DISSIMILATION OF NONNITROGENOUS COMPOUNDS BY ACETOBACTER PASTEURIANUM

by

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INTRODUCTION

During the past decade, the use of microorganisms, particularly bacteria, has contributed greatly in elucidating a number of biochemical pathways involved in mammalian metabolism.

Utilizing microbes offers many advantages over some other types of biological materials. They are vast in number in varied environments, resulting in organisms possessing diversified metabolic pathways.

The unicellular organism is a much simpler form of life, possessing a less complicated makeup of biological processes. This is quite useful in that specific reactions in the major pathways such as the tricarboxylic acid cycle and the Embden-Meyerhof pathway can be studied thoroughly.

Sometimes in bacterial systems, a major oxidative pathway such as the tricarboxylic acid cycle may show very little sign of activity. The lack of activity of this major pathway may lead to discovery of other important biological pathways. This has been shown in *Acetobacter suboxydans*, (an organism without tricarboxylic acid cycle activity) by demonstrating the pentose cycle *in toto* (5, pp. 11-26).

An approach in studying these unusual organisms is by investigating a related species possessing the missing major pathway. Studies in this direction has been started in this work with the species, Acetobacter pasteurianum, an organism showing tricarboxylic acid cycle activity.

MATERIALS AND METHODS

Organisms

Acetobacter pasteurianum ATCC 6033 and Acetobacter suboxydans ATCC 621 were obtained from the American Type Culture Collection. Transfers were made weekly on glycerol-yeast extract agar slants.

Streptococcus faecalis 10C1 was kindly furnished by Dr. I. C. Gunsalus. This organism was kept on stab agar tubes. Transfers were made from the stock culture tube when needed.

Materials

CoA[1](75% purity), TPN[1], DPN[1], ATP[1] and glucose-6-phosphate were obtained from Pabst Laboratories. Pyruvic acid (sodium salt), glutathione, and fructose-1,6-diphosphate were purchased from Schwartz Laboratories. Dihydroxyacetone, oxalacetic acid and glucose-1-phosphate were

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[1] The following abbreviations are used:

ATP	adenosine triphosphate
BAL	2,3-dimercapto-1-propanol
CFE	cell-free extract
CoA	coenzyme A
DPN	diphosphopyridine nucleotide
µM	micromole
TCA	tricarboxylic acid
TPN	triphosphopyridine nucleotide
tris	tris (hydroxymethyl) aminomethane

products from Krishell Laboratories.

The other materials were purchased as follows: cocarboxylase: S. A. F. Hoffmann-LaRoche & Company, BAL[1]: Mann Research Laboratories, Trypsin (powder form, 1:110): Pfanstiehl Chemical Company, cytochrome-C: Sigma Chemical Company, α-ketoglutaric acid: General Biochemical Incorporated, and Fleichmann's yeast extract type 3: Standard Brands Incorporated.

Preparation of Cells

Cultivations of A. pasteurianum and of A. suboxydans were made in 20 liter carboys containing 10 liters of medium with the following composition: glycerol, 5%, yeast extract, 1%, K_2HPO_4, 0.5%, and small quantities of Dow anti-foam; pH 6.0.

The inocula for the medium were prepared by transferring cells from agar slants to 500 ml. Erlenmeyer flasks containing 100 ml. of the above medium. They were placed on shakers at room temperature for 36 hours of growth.

Ten liters of medium were inoculated with 200 ml. of actively growing cells. The inoculated batch was placed in a 30° C. incubator and was aerated vigorously with sterilized air. After 48 hours of growth, the culture was checked for contamination by microscopic and plate methods. Cells were separated on a Sharples centrifuge. They were

suspended into 300 ml. of 0.05 M phosphate buffer, pH 6.0, and shaken for one hour at room temperature. The cells were then collected by centrifugation on a Servall centrifuge and washed twice by resuspension in distilled water. The final suspension of cells was dried by lyophilization. Dry cells were stored at -10°C. Yields ranged from 5 to 8 grams per ten liters of medium.

Preparation of Cell-free Extracts

CFE[1] were prepared as follows: 2 gm. of lyophilized cells were suspended in 40 ml. of glass distilled water or 0.01 M phosphate buffer, pH 7.4 and treated at sonic frequencies in a Raytheon 200-watt 10-kc. magnetostriction oscillator. The cylindrical stainless steel cup containing the cell suspension was cooled by circulating water at 6-10° C. into the cooling jacket. The cell suspension was treated for a total of fifteen minutes (three five minute periods with two five minute intermissions) in order to keep the stainless steel cup from overheating.

The sonic treated cell suspension was then centrifuged for 90 minutes at 20,000 x g. The solid phase was discarded and the supernatant liquid was stored at -10° C.

