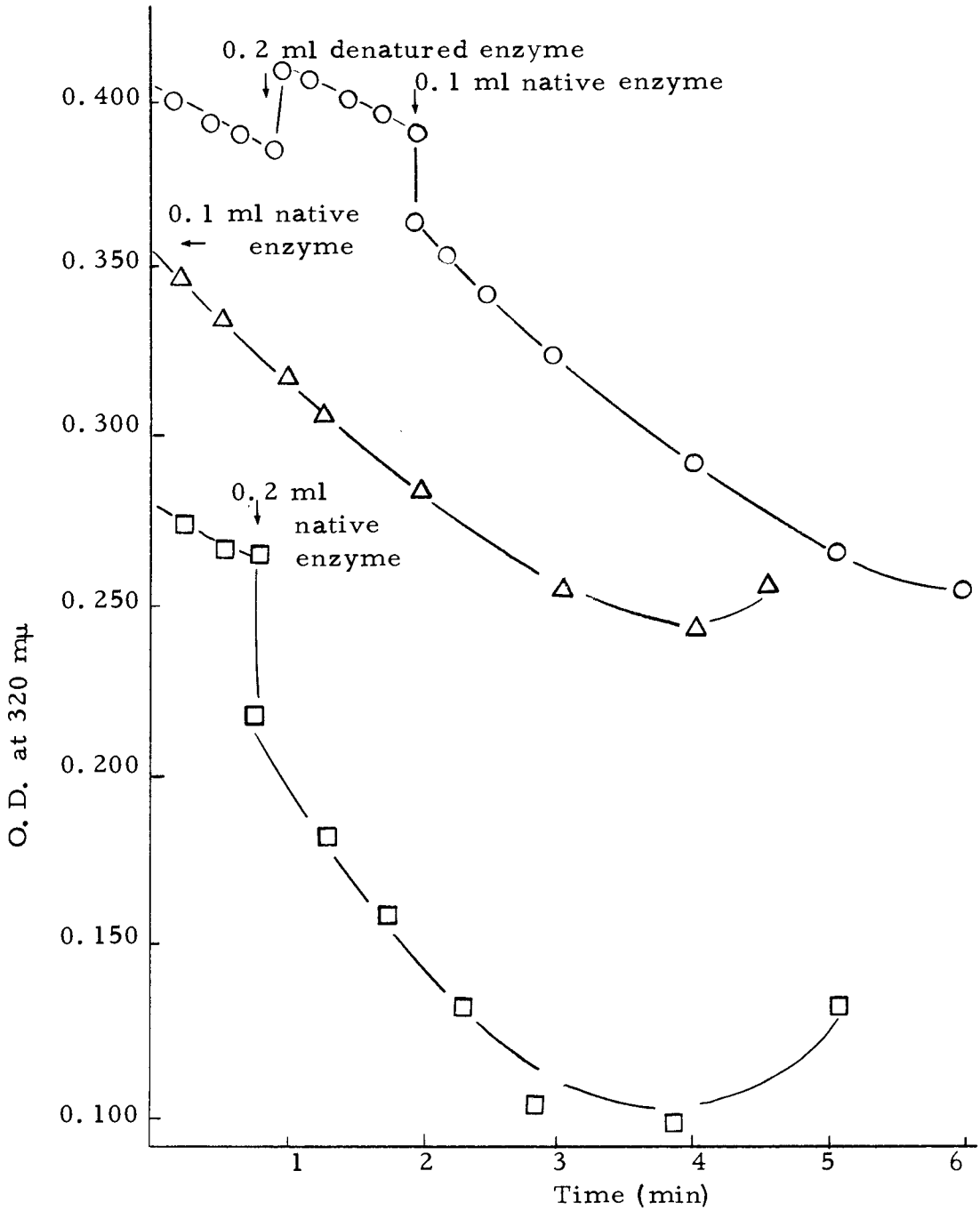


Figure 21. Oxidation of sulfite-PMS complex by Beggiatoa extracts. Reaction mixture contained in 3. 0 ml: 0. 5 μmole sulfite, 0. 1 mg PMS, 200 μmoles tris-HCl buffer (pH 7. 4) and enzyme (0. 1 mg protein per 0. 1 ml). Timing begun one min after addition of PMS and sulfite.

