THE CONTROL OF

ISOCITRATE METABOLISM IN *TETRAHYMENA PYRIFORMIS*

by

THOMAS E. DORSEY

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Chairman of Examining Committee

May 9, 1975
date

Executive Officer

Supervisory Committee

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of pyruvate kinase with the phenylhydrazine assay system were obtained by Dr. Hogg in Dr. Christian de Duve's laboratories at Rockefeller University while Dr. M. Muller, Dr. J. F. Hogg and Dr. C. de Duve were jointly working on the isolation of peroxisomes from Tetrahymena.

The members of my thesis committee, Dr. Diana Beattie, Dr. Charlotte Russell, Dr. John Berech, Dr. James Hogg and Dr. Aaron Lukton, gave many helpful suggestions and some valid criticism of this thesis during preparation. Any mistakes, however, in either fact or in proofreading are truly my responsibility.

Finally, it is impossible to thank Professor James Hogg sufficiently for his steadfast interest in my development while working in his laboratory. I am indeed grateful and privileged for having spent my critical training years with this man. His ability to vitalize and help organize an underdeveloped mind is unequalled in my experience. I shall fondly remember our many talks filled with ideas and suggestions which were of incalculable aid to me. I will be forever indebted to Professor Hogg for his scientific guidance, his philosophy and his friendship.

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GENERAL INTRODUCTION

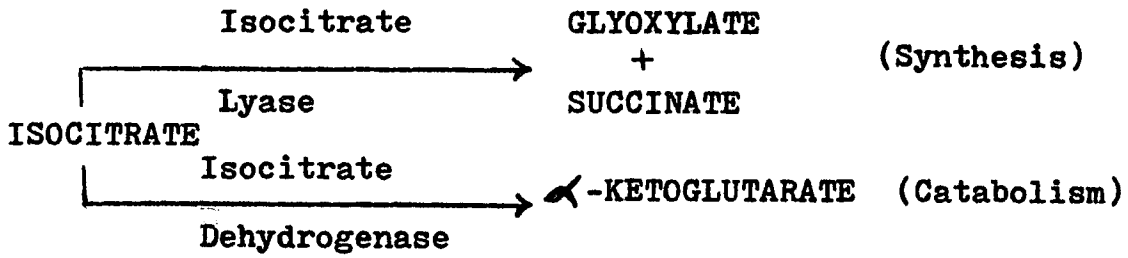
In planning this thesis, I have chosen to present the work in chapter form, each chapter appearing as it might be presented to a scientific journal. The sequence of the chapters follows the order in which the work was performed and in a sense represents the gradual enlargement of the scope of the original problem. At first we focused our attention on the single enzyme, isocitrate lyase, as a controlling factor in the regulation of isocitrate metabolism in Tetrahymena. We expanded our view of the problem to include not only the competing enzyme, isocitrate dehydrogenase, but the effect that organelle structure has on the regulation of isocitrate metabolism.

Chapter one of this thesis deals with the stabilization and partial purification of isocitrate lyase from Tetrahymena. This enzyme has long been suspected of being involved in the regulation of gluconeogenesis through its regulation of isocitrate metabolism and glyoxylate cycle. The proposed fine regulator for the activity of this enzyme (at least in bacteria) has been phosphoenolpyruvate, Kornberg, H. (1960). The work in chapter one shows that phosphoenolpyruvate is not a good inhibitor of the partially purified isocitrate lyase from Tetrahymena and thus less likely to be a metabolic regulator of the enzyme in this organism.

In chapter two, data are presented to show that phosphoenolpyruvate kinase, an enzyme which would break down

phosphoenolpyruvate, is present in high amounts in Tetrahymena under a variety of different growth conditions. In this chapter a new enzymatic assay for the kinase is given which is more sensitive and avoids the problems of a coupled assay system which has been used previously. With this new assay procedure the K_m (phosphoenolpyruvate) of phosphoenolpyruvate kinase is shown to be smaller than the concentration that will significantly affect the activity of isocitrate lyase. We must conclude therefore that phosphoenolpyruvate is probably not regulating isocitrate lyase and thus, isocitrate metabolism in Tetrahymena.

Although we have not completely excluded the possibility of regulation of the lyase on a purely enzymatic level, the intracellular distribution of the enzymes of the glyoxylate cycle very strongly suggests that regulation must be achieved at least in part by means of membrane transport, therefore we decided to look into the possible regulation of this enzyme, as well as the dehydrogenase at the organelle level. Chapters three and four deal with work done on the intact peroxisome from Tetrahymena pyriformis. Chapter three specifically gives information on the intraperoxisomal distribution of these enzymes which are at the branch point of metabolism where the isocitrate molecule will be either used for synthesis or catabolism.



In the fourth and final chapter the kinetics of the enzymes competing for isocitrate are given as they appear in the isolated peroxisome and in preparations from it. Interesting differences are found if the Lineweaver-Burk plots for the soluble and particulate enzymes are compared. These differences can start to explain some of the changes found in overall glycogen metabolism with changes in cellular conditions.

The enzymes involved in isocitrate metabolism were first reported to be incorporated into a special organelle in Tetrahymena in 1961 by Hogg and Kornberg. The name peroxisome was proposed and used by Christian de Duve, (1965). Since then, the peroxisome has been found in many species, from single cell organisms, Muller, M. (1969); to the complex cells of mammals, Leighton, F. et al. (1968), and higher plants, Breidenbach, W., Beevers, H. (1967); Tolbert, N. E., et al. (1969). The organelles from Tetrahymena have been purified to a considerable extent, Muller et al. (1968). Even the evolution of the peroxisome has not been neglected. The peroxisome is thought to be a primitive oxidative particle that preceded the mitochondrion as the cells' oxidative organelle, de Duve, C. and Baudhuin, P. (1966) and de Duve (1969). Figure one is a color xerox copy of an electron micrograph which shows the peroxisome as it appears in Tetrahymena pyriformis. Since a color had to be chosen, green was thought to be most appropriate in light of the high concentration of catalase in the particle.

Figure 1. Electron micrograph showing the peroxisome in Tetrahymena pyriformis

The author is indebted to Miklos Muller at Rockefeller University for allowing him to reproduce this photograph here and for having Helen Shio prepare the original slide for him.

I should like to conclude the general introduction by referring to a quote that William Jencks uses in his book CATALYSIS IN CHEMISTRY AND ENZYMOLOGY. Professor Jencks was referring to the state he felt that scientists were in as they probe the mechanism of enzyme action. In a way this quotation could equally refer to the situation many researchers occasionally find themselves in and hopefully try to avoid. Professor Jencks' analogy goes as follows: "At the present time we are in the positions of the drunk

on his hands and knees under the corner street light who, when approached by a citizen asking his intentions, replies that he is looking for his keys here, rather than in the poorly illuminated center of the block where they were lost, because the light is better at the corner." I can only hope that this statement has had a sufficiently sobering effect on the present author.

THE STABILIZATION, PARTIAL PURIFICATION AND PROPERTIES OF THE HIGHLY LABILE ISOCITRATE LYASE FROM TETRAHYMEA PYRIFORMIS

SUMMARY

Isocitrate lyase has been stabilized and partially purified from Tetrahymena pyriformis. 50% (v/v) aqueous glycerol with 0.01 M pyrophosphate was the best stabilizing agent at pH's 5.0-7.0. Mg^{++} destabilized the enzyme and dithiothreitol had no effect on enzyme stability. The partially purified enzyme was devoid of catalase, malate synthese and malate dehydrogenase activities but did contain NADP-linked isocitrate dehydrogenase. The Km with threo-D_s-isocitrate for purified isocitrate lyase is 1.0×10^{-4}. Phosphoenolpyruvate is not a significant inhibitor of the isocitrate lyase from T. pyriformis.