Analytical Methods

Protein Determination. Protein was determined by the modified Weichselbaum's method using crystalline egg albumin as the standard (14, pp. 16-20).

Manometric Techniques. Oxygen consumption or carbon dioxide formation was determined by conventional techniques using a circular Warburg apparatus.

Acetoin Formation. Acetoin was determined by the method of Westerfeld (15, pp. 495-502). Blanks were run for each determination.

Activation of Acetate. The method of Kitos et al. (9, pp. 236-237) was used with slight modifications. In a small test tube, a mixture of glutathione, 20 μM[1], and CoA, 2 μM, was incubated for ten minutes to keep the CoA in the reduced condition. ATP, 20 μM, $MgCl_2$, 20 μM, hydroxylamine, 200 μM, and tris[1], 100 μM, potassium acetate, 200 μM, CFE, 11 mg. protein, and water were then added to 2 ml. Subsequent steps were the same as those of Kitos et al. (9, pp. 236-237).

Lipoic Acid Assay. Lipoic acid was determined according to Gunsalus, Dolin, and Struglia (4, pp. 849-857) with modification. Fleischmann's yeast extract type 3 was used as the standard. The response from 1 mg. of yeast extract was used as one unit of lipoic acid.

Earlier work in this laboratory (10) found that in employing the assay system described by Gunsalus et al.

(4, pp. 849-857), it was difficult to obtain a standard curve comparable to the one obtained by the published method. Thus, results were erratic. By modifying the preparation of the enzymatically digested casein, standard curves with more spread were obtainable. By doubling the concentration of the enzyme-digested casein, consistent results were always obtained.

It was found that the preparation of enzymatic digested casein and its concentration was critical. The following method of digestion proved satisfactory; 50 gm. of vitamin-free casein were added to 490 ml. of water and the mixture was placed on a shaker for 15 minutes. The solution was adjusted to pH 8.0; 10 ml. of 5% trypsin solution was added. After the mixture was shaken for 10 minutes, it was transferred to a 1000 ml. flask and placed in a 37° C. bath. A thin layer of toluene was added. The flask was fitted with a stirrer and the mixture was very slowly stirred. After 24 hours of incubation, the contents were transferred to a large separatory funnel and the toluene removed. The mixture was adjusted to pH 6.0 and filtered with mild suction through a layer of cotton on a Buchner funnel. 25 gm. of Norite were added to the mixture and shaken for 30 minutes. The Norite was removed by filtration and the digested mixture was adjusted to pH 3.8. A second treatment with 10 gm. of Norite was performed in

a similar way. The mixture was preserved with toluene and stored at 0-5° C.

RESULTS AND DISCUSSION

Survey of Oxidation

A survey of oxidation of various substrates by A. pasteurianum was first studied with lyophilized cells. The results are summarized in table I. Some substrates were readily oxidized so the duration of the experiments was not the same for all compounds studied.

The oxygen consumption was approximately 9 μatoms per molecule of glucose. Less oxygen was consumed by the phosphorylated sugars. The slow oxidation was presumably due to the fact that the permeability became the limiting factor, since the phosphorylated compounds are more polar than the free sugars.

The rate and total amount of oxygen consumption was much slower for dihydroxyacetone than glycerol. Ethanol and acetaldehyde were readily oxidized, although the values obtained did not approach the theoretical value calculated for the complete oxidation to carbon dioxide and water. However, ethanol was oxidized well beyond the acetic acid stage, and lyophilized cells were able to oxidize acetate (cf. table I). This is not true with all species of Acetobacter: in A. suboxydans, resting cells can not attack acetic acid under the conditions tested (7, pp. 581-584).

Table I shows that TCA[1] and dicarboxylic acid cycle intermediates, with the exception of citric acid, are readily oxidized. However, the rates of oxidation for various substrates were not the same. This could be due to many factors which were not easily controlled under the experimental conditions. It is interesting to note that citric acid was only slightly oxidized by the lyophilized cells of A. pasteurianum. Campbell and Stokes, (1, pp. 853-858), have found that citric acid was not oxidized by fresh cells of Pseudomonas aeruginosa but easily oxidized by lyophilized cells. They have interpreted that the difference of oxidative behavior in these two types of cells is due to the alteration of the cell wall by lyophilization.

Citric Acid Oxidation

In order to rule out the permeability factor involved in citric acid oxidation by A. pasteurianum, a cell-free extract was used. In a system containing 10 μM citric acid, 1 mg. mammalian cytochrome-C, 2 mg. TPN, 1 mg. DPN, 0.5 mg. cocarboxylase, 150 μM phosphate buffer, pH 6.0, 20 μM $MgCl_2$, and CFE (14 mg. protein), citric acid was not oxidized. The addition of 5 mg. Armour liver concentrate to the system resulted in a slight oxidation equivalent to 0.4 μatoms per molecule of citric acid.