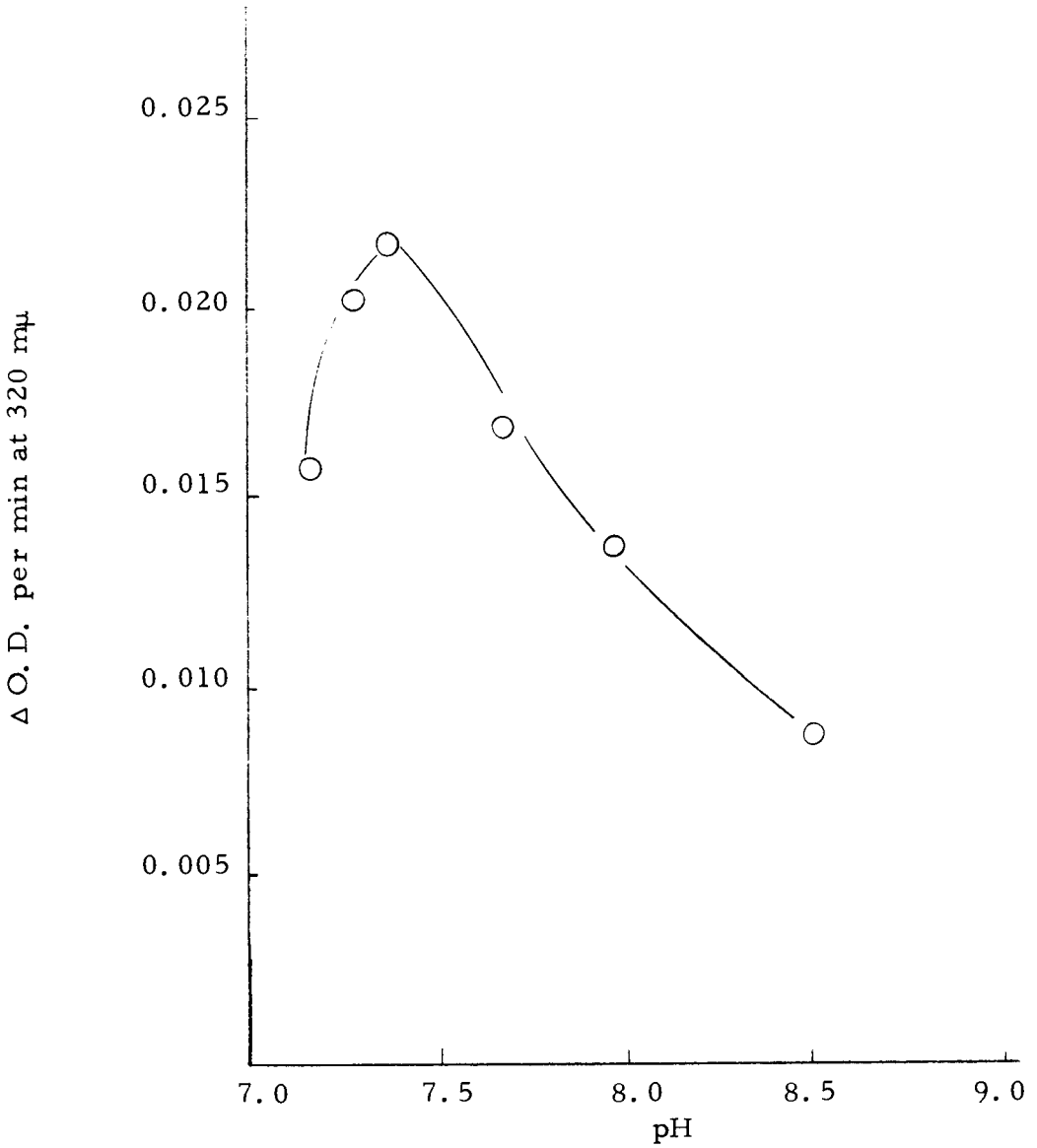


Figure 22. Effect of pH on oxidation of sulfite-PMS complex. Reaction mixture contained in 3.0 ml: 0.5 μmole sulfite, 0.1 mg PMS, 200 μmoles tris-HCl buffer (pH 7.4) and 0.12 mg protein.