INTRODUCTION

Isocitrate lyase (EC 4.1.3.1) is an acetate-inducible enzyme present in a number of microorganisms. It has been purified to some extent from different sources by several investigators: (Olsen, 1959, yeast); (McFadden and Howes, 1963, Pseudomonas indigofera); (Turien and Kobr, 1965, and Johanson et al. 1974, Neurospora crassa); and (John and Syrett, 1967, Chlorella pyrenoidosa). The enzyme's key position in glyconeogesis makes it a desirable enzyme to purify so that the regulation of the glyoxylate cycle and thus glyconeogenesis can be better understood, Hogg (1969).

A stumbling block to purification was the extreme lability of this enzyme from Tetrahymena. This paper describes the stabilization, partial purification and some of the properties of isocitrate lyase from Tetrahymena pyriformis.

METHODS AND MATERIALS

The procedure for growth and maintenance of Tetrahymena pyriformis is given by Wu and Hogg (1952). The medium contains 10.0 gms proteose-peptone, 1.0 gm sodium acetate, 1.0 gm glucose, 1.0 gm K_2HPO_4 and 0.1 gm of yeast extract per liter of solution. The pH of the solution is 7.2. Growth was allowed to proceed to the stationary phase (4-7 days) before harvesting.

The cells were harvested in a modified plankton centrifuge described by Conner et al. (1966). By appropriately adjusting the flow of the cell culture and the centrifuge speed, all the cells could be harvested without damage. Cells were routinely checked during harvesting with a phase contrast microscope. After collecting the cells, they were washed with Ryley-Ringer phosphate solution (Ryley, 1952) equal in volume to the original volume of medium. The cell suspension was adjusted to a density of 10% v/v using Constable protein tubes for determing cell volume. Finally the cells were freeze-dried in an all glass Kontes lyophilizer and stored at -20° C.

extracts were made by suspending freeze-dried cell powders in 50% v/v glycerol, made to 0.1 M in potassium

phosphate buffer pH 6.9, in a precooled Brendler homogenizer tube and grinding with a teflon pestle for one minute at 1000 rpm. At dried cell concentrations greater than 12.5 mg/ml, the enzyme extraction was not complete. The homogenates were centrifuged at 20,000 x g for 20 minutes in a Sorvall RC-2 super centrifuge with a SS-34 angle head rotor and the extract decanted. Additions of aqueous solutions to the extracts to test for their effect on enzyme stability were followed by an equal volume of pure glycerol.

Isocitrate lyase was assayed by the spectrophotometric method of Dixon and Kornberg (1959) and by the colorimetric method of McFadden and Howes (1960). Malate synthase was determined spectrophotometrically by the procedure of Dixon and Kornberg (1959). Malate dehydrogenase and NADP-linked isocitrate dehydrogenase were assayed according to Kornberg (1955). Catalase was assayed as described by Luck, H. (1963). The specific activity for isocitrate lyase is defined as micromoles of glyoxyate formed/hr/mg protein. Spectrophotometric assays were run in a Gilford model 2400 spectrophotometer with the cuvette temperature controlled at 30^{0} C by a Lauda water bath.

CM and DEAE ion exchange celluloses (Bio-Rad) were prepared according to the methods of the manufacturer and were equilibrated with the appropriate buffer.

Protein was determined by the method of Lowry et al. (1951), after a precipitation and wash in cold 2% perchloric acid.

MATERIALS

With the exception of proteose-peptone (Difco), all biochemicals and potassium pyrophosphate were purchased from Sigma Chemical Co. St. Louis, Mo.. Other inorganic chemicals were Baker Analyzed grade from J. T. Baker Chemical Co.. Glyoxylate phenylhydrazone to be used as a standard in the colorimetric assay for isocitrate lyase was prepared according to the procedure of El Hawary and Thompson (1953).

RESULTS

Crude aqueous extracts of isocitrate lyase from lyophilized cell powders of Tetrahymena pyriformis in 0.1 M potassium phosphate or imidazole -HCl buffer pH 6.9 are active for less than 24 hours at 5^{o} C in our laboratory. Equivalent extracts in 50% (v/v) aqueous glycerol under the same conditions are stable for 2 to 3 days. This stabilization occurs because of the solvent effect in glycerol rather than the elimination of a destabilizing agent during extraction with 50% glycerol because a 50% glycerol extract made by 1:1 combination of pure glycerol and a buffer extract is also stable for 2 to 3 days. We have also found that other organic polyalcohols such as sucrose or ethylene glycol preserve isocitrate lyase activity to some extent if they are present at like concentrations.

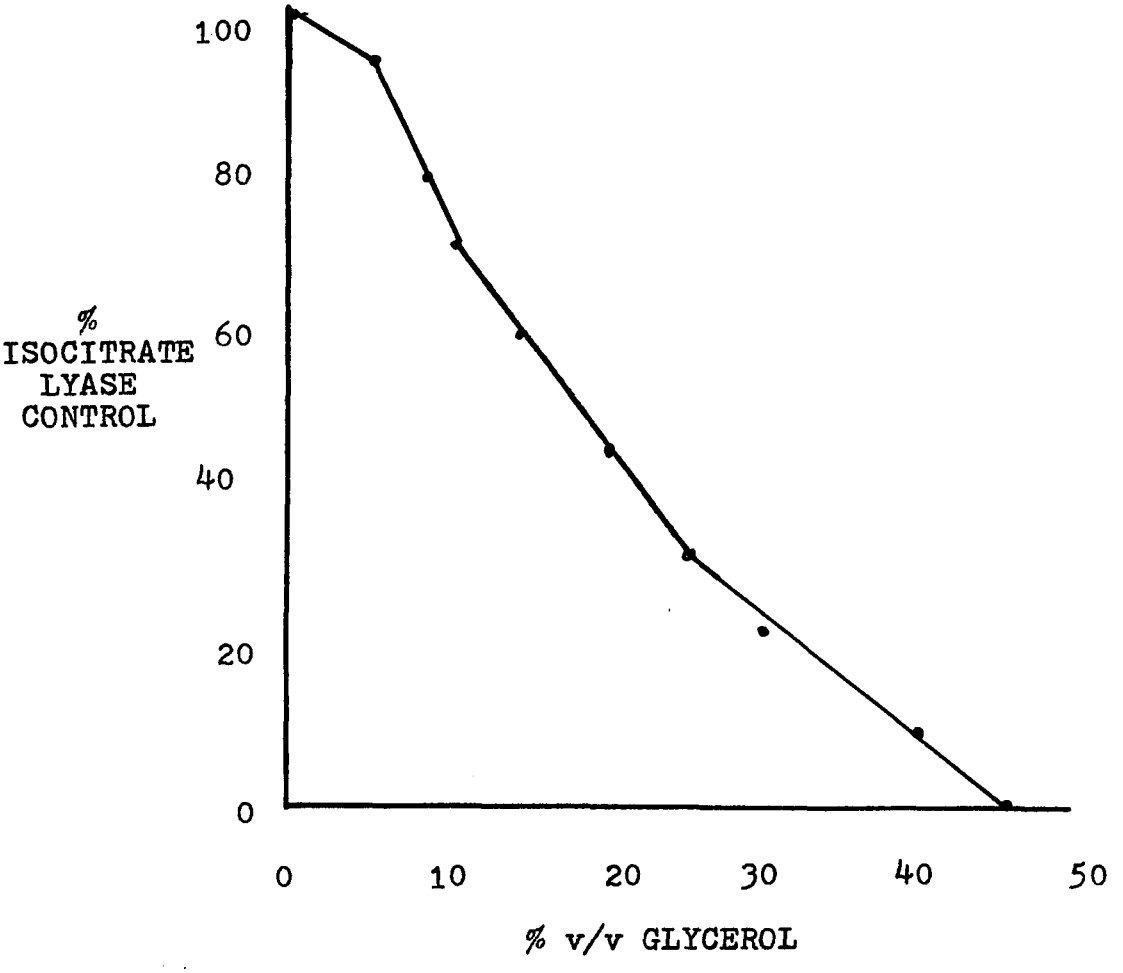
Changing the solvent from water to 50% v/v glycerol in the assay has a pronounced effect on the activity of iso-

citrate lyase as demonstrated in figure 1. As the glycerol concentration in the assay medium is increased to 50% (v/v), the isocitrate lyase activity steadily decreases to zero. This lowering of activity is not glycerol interference with the chemical assay procedure since a known amount of glyoxyate gives an immediate and complete phenylhydrazone reaction in the assay system with 50% glycerol present. Most likely the glycerol is either dehydrating the active site or changing the conformation of isocitrate lyase so that the enzyme is incapable of catalysis by either dehydration or specific chemical interaction.