These results indicated the non-oxidizability was

either due to the absence of appropriate enzymes in the cells or these enzymes were removed by the preparation of cell-free extracts. Thus, cell homogenates were used to determine these two possibilities. Cell homogenates were made by sonic disintegrating lyophilized cells suspended in 0.01 M phosphate buffer, pH 7.4. It was interesting to note that fresh preparations readily oxidized citric acid as shown in figure 1. The oxidative behaviors at pH 6.0 and 8.0 were practically the same. Judging from the values of oxygen consumption, citric acid was completely oxidized.

These results strongly suggest that the TCA cycle is present in A. pasteurianum, in contrast to A. suboxydans (7, pp. 581-584).

Pyruvic Acid Oxidation

In studying pyruvic carboxylase in A. suboxydans, King and Cheldelin have observed a lag period in oxygen consumption in the oxidation of pyruvic acid but not in acetaldehyde (6, pp. 821-831). Also, no lag period in carbon dioxide formation is observed. Similar results were found in experiments on A. pasteurianum CFE. The results are summarized in table II and figure 2.

During the first fifteen minutes as shown in table II, pyruvic acid showed no oxygen consumption. Gradual oxygen consumption occurred after fifteen minutes,

indicating a lag period during the initial period. For acetaldehyde, oxygen consumption was consistent without an initial lag period. Also, a consistent carbon dioxide evolution was observed in pyruvic acid dissimilation (figure 2).

With similar experimental conditions indicated under table II, except with substrate concentration equal to 10 μ moles, the oxygen consumption leveled off after 45 minutes for pyruvic acid. A ratio of one μatom per μmole of substrate was observed. For acetaldehyde, oxidation practically stopped after fifteen minutes with a 0.6 ratio. Acetic acid was not oxidized by cell-free extract in the presence or absence of catalytical amounts of oxalacetic acid.

These results suggest that the pyruvic decarboxylation in A. pasteurianum CFE is the type of simple decarboxylation. The acetate oxidation enzymes do not exist in the extract and presumably are removed during the preparation.

The Dimedone Effect. To further substantiate the evidence for a simple pyruvic carboxylase activity, the oxidation of pyruvic acid was tested in the presence of dimedone. Results similar to the pyruvic carboxylase system in A. suboxydans were observed (6, pp. 821-831), i.e., increasing concentrations of dimedone forced a

decrease in oxygen consumption with pyruvic acid (figure 3) and acetaldehyde (figure 4). 8 mg. of dimedone in the system reduced acetaldehyde oxidation to three-fourths value. 20 mg. of dimedone inhibited the oxidation almost completely. On the other hand, 20 mg. dimedone did not decrease the formation of carbon dioxide from pyruvate.

Acetoin Formation. The pathways of the intermediate, acetaldehyde, formed from pyruvic acid in microorganisms have been reviewed extensively by Gunsalus, Horecker, and Wood (3, pp. 79-128). The acetaldehyde may react with another molecule of acetaldehyde or pyruvic acid to form acetoin.

The acetoin formation was studied by A. pasteurianum CFE. 6 µmoles of acetoin were formed from pyruvic acid at a level of 50 µmoles per 2.8 ml. reaction volume after four hours, (table III). At this concentration of acetaldehyde, no acetoin was formed. However, higher concentrations of acetaldehyde did give acetoin. The amount of acetoin formed from acetaldehyde was approximately two-thirds of that formed from pyruvic acid. These results indicate that the acetaldehyde intermediate formed from pyruvic acid may follow an acyloin condensation with free acetaldehyde to acetoin as postulated by Neuberg and Hirsch, (11, pp. 282-310).

Arsenite Inhibition. The pyruvate oxidation by

A. pasteurianum extracts was found to be arsenite sensitive. The degree of inhibition was proportional to the concentration of arsenite in the range from 0.1 μmole to 100 μmoles per 2.8 ml. A monothiol, glutathione, did not show reversal at concentrations of 10, 100, or 1000 μmoles per flask. Stocken and Thompson (13, pp. 535-548) used a dithiol, BAL, to reverse the arsenite inhibition. It has been also found that BAL, but not monothiols, can reverse the lipoic acid dependent system in microorganisms (2, pp. 113-136). BAL was, therefore, tested as a reversing agent. As shown in table V, BAL did not reverse the arsenite inhibition. Instead, BAL inhibited the oxygen consumption even in the absence of arsenite.

The arsenite effect on the carbon dioxide formation was tested under a nitrogen atmosphere. The system used was the same as that of table IV, except 100 μmoles of arsenite were used and KOH was omitted from the center wells. It was found that arsenite did not inhibit the decarboxylation.