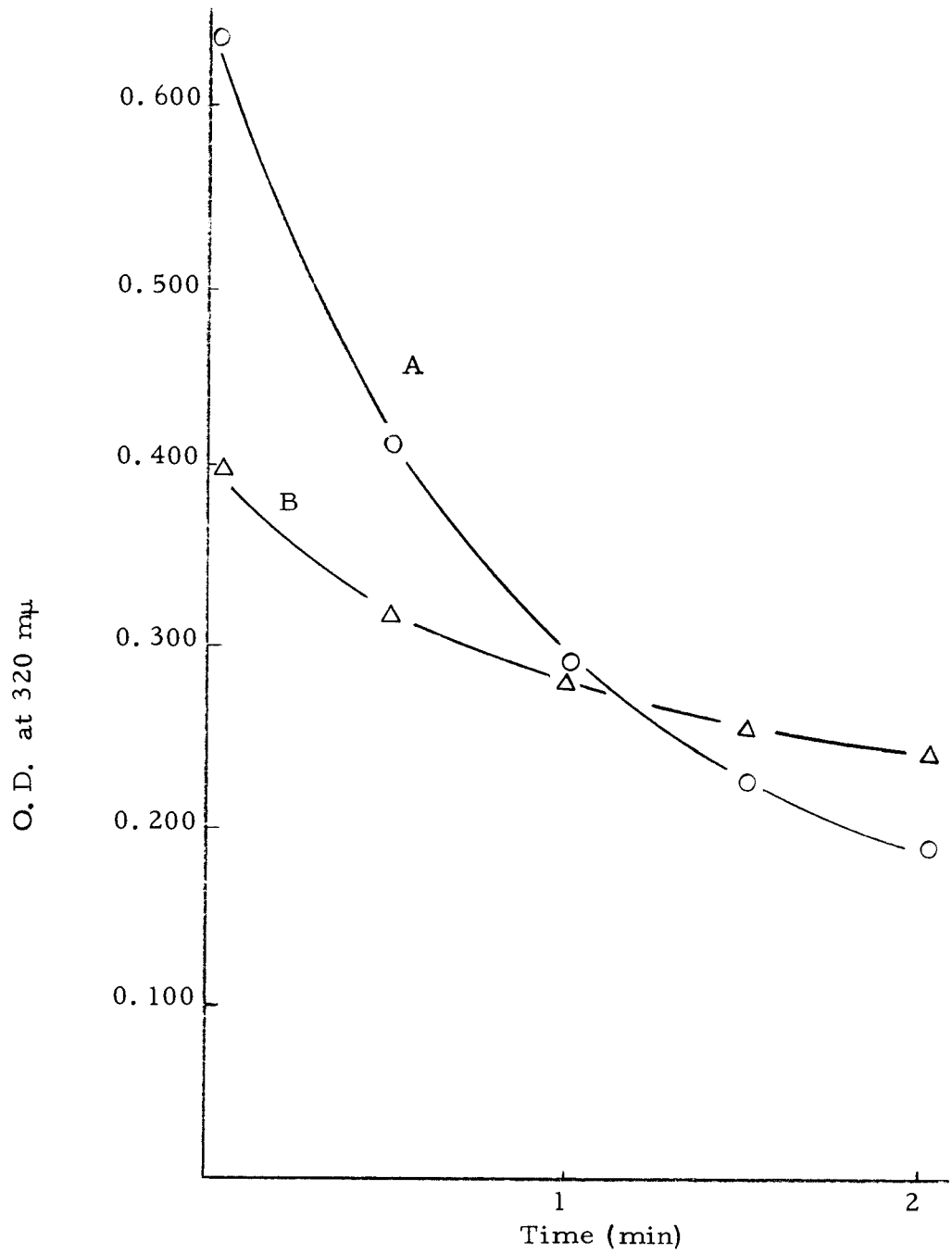


Figure 23. Effect of cell-free extracts of Beggiatoa on reduction of PMS by ascorbic acid. The reaction mixture contained in 3.0 ml: 0.5 μmole ascorbic acid, 0.1 mg PMS and 200 μmoles tris-HCl buffer (pH 7.4). Curve B had 0.2 mg protein added.

Table 7. Oxidation of Hydrogen Sulfide by Beggiatoa Extracts.