The effect of different chelating agents on the stability of isocitrate lyase in 50% glycerol, 0.1 M potassium phosphate buffer pH 6.9 at 5° C can be seen in figure 2. No change in stability occurs when crude extracts are made 0.1 M in sodium citrate or 0.1 M in EDTA. Extracts containing 0.01 M potassium pyrophosphate however, are still 50% active after 2 weeks at 5° C. Little activity is lost at all over the period of a year if the glycerol-pyrophosphate extracts are kept at -20° C even though the extracts are still liquid at this temperature.

The addition of 0.001 M Mg^{++} increases the rate of isocitrate lyase loss in 50% v/v glycerol extracts at pH 6.9 and 5° C as shown in Figure 3. Although the control tube loses 70% of its activity in 48 hours, adding .001 M Mg^{++} enhances the loss so that the tubes containing added Mg^{++} are only 25% as active as the controls. Control tubes would

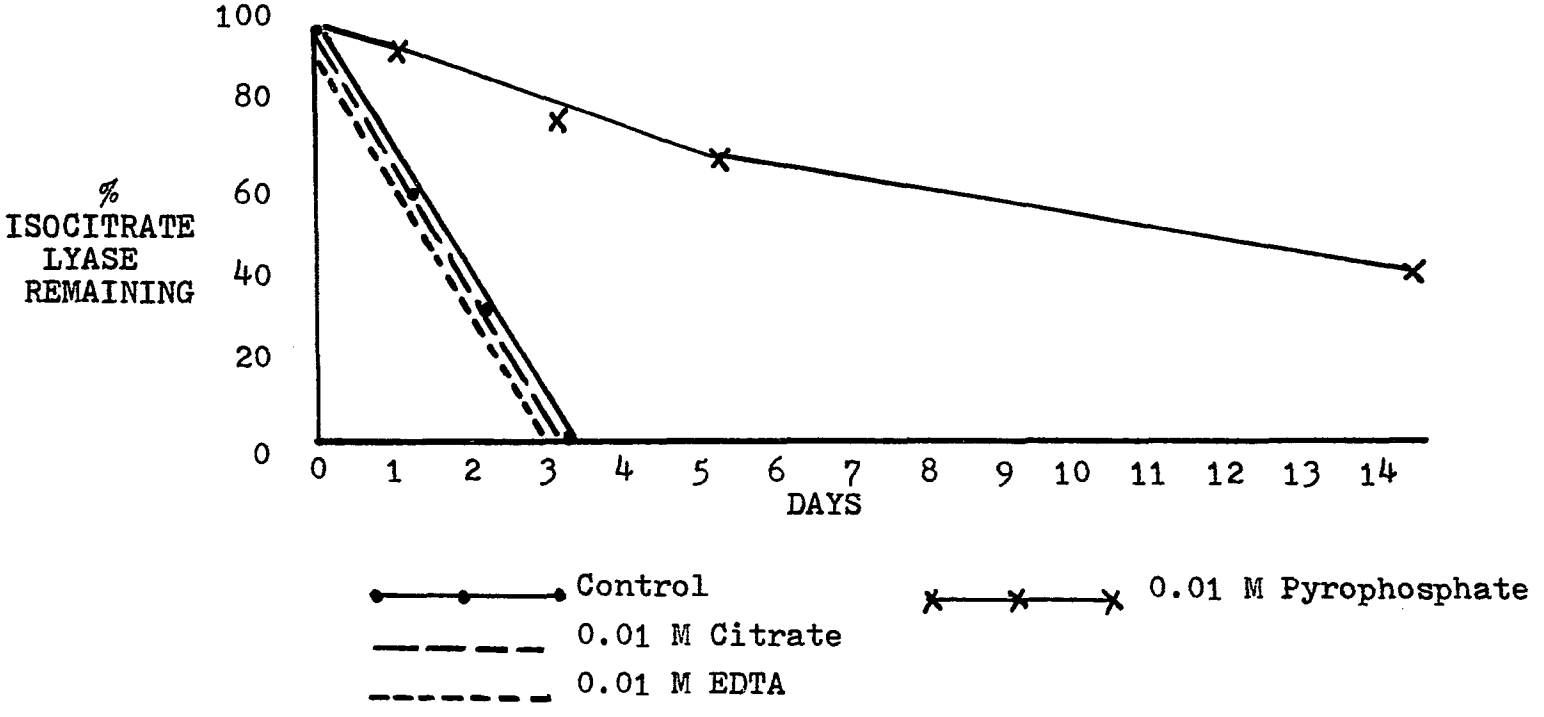
THE EFFECT OF GLYCEROL ON THE ISOCITRATE LYASE ENZYME REACTION



Extracts were prepared as described in Methods. The spectrophotometric assay was used to determine isocitrate lyase activity. Initial rate is 0.234 Δ A/ min/0.2 ml. See Appendix for the enzyme assay.

Figure 1

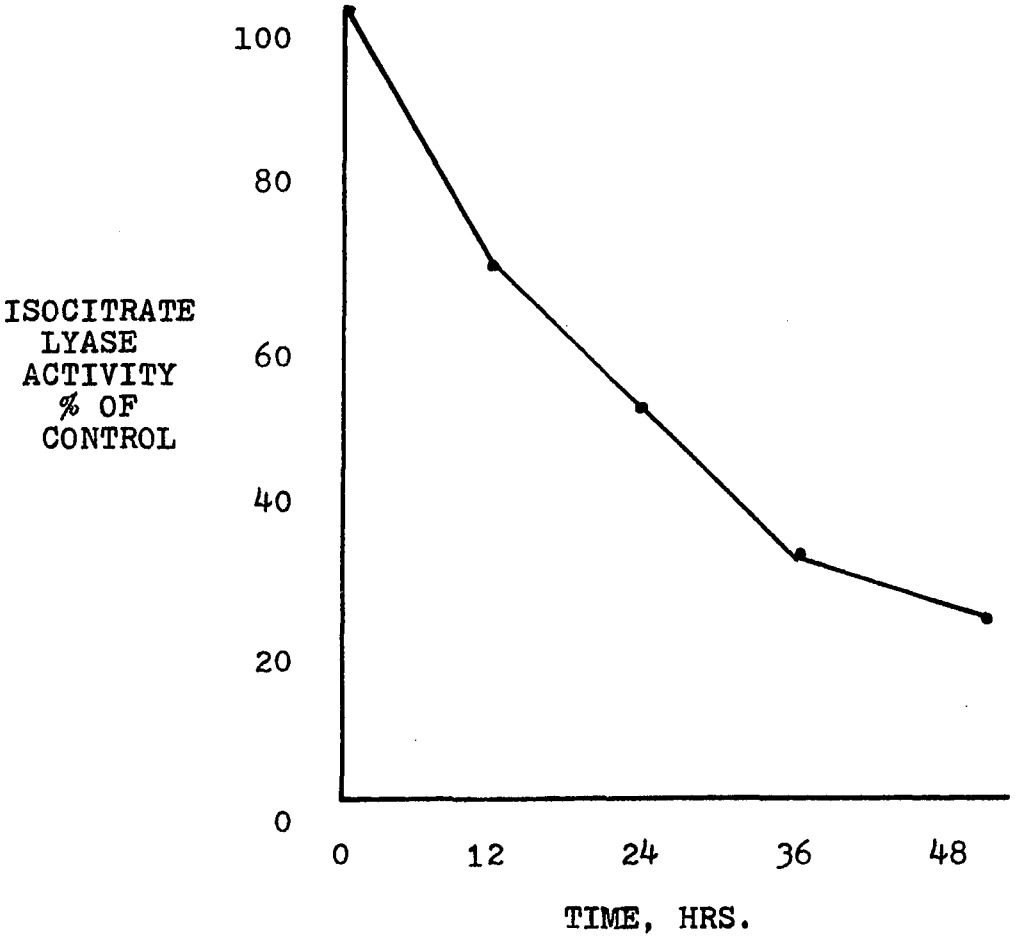
THE EFFECT OF VARIOUS CHELATING AGENTS ON THE STABILITY OF ISOCITRATE LYASE IN 50% GLYCEROL, pH 6.9 AT 5° C.