All these results indicate that the pyruvate decarboxylation in A. pasteurianum CFE was not lipoic acid dependent and followed the same type that occurs in A. suboxydans. However, resting cells of A. pasteurianum could oxidize pyruvate with about 5 atoms of oxygen per molecule of substrate (cf. table I). Thus, A. pasteurianum

is one of the very few organisms which possess two different pathways for pyruvate decarboxylation.

Lipoic Acid Studies

A. pasteurianum and A. suboxydans were found to contain 45 units and 15 units of lipoic acid respectively per mg. of protein of CFE.

Various methods were tried to remove lipoic acid from the CFE systems. The alumina adsorption methods used by Seaman (12, pp. 731-733) resulted in approximately less than 40% removal when the CFE was passed through a column charged with adsorptive alumina. Treatment with the OH^- form of Dowex-1 resulted in about 30% removal.

Dialysis of CFE against 0.01 M versene removed 20% of the cofactor. When cysteine-KCl solution was used as the dialyzing agent, at varying pH 6.0, 8.0, 8.5, and 9.0, none of the cofactor was displaced. Upon increasing the concentration of cysteine to 0.01 M at pH 8.0, approximately 40% removal was possible in the case of A. pasteurianum CFE. Cysteine-KCl dialysis followed by alumina adsorption did not result in any improvement.

In view of the behavior of the pyruvate decarboxylation, the function of lipoic acid in A. suboxydans must be in systems other than pyruvate oxidation. In A. pasteurianum, lipoic acid may play a role in the pyruvate

oxidation by resting cells but not by CFE. More decisive information can be obtained only when enzyme preparations free from lipoic acid are available.

Acetate Activation

A. pasteurianum CFE catalyzed the formation of acetyl-CoA. The pH effect on the formation is shown in table VI.

With increasing concentration of ATP and CoA, increased formation of acetyl-CoA was observed. Since phosphatase activity might have been present in the system, fluoride was added to inhibit the phosphatase action on ATP. However, no significant effect by fluoride was observed.

The significance of acetyl-CoA formation in CFE of A. pasteurianum as well as in A. suboxydans is not clear. Since extracts from neither organism could oxidize acetate, it is not likely that the acetate is led to the TCA cycle. However, fatty acid synthesis which has not been studied in these organisms may be related to acetyl-CoA formation.

SUMMARY

Acetobacter pasteurianum lyophilized cells oxidatively dissimilated a variety of substrates. Of the TCA cycle intermediates tested, only citric acid could not be oxidized. However, cell homogenates rapidly oxidized citric acid.

Oxidation of pyruvate by *A. pasteurianum* CFE occurred with a lag period in oxygen consumption but not in carbon dioxide evolution. No lag period was observed in acetaldehyde oxidation. Dimedone inhibited both acetaldehyde and pyruvate ovidations, but did not inhibit the carbon dioxide formation from pyruvate.

Acetoin was formed from high concentrations of pyruvate and acetaldehyde, whereas at low concentrations, acetoin was formed only from pyruvate by *A. pasteurianum* extracts.

Arsenite inhibited the oxygen consumption in pyruvate oxidation, but not carbon dioxide formation. The inhibition was not reversed by glutathione or BAL.

A. pasteurianum and *A. suboxydans* contained 45 units and 15 units of lipoic acid respectively, per mg. of protein of CFE.

Like *A. suboxydans*, *A. pasteurianum* CFE catalyzed the formation of acetyl-CoA.

TABLE I

Oxidizability of TCA intermediates and others by lyophilized A. pasteurianum cells

Substrate	Duration of experiment	µAtoms oxygen / µmoles substrate
Glucose	300	9.2
Dihydroxyacetone	300	1.2
Glycerol	180	4.4
Ethanol	60	4.5
Acetaldehyde	120	3.5
Fructose-1,6-diphosphate	300	3.5
Glucose-6-phosphate	300	4.4
Glucose-1-phosphate	300	5.5
Acetic acid	300	2.7
Pyruvic acid	80	4.5
Citric acid	300	0.15
α-Ketoglutaric acid	300	3.6
Oxalacetic acid	120	3.5
Fumaric acid	300	5.4
Succinic acid	300	6.3

The system contained 10 µmoles substrate; 10 µmoles $MgCl_2$; 150 µmoles phosphate buffer, pH 6.0; and 10 mg. lyophilized cells to a total volume of 2.8 mls. 0.2 ml. of 10% KOH was placed in the center well. Temperature, 30° C.