Protein	Incubation time	Residual H_2S	Abiological and biological H_2S oxidation	Biological H_2S oxidation
(mg)	(min)	(μmoles)	(μmoles)	(μmoles)
0.1 (denatured)*	0	0.200	0.000	0.000
0.1 (denatured)*	10	0.168	0.032	0.000
0.1	10	0.050	0.150	0.118
0.2	10	0.015	0.185	0.153
0.3	10	0.000	0.200	0.168

The reaction mixture contained in three ml: 0.2 μmoles sulfide, 600 μmoles tris-HCl buffer (pH 7.2), and enzyme. H_2S concentration determined by the non-enzymatic reduction of DCIP.

*Heated to 100 C for ten min.

extracts for one hr prior to addition of sulfide increased intitial sulfide oxidation.

DISCUSSION

Growth of Beggiatoa

Faust and Wolfe (24) noted that their pure cultures of Beggiatoa required oxygen for growth but would not grow at the surface of semi-solid medium. None of their cultures required hydrogen sulfide for growth, but all were stimulated by low levels of sulfide. These characteristics correspond to those of the Beggiatoa strain used in this study. Photomicrographs of the strains used by Faust and Wolfe (24) and Morita and Stave (40) appear similar to Figs. 7 and 8.

Winogradsky (66, 67, p. 1-149) classified Beggiatoa according to the diameter of the trichomes. Based on this classification Maier (37) considered the Beggiatoa used in this study to be B. leptomitiformis. Faust and Wolfe (24) preferred not to use this type of classification and named their strains B. alba rather than B. leptomitiformis, a name which they considered to have no real significance.

The Beggiatoa used in this study was observed to vary its diameter according to growth conditions. When grown on agar supplemented with catalase, some trichomes were double the diameter of other trichomes. Therefore, because of the lack of any good classification of Beggiatoa, no species name was given to the strain employed.

Prior to exposure to hydrogen sulfide, Beggiatoa trichomes

contain lipid granules that readily stain with sudan black. After exposure to hydrogen sulfide these granules lose their affinity for sudan black, and new smaller granules appear within 15-20 min. Winogradsky (66) observed granules appearing within three-four min. The rapidity with which sulfur granules appear after exposure to hydrogen sulfide suggests that useful energy is not derived by the cell from this process.

Faust and Wolfe (24) pointed out that the hay enrichment procedure of Cataldi (11) selects only those strains of Beggiatoa which can grow without hydrogen sulfide. Since this procedure has been used to obtain most of the pure strains isolated since 1940, it is not surprising that none of these strains were autotrophic.

Kiel (33) employed tufts of Beggiatoa taken from natural environments and used inorganic medium of known composition throughout the isolation procedure. The validity of his results must be evaluated on strains isolated in a similar manner and not compared to strains obtained by isolation procedures that are quite selective for heterotrophs. Until such investigations are performed, any opinion as to the autotrophic nature of Beggiatoa must remain speculative.

Winogradsky (67) noted that distilled water rapidly lysed Beggiatoa and that trichomes lysed on solid surfaces. Faust and Wolfe (24) later found that the incorporation of calcium salts greatly reduced lysis. In the present study it was observed that cells grown

in the presence of catalase were much more resistant to lysis during centrifugation and washing than were cells grown without catalase.

If Beggiatoa is related to Oscillatoria as Bücher (6), Cohn (14), Guilliermond (26), Hübner (29), Maier (37) and Pringsheim (46) contend, an interesting parallel can be drawn. Spirulina albida which differs from Beggiatoa mainly in its spirally shaped morphology was found by Dyar (21) associated with Beggiatoa and Oscillatoria in a sulfur spring. He found that on blood agar S. albida grew profusely and glided away from contaminating organisms, and he was thus able to obtain pure cultures. He determined that it was the erythrocytes and not the plasma that stimulated growth. The presence of aerobic bacteria also stimulated growth when mixed with S. albida. The stimulation of growth by erythrocytes and aerobic bacteria may have been due to the large amounts of catalase present in these cells.

The use of catalase in culture medium has extended the length of time that can elapse between transfers from one week to two months. Catalase, when added to medium, increased cell yields to the same order of magnitude as other hetertrophs.

It is interesting to note that Winogradsky (67) found the survival time of Beggiatoa in the absence of hydrogen sulfide to be about eight days. This is the same survival time for cultures grown in liquid medium without catalase.