Glycerol extracts were prepared as described in Methods. The spectrophotometric assay was used to determine enzyme activity.

Figure 2

THE EFFECT OF MAGNESIUM IONS ON THE STABILITY OF ISOCITRATE LYASE IN 50% GLYCEROL, pH 6.9 PHOSPHATE AT 5° C.



For this experiment, one portion of a glycerol extract was made0.001 M in $MgCl_2$. See Methods for preparation of extracts. The activity of isocitrate lyase was determined spectrophotometrically. Initial rate is 0.116 ΔA/min/0.2 ml.

Figure 3

of course contain some Mg^{++} from the wash liquid as well as from the cells themselves.

Table 1 shows that 0.01 M potassium pyrophosphate, 0.01 M potassium fluoride and 0.01 M trisodium ATP are all excellent preservers of isocitrate lyase activity in buffered glycerol solutions. Since these compounds are all complexing agents for Mg^{++}, it appears likely that they are acting by removing this ion from solution. The fact that fluoride ions also protect the lyase from breakdown seems to indicate that the pyrophosphate and ATP are also preserving the lyase activity with their complexing ability rather than by a mechanism such as phosphorylation of the enzyme or an allosteric interaction with it.

Dithiothreitol at 0.01 M concentration had no effect on the stability of isocitrate lyase, as indicated in Figure 4. Other thiols such as cysteine, glutathione and dithioerythritol were also tested and gave no protection. Thus it appears that, although a very reactive thiol group is necessary for enzyme activity (Hogg, 1969), this group is not responsible for the extreme instability of the enzyme.

The optimal pH for stability of the enzyme appears to be approximately pH 6.0 from data given in Figure 5. Slightly alkaline solutions of the enzyme are extremely unstable and pyrophosphate has little stabilizing effect on the enzyme in this range. The data also shows that at pH's 5.0 through 7.0 pyrophosphate is an important stabilizer for isocitrate lyase in glycerol solutions.

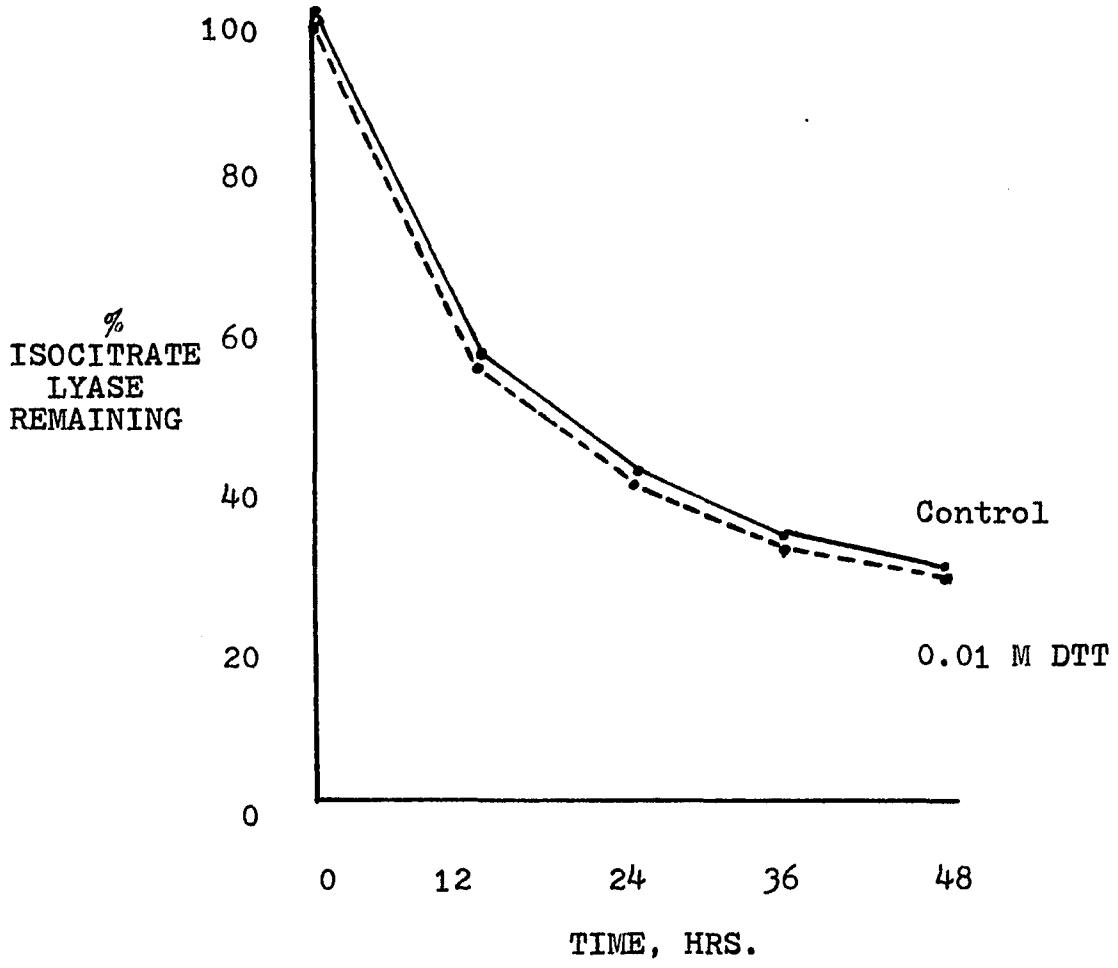
THE EFFECT OF SOME MAGNESIUM ION COMPLEXING AGENTS ON THE STABILITY OF ISOCITRATE LYASE IN 50% GLYCEROL, pH 6.9 PHOSPHATE AT 5° C.

EXPERIMENT	% OF ORIGINAL ISOCITRATE LYASE ACTIVITY REMAINING AFTER 48 HRS.
CONTROL	14%
0.01 M PYROPHOSPHATE	101%
0.01 M FLUORIDE	99%
0.01 M ATP	91%

Extracts containing glycerol were prepared as previously described. Isocitrate lyase activity was measured spectrophotometrically.