TABLE II

Oxidation of pyruvic acid and acetaldehyde by CFE of A. pasteurianum

	µAtoms of oxygen/µMoles of substrate	
Time (Minutes)	Pyruvate	Acetaldehyde
0	0.0	0.0
15	0.0	0.10
30	0.09	0.21
45	0.20	0.32
60	0.33	0.42
75	0.43	0.49

The system contained 1 mg. of DPN; 20 µmoles of $MgCl_2$; 0.5 mg. of co-carboxylase; 150 µmoles phosphate buffer, pH 6.0; CFE, 14 mg. protein and 50 µmoles of pyruvate or 47 µmoles of acetaldehyde to a volume of 2.8 ml. 0.2 ml. of 10% KOH was placed in the center well. Temperature 30° C.

TABLE III

Acetoin formation by CFE of A. pasteurianum from pyruvic acid and acetaldehyde

Substrate concentration	Duration of Experiment	Acetoin formed from Pyruvic Acid	Acetoin formed from Acetaldehyde
50 µmoles	240 minutes	6 µmoles	--
450	120	33	19

The system contained substrate; 150 µmoles phosphate buffer, pH 6.0; 20 µmoles $MgCl_2$; 1 mg. DPN; 0.5 mg. co-carboxylase and CFE, 11 mg. protein to a total volume of 2.8 ml. 0.2 ml. of 10% KOH was placed in the center well. Temperature, 30° C.

TABLE IV

Inhibition of pyruvate oxidation by arsenite in A. pasteurianum extracts

μmoles arsenite	μliters oxygen consumed
0.0	645
0.1	628
1.0	569
10.	433
100.	252

The additions to the flask were the same as table III, except for pyruvate, 50 μmoles and varying concentrations of arsenite.

TABLE V

The effect of BAL on arsenite inhibition with *A. pasteurianum* extracts

Additions	µliters oxygen consumed
No arsenite	804
plus 100 µmoles arsenite	630
0.1 µmoles BAL	393
0.1 µmoles BAL plus 100 µmoles arsenite	652
1.0 µmoles BAL	340
1.0 µmoles BAL plus 100 µmoles arsenite	319

The conditions of the experiment were the same as table IV, except 100 µmoles pyruvate were added to the flask and BAL was added into the main compartment from the side arm.