The absorption spectra of Beggiatoa extracts grown without

catalase, and the action of catalase on these extracts, strongly indicates that a peroxide is present and accumulates with the age of the culture. The peroxide could be responsible for the oxidation of hydrogen sulfide by Beggiatoa. Sulfur granules would be produced near areas of oxidation and peroxide production. The stimulatory effect of hydrogen sulfide, yet the failure of this compound to be used as an energy source, could thus be explained. Cysteine and semi-solid medium may stimulate growth by providing conditions so that the rate of peroxide formation is minimal and is abiologically removed.

The absorption spectra also indicates that the cytochrome system is lacking or in very low concentration. The oxidative mechanism may depend on flavin-linked systems such as is the case with the cytochrome negative lactobacilli (19). The production of hydrogen peroxides by these microaerophilic cultures and the stimulation of growth by the addition of catalase(5, 18, 25, 38) has been well documented. Holman (28) reported a similar effect of catalase on clostridia, but Mateles and Zuber (38) demonstrated that this observation was due to impurities in the liver extract Holman (28) used as the source of catalase.

With the exception of the autotrophic strains used by Winogradsky (65, 66, 67) and Kiel (33), the characteristics of the Beggiatoa strain employed in this investigation correspond very closely to

other strains reported in the literature (2, 4, 6, 7, 10, 11, 14, 17, 21, 22, 23, 24, 26, 27, 29, 30, 35, 37, 39, 40, 44, 46, 52, 53, 56, 59, 61, 62, 63, 68) and the stimulation by catalase may be a general phenomenon for heterotrophic strains.

Metabolism of Beggiatoa

The inability of Beggiatoa to metabolize glucose is consistent with the absence of key glycolytic enzymes in cell-free extracts. Likewise, the absence of aconitase, condensing enzyme, α-ketoglutaratic dehydrogenase and fumarase, as well as the lack of CO_2 production by growing cultures, rules out the operation of the citric acid cycle. It is possible, however, to devise a scheme employing the enzymes found that is consistent with the data observed. The proposed scheme is summarized in Fig. 24.

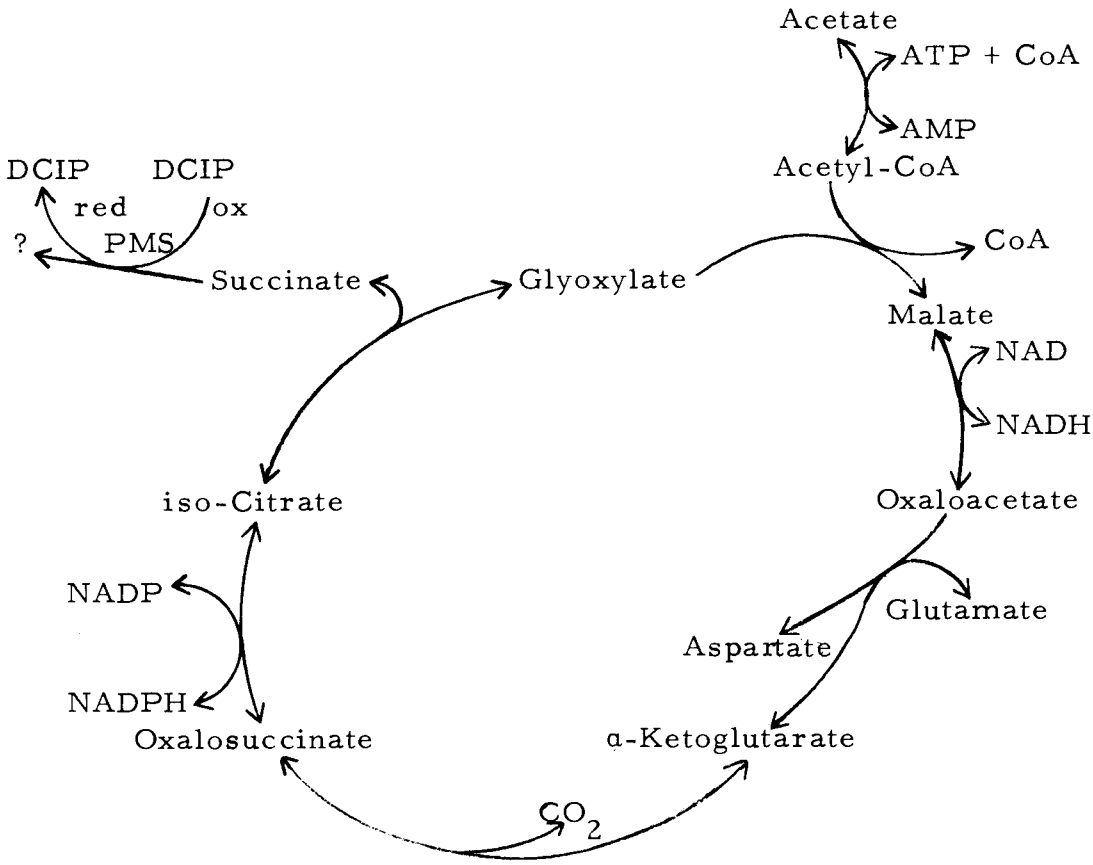
The first enzyme in this system, acetyl thiokinase, is similar to acetyl thiokinase found in other sources (31). The activity observed without the added CoA undoubtedly is due to the CoA in crude extracts. Acetyl kinase, which would catalyze the following reaction, ATP + acetate $\rightleftharpoons$ acetylphosphate + ADP, could not be demonstrated with certainty.