Table 1

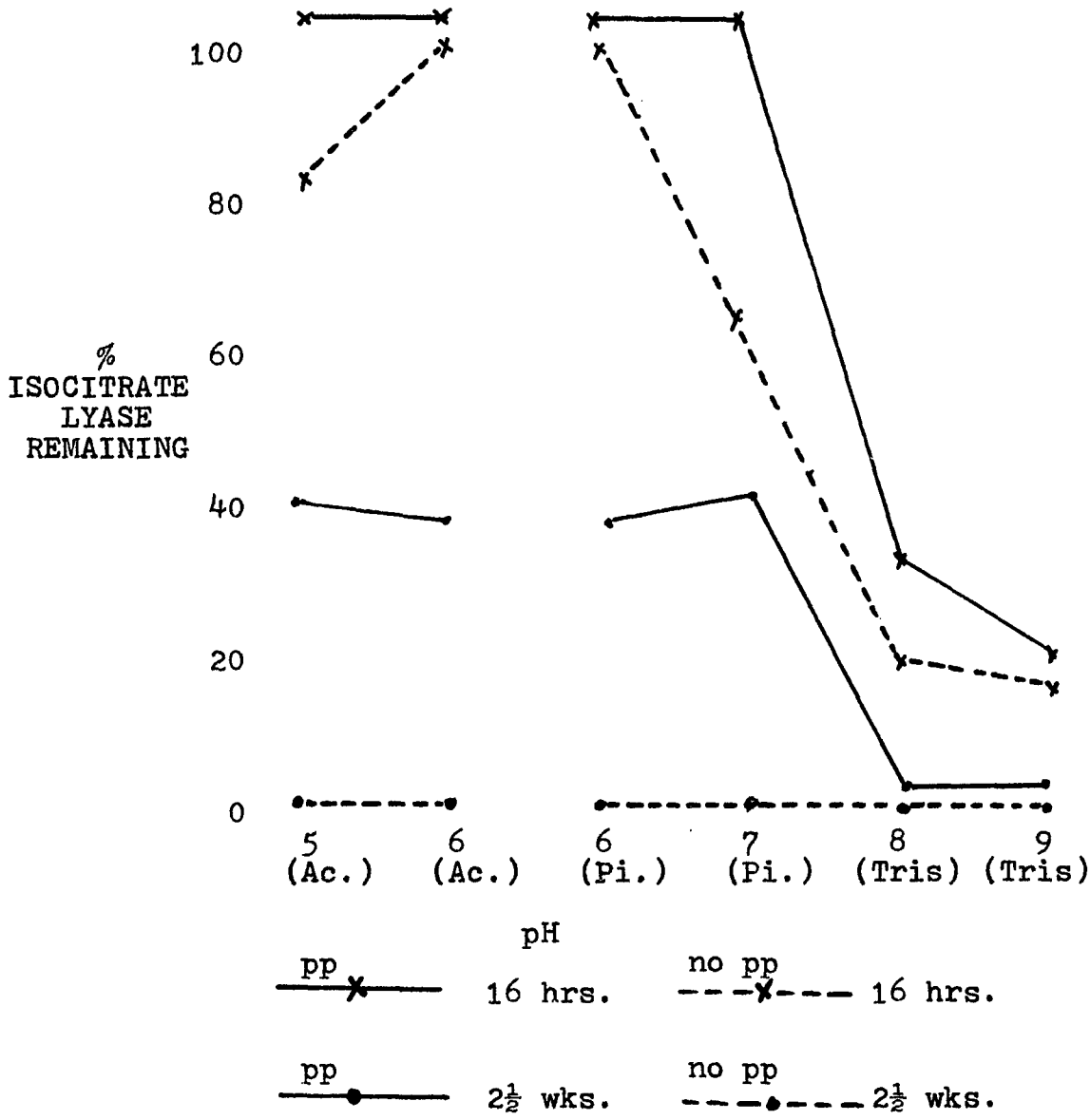
THE EFFECT OF DITHIOTHREITOL ON THE STABILITY OF ISOCITRATE LYASE IN 50% GLYCEROL, pH 6.9 PHOSPHATE AT 5° C.



See the Methods section for the description of the extract preparation and for the spectrophotometric assay for isocitrate lyase. Initial rate is 0.152 $\Delta A/mn/0.2$ ml.

Figure 4

THE EFFECT OF pH ON ISOCITRATE LYASE STABILITY IN 50% GLYCEROL WITH AND WITHOUT 0.01 M PYROPHOSPHATE AT 5° C.



The extracts were prepared as described previously. The buffers were 0.01 M concentrations. Enzyme assayed spectrophotometrically.

Figure 5

Table 2 shows the results from the partial purification procedures that were run at 0-5° C. An extract was made by homogenizing 12.5 mg of freeze-dried cells per ml of 50% v/v glycerol, 0.001 M potassium phosphate, 0.0008 M potassium malate, 0.0008 M potassium pyrophosphate buffer solution at pH 4.0 in a cold homogenizing tube and centrifuging the homogenate in a Sorvall RC-2 supercentrifuge with an SS-34 rotor for 20 minutes at 20,000 x g. The pellet contained no isocitrate lyase activity and was discarded. One half g CM-cellulose was added per 10 mls of the extract, after which the thick slurry was filtered over cheesecloth. The resulting solution was adjusted to pH 6.9 with 1.0 M K_2HPO_4. In the final step DEAE cellulose washed with 0.001 M potassium phosphate buffer pH 6.9 was added to the solution, 0.5 gms/ml, and filtered as before. The glycerol concentration was adjusted back to 50% v/v using the density graph in Figure 6. This was necessary since the celluloses were not previously washed with 50% v/v glycerol and the lyase still requires the high concentration of glycerol for stability. We routinely determined glycerol densities by using a 500 microliter pipet as a pycnometer. After adjusting the glycerol concentration, 1.0 M potassium pyrophosphate (adjusted to pH 6.0 with H_3PO_4) was added to bring the product to 0.01 M pyrophosphate before storing at -20° C. The final product has no detectable catalase, malate synthase or malate dehydrogenase activities but is heavily contaminated with NADP-linked isocitrate dehydrogenase (specific activity

PARTIAL PURIFICATION OF ISOCITRATE LYASE
IN 50% GLYCEROL

PURIFICATION STEP	SPECIFIC ACTIVITY	TOTAL UNITS OF ACTIVITY	TOTAL PROTEIN (MGS)	PERCENT RECOVERY
	micromol/hr/ mg protein			
EXTRACTION	6.0	185	30.8	100%
CM CELLULOSE	8.2	87	10.6	47%
DEAE CELLULOSE	53.5	79	1.5	43%

See the Methods section for details of the purification steps. The final specific activity is about a 90-fold purification over ordinary aqueous extracts. Initial volume is 25.0 ml. The assay medium contains: 66 mM potassium phosphate buffer pH 6.9, 5 mM magnesium chloride, 3.3 mM phenylhydrazine-HCl (recrystallized), 50 micromolar EDTA, and 3.3 mM threo-D_s-isocitrate. The increase in absorbance is measured at 324 nm after the addition of 3.3 mM threo-D_s-isocitrate.