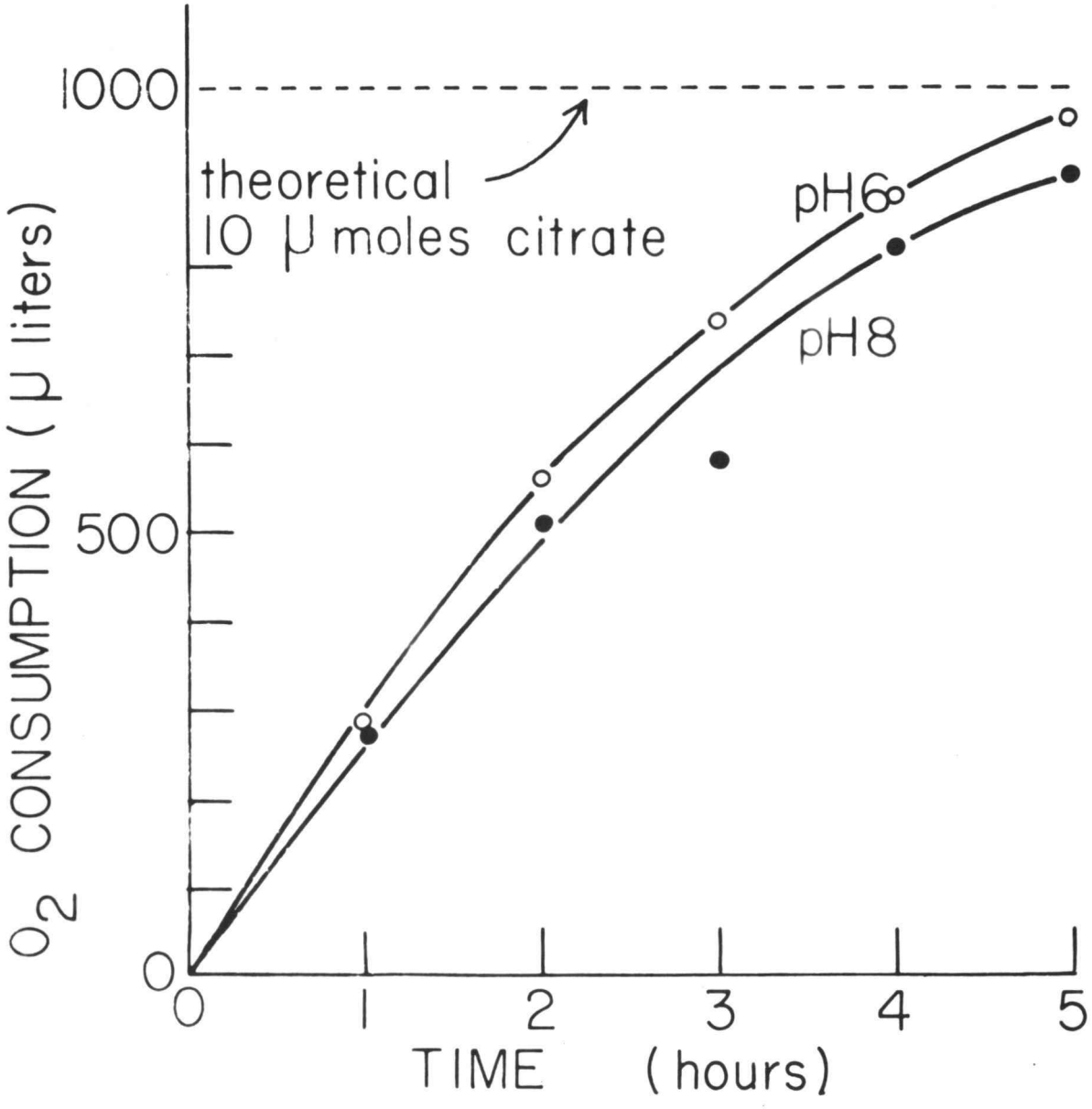
TABLE VI

The effect of pH (tris buffer) on the formation of acetyl-CoA by A. pasteurianum extracts

pH	7.2	7.6	8.0	8.2	8.4	8.8
µmoles hydroxamic acid formed	1.7	1.7	0.2	1.2	1.4	0.6

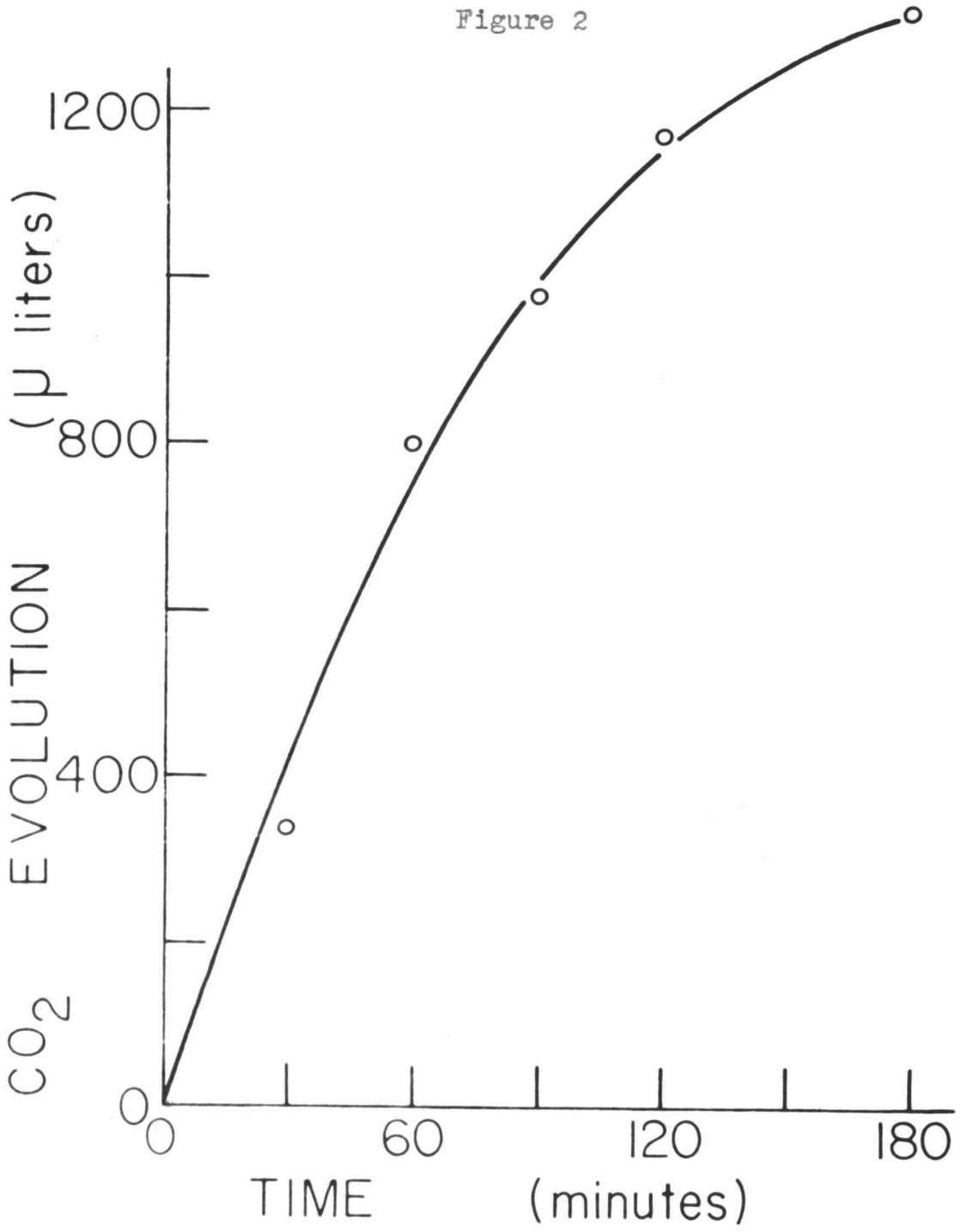
The system consisted of 20 µmoles glutathione; 20 µmoles CoA; 20 µmoles ATP; 20 µmoles $MgCl_2$; 200 µmoles hydroxylamine; 100 µmoles tris; 200 µmoles potassium acetate; CFE, 11 mg. protein to a total volume of 2 ml. Incubated at 37° C. for one hour.