Acetyl-CoA may have numerous fates in Beggiatoa. It was of interest, however, to determine how acetyl-CoA may be used to produce four carbon carboxylic acids without key enzymes involved in

FIGURE 24

Proposed System for Utilization of Acetate

by Beggiatoa

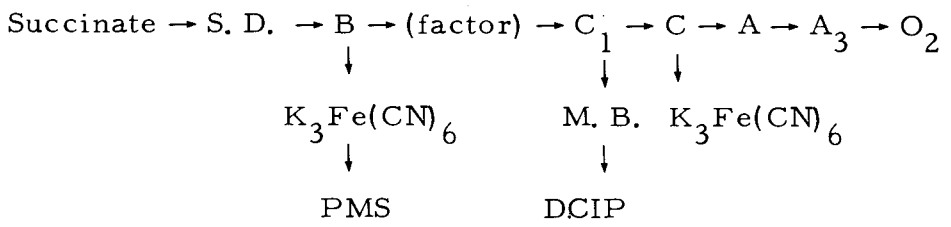


the citric acid cycle and glyoxylate bypass.

The reversible NADP-linked isocitrate dehydrogenase fixes CO_2 in the direction marked in the proposed scheme. The fact that Beggiatoa does not produce CO_2 during growth indicates that the normal direction of this reaction is toward isocitrate production.

The evidence that succinate is produced from isocitrate is provided by the ability of isocitrate to replace succinate in dialized extracts for reduction of DCIP. The process which occurs is the production of succinate and glyoxylate from isocitrate and the subsequent oxidation of succinate. The absorption spectra of the phenylydrazones of the keto acids indicates that glyoxylate is the other product produced.

Malic synthetase and malic dehydrogenase appear similar to enzymes isolated from other sources. The oxidation of succinate is, however, different from the classical succinic dehydrogenase. The oxidation of succinate is not associated with cytochrome b or an electron transport particle as it is in many organisms (55, 57). Methylene blue and ferricyanide fail to act as hydrogen acceptors for the oxidation of succinate by Beggiatoa. This correlates with the observation that purified succinic dehydrogenase fails to react with methylene blue and reacts poorly with ferricyanide (55). Slater (57) suggested that succinate acts with artificial acceptors in the following manner:



Since this suggestion it has been demonstrated that PMS reacts directly with succinic dehydrogenase (55). The absence of cytochromes in Beggiatoa, however, could explain why methylene blue and ferricyanide would not act as hydrogen acceptors for succinate oxidation by Beggiatoa.

The pH optimum for the oxidation of succinate (pH 8.7) was higher than that reported for heart (pH 7.5) and yeast (pH 7.8) succinic dehydrogenase (57). Fumarate was not detected as an end product of succinate oxidation, but this assay was hampered by the presence of PMS and DCIP in the reaction mixture. These observations indicate that succinate oxidation differs considerably from the classical succinic dehydrogenase.

The fate of the reduced coenzymes produced in the cycle (from malate and succinate oxidation) and the source of reduced NADP (for isocitrate dehydrogenase) is not known. The system presented, however, can only be considered a small portion of the total metabolic process that is occurring. Pyridine nucleotide transhydrogenase could generate the NADPH required for isocitrate dehydrogenase from NADH (16, 32). The lipoic dehydrogenase and DCIP-linked

diaphorase represent other possible fates for reduced coenzymes.