Table 2

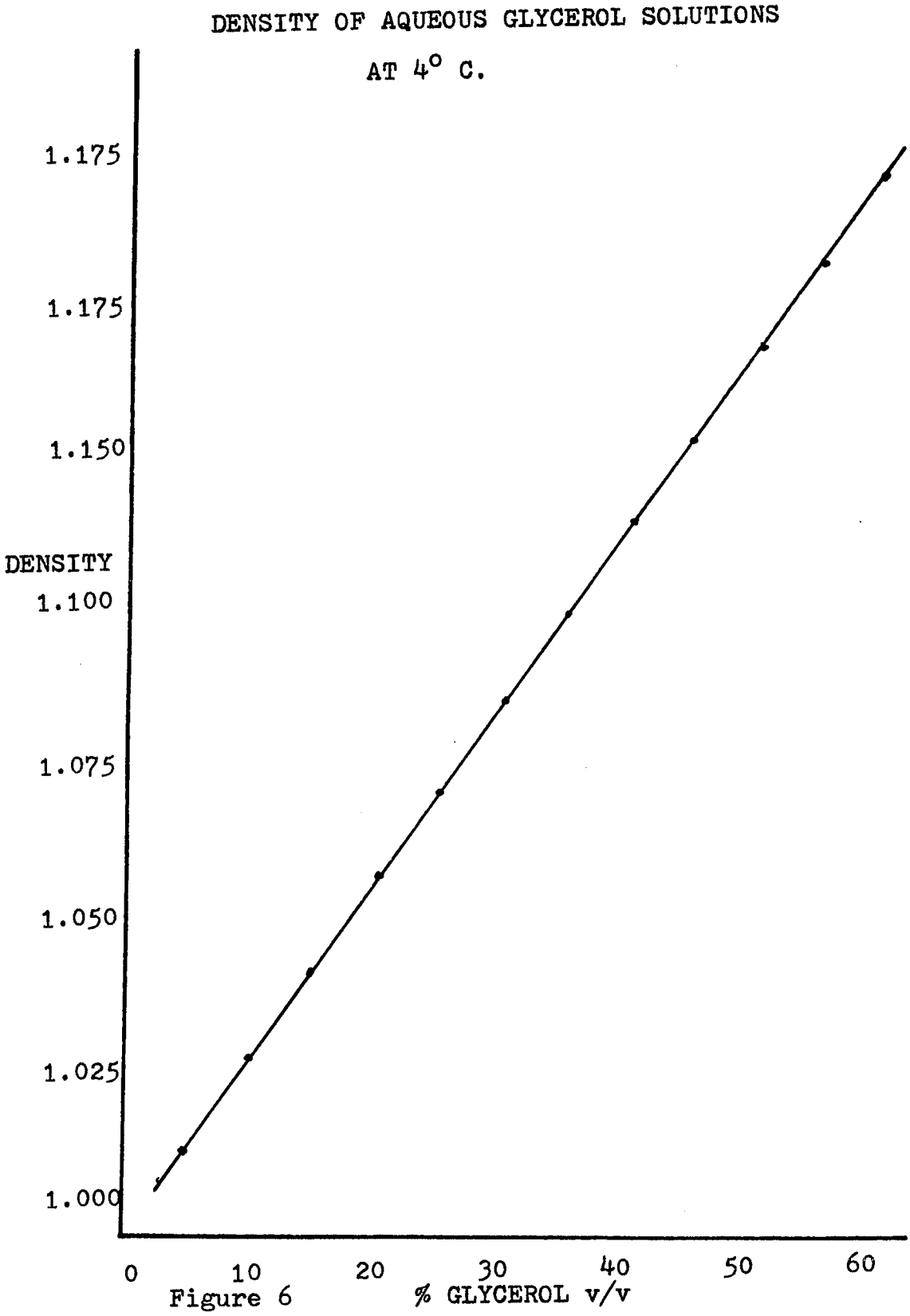

DENSITY OF AQUEOUS GLYCEROL SOLUTIONS
AT 4° C.
1.175
1.175
1.150
DENSITY
1.100
1.075
1.050
1.025
1.000
0
10
20
30
40
50
60
% GLYCEROL v/v

Figure 6

1.3 micromol/min/mg prot.)

The activity vs temperature plot is given for the purified isocitrate lyase in Figure 7. The data indicates a temperature optimum of 46° C for enzyme assay which does not change whether the enzyme is isolated from log phase or stationary phase cells.

The following table 3 shows a modest inhibition of purified isocitrate lyase by two relatively high concentrations of phosphoenolpyruvate (PEP). We have tested crude glycerol extracts of isocitrate lyase and found that they are inhibited as much as 50% at 5.0 millimolar PEP concentrations. The colorimetric assay (McFadden and Howes, 1960) was used for studying the effects of PEP on isocitrate lyase because the assay mixture is less complex and allows variation of pH.

DISCUSSION

Isocitrate lyase from T. pyriformis is much less sensitive to phosphoenolpyruvate than previously described by Kornberg et al. (1960), Ashworth and Kornberg (1963) and Syrett and John (1968) for other organisms. The fact that purified samples of the Tetrahymena enzyme are only inhibited 33% at 0.005 M PEP concentration, pH 7.5 and 50% at 0.005 M PEP at pH 6.9, makes PEP a poor candidate for metabolic regulation of isocitrate lyase in Tetrahymena. The physiological concentration of PEP will probably never reach such high concentration at the peroxisome where isocitrate

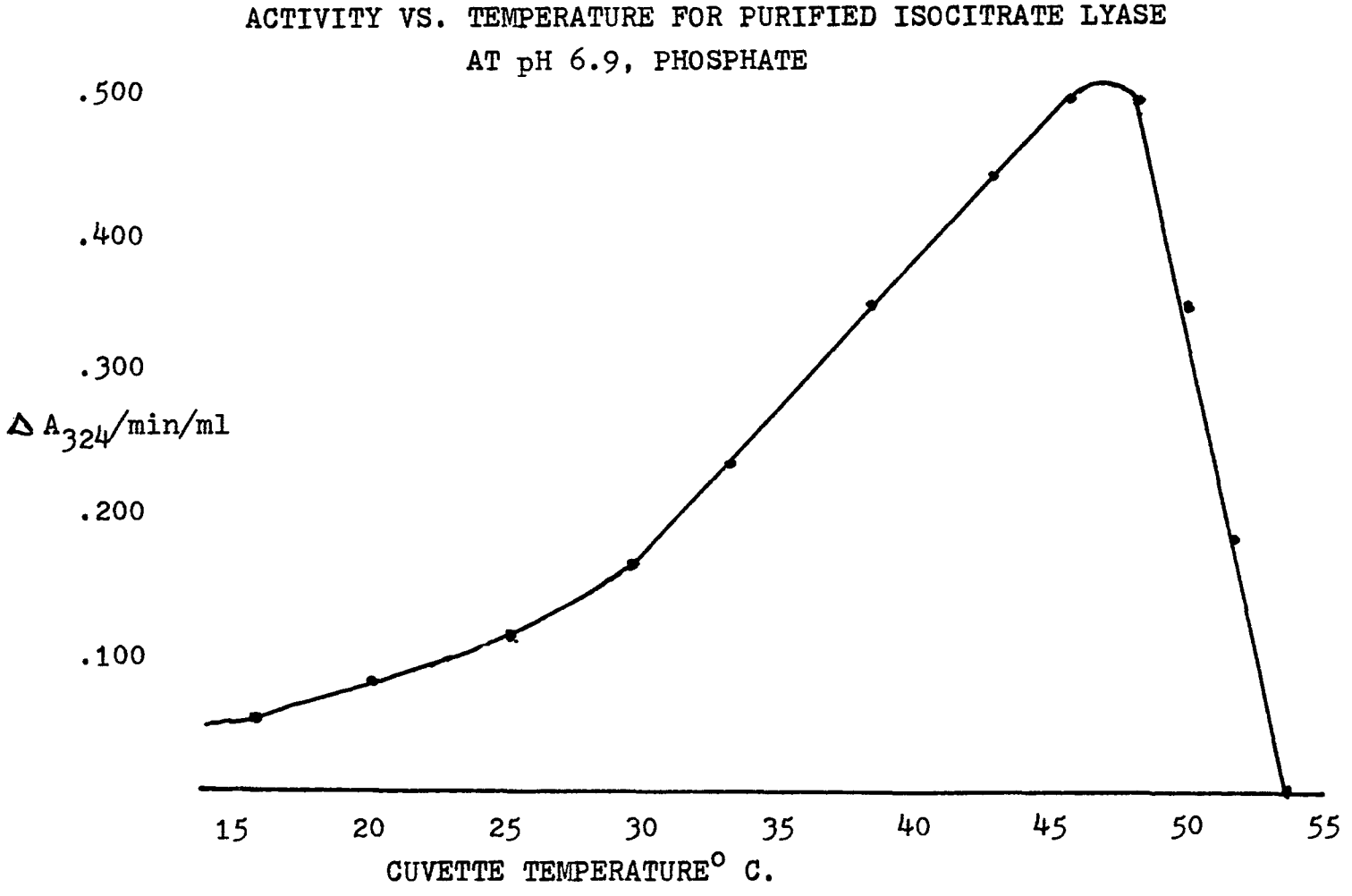


Figure 7 The temperature given is the assay solution temperature at the end of assay as determined with a thermister probe.

THE INHIBITION OF PURIFIED ISOCITRATE LYASE
BY PHOSPHOENOLPYRUVATE AT pH 7.5

PEP CONCENTRATION	% INHIBITION OF T. PYRIFORMIS ISOCITRATE LYASE
1.5mM	12.2%
5.0mM	32.2%

The colorimetric assay was used to determine the isocitrate lyase activity in these experiments. All assays were determined by color absorbance between 0.100 and 0.400 absorbance units using purified isocitrate lyase of specific activity 35-40 micromoles/hr/mg protein.