Figure 1



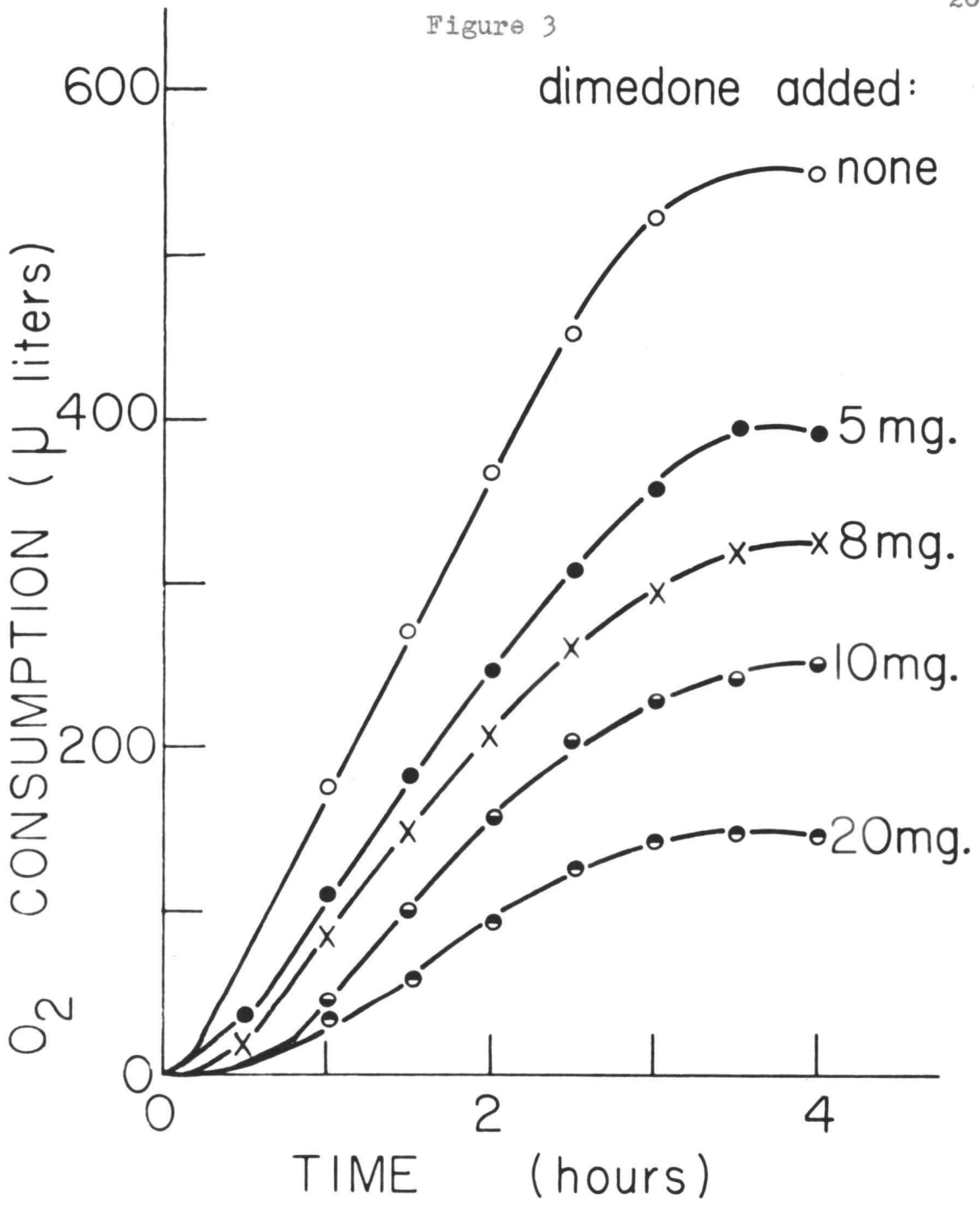
Oxidation of citric acid by A. pasteurianum cell homogenate. The system contained 10 μmoles citric acid, 20 μmoles $MgCl_2$, 150 μ moles phosphate buffer, pH 6.0 or 8.0, and 1 ml. of cell homogenate to a total volume of 2.8 ml. 0.2 ml. of 10% KOH was placed in the center well. Temperature, 30° C.