The proposed system does not yield energy to the cell. Doudoroff (20) has advanced the hypothesis that under aerobic conditions aerobes are limited in natural conditions by limited amounts of carbon compounds and not energy. Beggiatoa appears to have high energy when found in nature and when grown under laboratory conditions as evidensed by the large lipid stores and high endogenous metabolism. The stimulation of growth by acetate may be due to the use of this compound to build needed carbon skeletons. It is also possible that the fate of the carboxylic acids produced in this system generate energy at later steps in their metabolism. Most of the compounds could leave the proposed system and become involved in numerous side reactions.

In the malate synthetase system, oxaloacetate was a very poor acetyl acceptor in comparison to glyoxylate (Table 6). Whether this activity was due to impurities in oxaloacetate, the presence of condensing enzyme in the extract, or the specificity of malate synthetase was not determined. It appears unlikely that the activity was due to the presence of condensing enzyme since aconitase and citratase were not detectable in extracts, and citrate appeared toxic to growing cultures. The toxicity of citrate to growing cultures may be due to chelation of essential divalent ions. Citrate produced in vivo could be metabolized via a different pathway and not accumulate to toxic

levels.

The reaction of Beggiatoa extracts of sulfite, hydrogen sulfide and ascorbic acid may be correlated to peroxide production. The trichomes were washed prior to the extraction of enzymes. Thus, these preparations lacked catalase activity, and peroxides were free to accumulate. The ability of PMS to transfer electrons from Beggiatoa enzymes to DCIP was a consistent observation throughout this investigation. PMS is known to carry electrons from flavoproteins to suitable acceptors with hydrogen peroxide as an endproduct (19). It appears possible that the observed oxidations of sulfite, sulfide and ascorbic acid may be associated with flavin-linked peroxide formation.

The absence of certain enzymes from Beggiatoa may be related to its natural habitat. In nature Beggiatoa is associated with the presence of hydrogen sulfide. This compound appears to have ready access to the interior of the cell where it is oxidized. Many of the enzymes that are absent from Beggiatoa are either inhibited by hydrogen sulfide or are associated with a sulfide-sensitive system.

Evolutionary patterns would eliminate strains inhibited by hydrogen sulfide and favor strains which employ metabolic systems that could tolerate or even benefit from the presence of hydrogen sulfide. It is interesting to note that many of the enzymes found in Beggiatoa are stimulated by, or require reduced compounds such as, cysteine,

glutathione or hydrogen sulfide.

The proposed system accounts for all of the observed information and presumably may account for a considerable portion of the acetate utilized by Beggiatoa. Beggiatoa grows in vitamin-free medium and also grows, but very poorly, in a mineral-salts medium with acetate and histidine as the only organic compounds. Histidine metabolism in mammalian tissues yields a-ketoglutarate and could thus supply this compound to the cycle proposed for acetate utilization.

This culture of Beggiatoa will be deposited with the American Type Culture Collection.

SUMMARY

The addition of catalase to stock cultures increased the time of culture viability from one week to two months. It also produced a 24-fold increase in wet weight and 93-fold increase in malic dehydrogenase activity. Cultures grown without catalase exhibited an absorption peak characteristic of peroxides. This absorption peak was removed by addition of catalase either during or after growth. Oxygen was an absolute necessity for growth, but carbon dioxide was not produced. Citrate and sulfite were inhibitory to growth whereas malate and acetate stimulated growth at low levels. Glucose and thosulfate were not oxidized and cytochromes were not detectable by spectrophotometric analysis.

A proposed system which would permit acetate incorporation into four carbon compounds without the presence of key enzymes of the citric acid cycle or glyoxylate bypass was described. The proposed system is similar to the glyoxylate bypass in that malate is produced from glyoxylate and acetyl-CoA. It differs from both the citric acid cycle and the glyoxylate bypass since citrate and fumarate are not involved.

The oxidation of succinate differs from previously described succinic dehydrogenases since methylene blue or ferricyanide would not act as hydrogen acceptors. Cytochrome b was lacking and the pH optimum (8. 7) was considerably higher than the optimum

for classical succinic dehydrogenases (7.8-8.0).

Sulfite, sulfide and ascorbic acid oxidation appeared to be correlated with peroxide production by the organism.

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