Table 3

lyase is located (Muller et al., 1968) because the PEP would have to survive the relatively high concentration of pyruvate kinase in the cytosol, Warnock and van Eys (1962), Dorsey and Hogg (a). PEP is a strong inhibitor of isocitrate lyase in Eschericia coli, a microorganism devoid of the organellar structure present in Tetrahymena pyriformis; perhaps the regulation of isocitrate lyase and thus of the glyoxylate cycle is at the enzyme level in E. coli and at the organelle level in Tetrahymena.

Based on stability studies, the isocitrate lyase isolated from Pseudomonas indigofera seems to be different from that isolated from Tetrahymena since the former is reported to be stabilized by $MgCl_2$, EDTA and β-mercaptoethanol whereas the latter is destabilized by $MgCl_2$ and its stability unaffected by EDTA and β-mercaptoethanol. Of course, if the instability of one or both of the enzymes is due to proteolytic breakdown rather than chemical instability of the enzyme the isocitrate lyase from the two sources could still be similar. The Km values for the two enzymes are quite similar; 1.0×10^{-4} for the enzyme from Tetrahymena and $.82 \times 10^{-4}$ for the enzyme from Pseudomonas.

Although the purification of isocitrate lyase presented in the paper provides only an overall 10 fold purification, the final specific activity is a 90 fold purification over ordinary aqueous extracts. The reason for this is that the 50% glycerol extraction is selective and provides a 10-fold purification over aqueous extracts. The specific activity

of isocitrate lyase from aqueous extract is 0.60 micromoles glyoxylate/hr/mg protein as given by Hogg and Kornberg (1963) using the same assay as the present paper which gives a specific activity of 6.0 micromoles glyoxylate/hr/mg protein in crude 50% glycerol extracts. The overall recovery of the purified enzyme is good and highly reproducible. Since the first extraction is virtually complete we are obtaining a purified sample which is representative of the total enzyme present in the protozoan.

There are several common purification procedures which are not suitable for use in 50% glycerol such as solvent precipitation and $(NH_4)_2SO_4$ precipitation. Using column chromatography is quite difficult in 50% glycerol and there is no advantage in the degree of purity achieved over the bath procedure used in our method of purification. We can easily and quickly perform this purification in only a few hours and the 90-fold purification over aqueous extrasts compares well with the 80-fold purification recently published by Johanson et al. (1974).

The question of why isocitrate lyase from T. pyriformis is so unstable is still unanswered but we have shown the conditions for maintaining stability in solution are 50% glycerol 0.01 M K^+pyrophosphate between pH's 5-7 at -20° C. it is interesting to note that the enzyme still requires the glycerol and pyrophosphate even after the 90-fold purification.

A MORE SENSITIVE NON-ENZYMATICALLY COUPLED ASSAY FOR PYRUVATE KINASE

The very elegant assay procedure of Dixon and Kornberg (1959) for isocitrate lyase (EC 4.1.3.1) which depends upon measuring glyoxylate production as the phenylhydrazone may be adapted for the assay of pyruvate kinase (EC 2.7.1.40) by substituting phosphoenolpyruvate (PEP) and adenosine diphosphate (ADP) for isocitrate. Also cysteine was omitted, as indeed it has been done in subsequent modifications of the lyase assay. This assay procedure is superior to the LDH coupled assay proposed by McQuate and Utter (1959) in that it is a non-enzymatically coupled assay and because it is four times as sensitive. A disadvantage of the assay is that it can not be used if high amounts of phosphoenolpyruvate carboxykinase or other enzyme is present to yield a product that readily forms a phenylhydrazone.

The pyruvate kinase activity is measured by the increase in absorbance at 324 nm due to the formation of the pyruvate phenylhydrazone. The assay is determined in 66 mM potassium phosphate pH 6.9, 5.0 mM $MgCl_2$, 6.6 mM phenylhydrazine HCl, 1.6 mM potassium phosphoenolpyruvate and 0.0125 -0.500 ml enzyme solution in a three milliliter cuvette with a one centimeter light path. The reaction is started by the addition of 3.3 mM ADP. Table I shows the assay requirements. The absorbance change for one micromole pyruvate in this assay (total volume of 3.00 milli-

PHENYLHYDRAZINE ASSAY FOR PYRUVATE KINASE

ASSAY SYSTEM	ΔA_{324}/min/0.025 ml*
COMPLETE	0.277
MINUS MAGNESIUM IONS	0.004
MINUS ADP	0.004
MINUS PEP	0.000
MINUS PHENYLHYDRAZINE	0.000

*Freeze-dried cell powder from T. pyriformis was extracted by addition of cold 0.01 M potassium phosphate buffer pH 6.9 and grinding in a Brendler homogenizer. The homogenate was centrifuged at 10,000 x g for 10 minutes to form a soluble extract. Extracts contain approximately 6.9 mg/ protein/ml.

Table 1

liters) is 3.00.

The assay procedures were checked on extracts of lyophilized cells of Tetrahymena pyriformis harvested from 2 or 4 day cultures grown in proteose-peptone media with and without glucose and acetate as indicated (Table II). The specific activities average 28.8 micromoles/hr/mg protein which is approximately three times the value given by Warnock and van Eys (1962) who used the LDH coupled assay. To assess the effect of CO_2 requiring enzymes such as PEP-carboxykinase (EC 4.1.1.32) which also react with PEP to give a product that forms a phenylhydrazone, the extracts were assayed after saturating all solutions except enzyme extract with N_2, CO_2 or O_2 in an anerobic cuvette (Table III). The reactions in N_2 and O_2 were allowed to proceed sufficiently to use up the CO_2 that might be present in the enzyme extracts. The activities agree well with the control: 95% of control for assays run under N_2; 93% for O_2 and 88% for CO_2. No increase in rate was noticed. However, the slightly lower activity might be due to an inhibitor present in the N_2 and O_2. The lower rate with CO_2 is due to a shift in pH.

Lineweaver-Burk plots for pyruvate kinase at 30° C. using the phenylhydrazine assay agree with those obtained with the coupled lactic dehydrogenase assay (Shrago, 1972). The K_m is 5.0×10^{-4} M for ADP (figure 1) and 1×10^{-4} M for PEP (Figure 2). The Lineweaver-Burk plot for PEP is as it is with the enzyme-coupled assay; non-linear at concen-

SPECIFIC ACTIVITY OF PYRUVATE KINASE IN *TETRAHYMENA PYRIFORMIS* UNDER VARYING GROWTH CONDITIONS USING THE PHENYLHYDRAZINE ASSAY

GROWTH CONDITIONS	SPECIFIC ACTIVITY micromoles/hr/mg protein
PPGA* 1 day	27.3
PPGA* 4 days	32.4
PPGA* 6 days	29.4
PPA* 2 days	26.4

*The stock culture medium (PPGA) contains: proteose-peptone (Difco), 1.0%; D-glucose, 0.1%; sodium acetate, 0.1%; K_2HPO_4, 0.1% and yeast extract, 0.01%. The cultures were grown at 25° C. in 500 ml lots in 2 liter penicillin flasks. They were inoculated with 5.0 ml of an actively growing stock culture and incubated without shaking. The extracts were prepared as in Table 1.