Figure 2



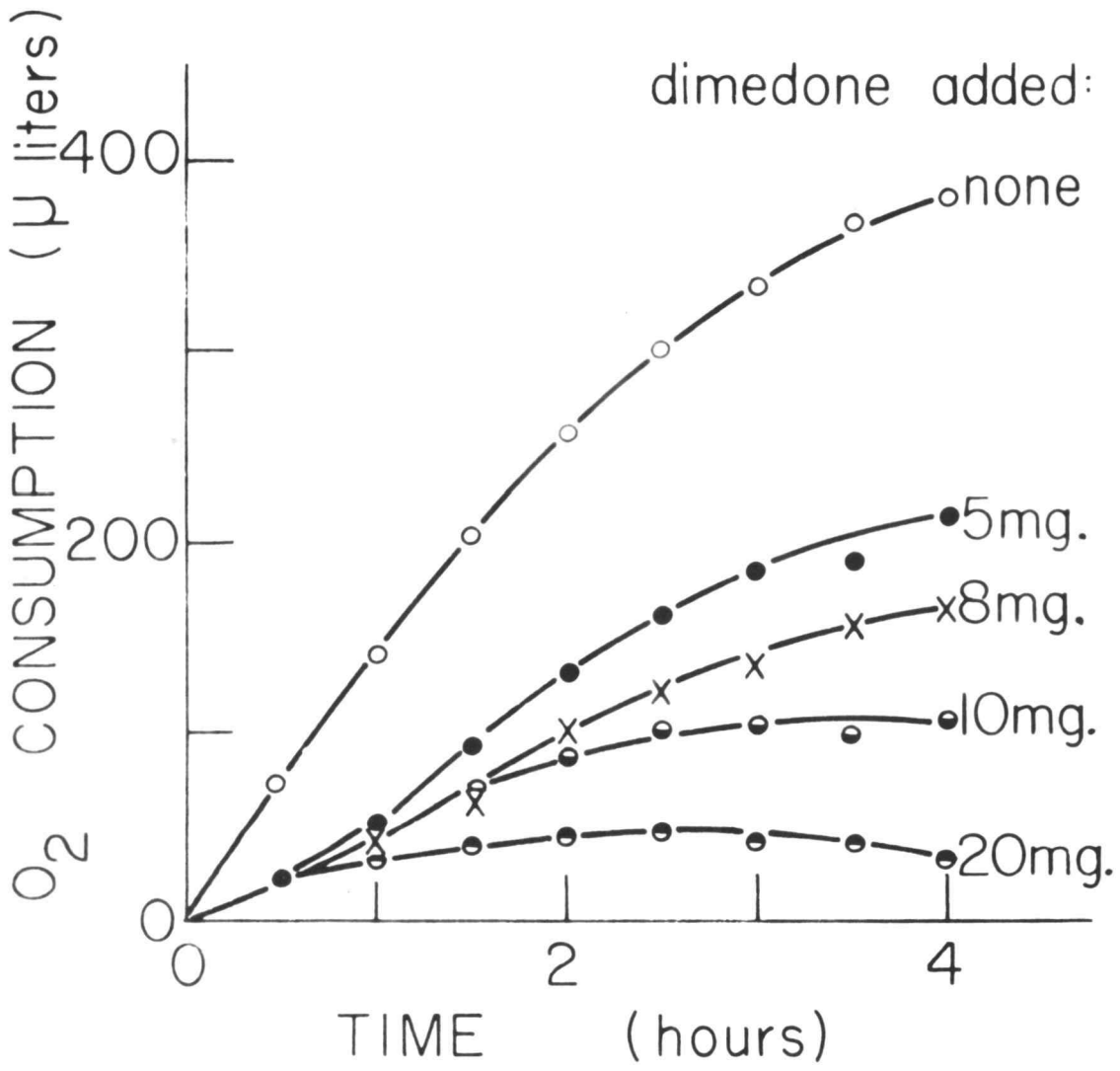
Carbon dioxide evolution from pyruvic acid by A. pasteurianum CFE. The system was the same as that of table II.

Figure 3



The effect of dimedone on the oxidation of pyruvate by A. pasteurianum CFE. The same system was used as shown by figure 2.

Figure 4



The effect of dimedone on the oxidation of acetaldehyde by A. pasteurianum CFE. The same system was used as shown under figure 3, except 47 μmoles of acetaldehyde was used as the substrate.

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