Table 2

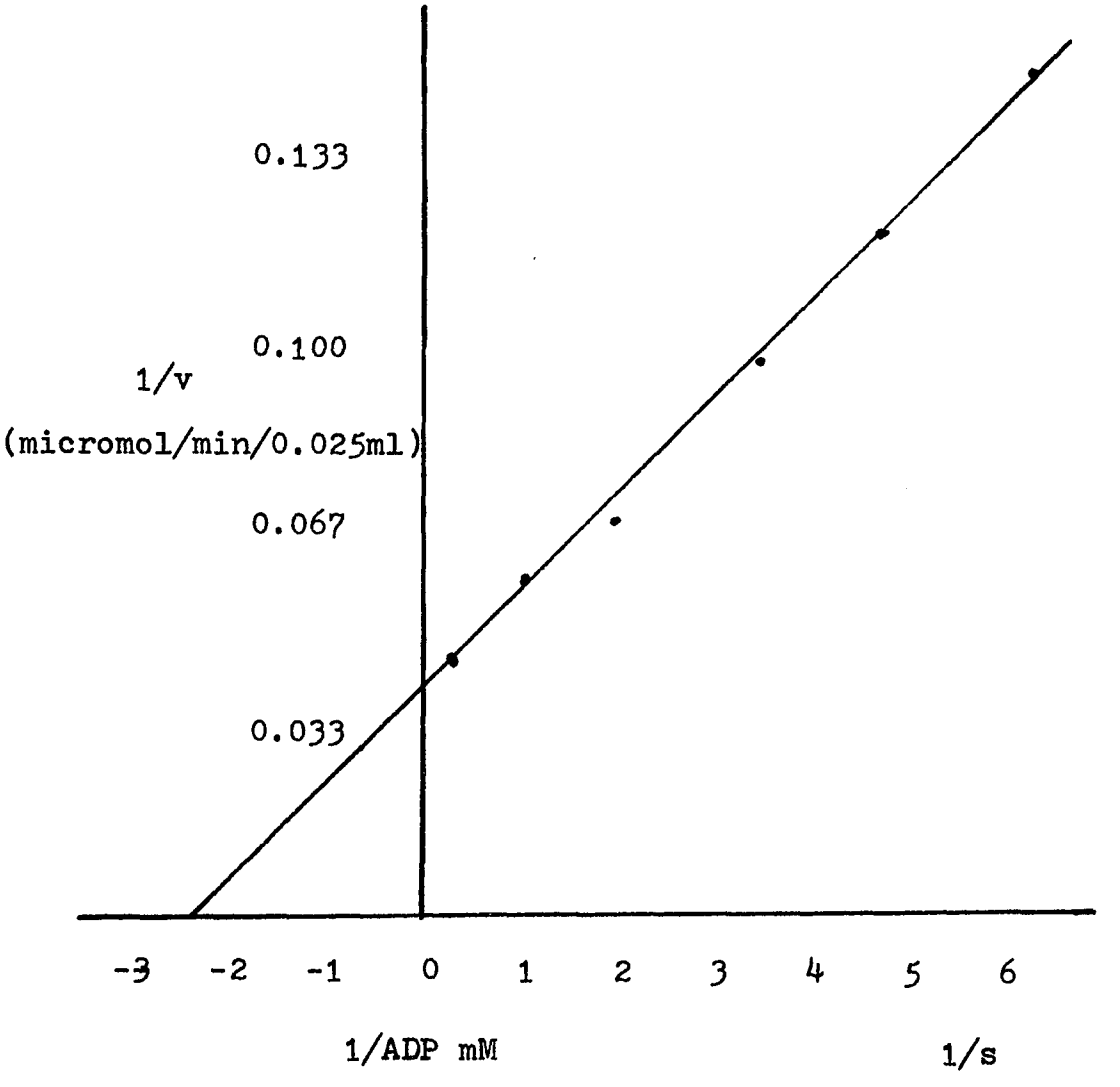
PHENYLHYDRAZINE PYRUVATE KINASE ASSAY

T. PYRIFORMIS EXTRACTS ASSAYED IN	% WITH RESPECT TO AIR
AIR	100%
O_2	95%
N_2	93%
CO_2	88%

Extracts were prepared as described under Table 1.

Table 3

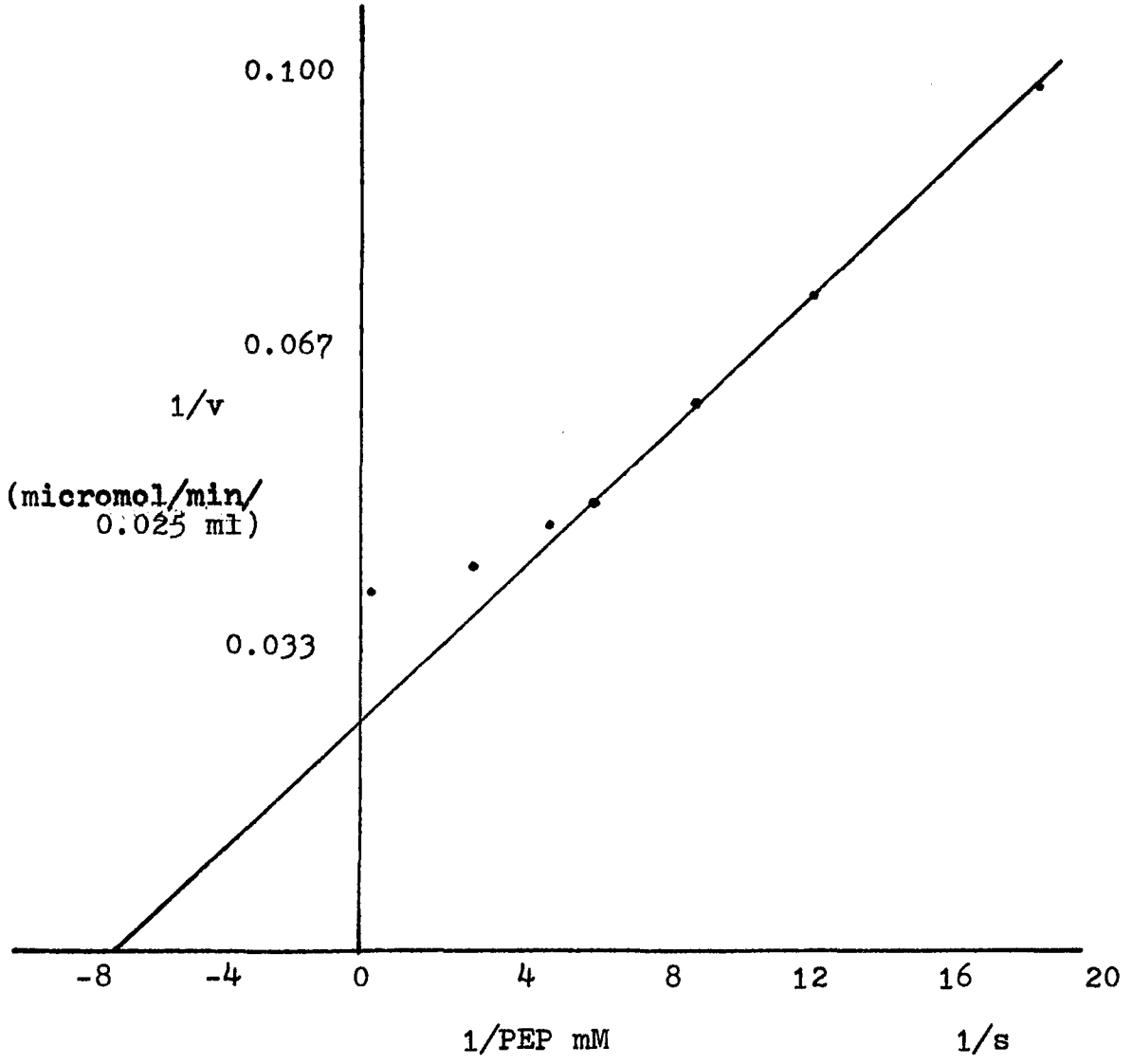
LINEWEAVER-BURK PLOT FOR PYRUVATE KINASE AT pH 6.9
PHOSPHATE ADP VARIED USING PHENYLHYDRAZINE ASSAY
AT 30^0 C.



Extracts were made as described in Table 1. The K_m for ADP is 5.0×10^{-4}.

Figure 1

LINEWEAVER-BURK PLOT FOR PYRUVATE KINASE AT pH 6.9
PHOSPHATE AND PEP VARIED USING PHENYLHYDRAZINE ASSAY
AT 30° C.



Extracts were made as described in Table 1. The K_m for PEP is 1.0×10^{-4}.

Figure 2

trations above 2 x 10^{-4} M, because of substrate inhibition.

Using zonal sucrose density gradient separation techniques for the organelles in Tetrahymena as described by Muller et al. (1968), the distribution of the pyruvate kinase was determined with the phenylhydrazine assay and found to be in the soluble fraction, thus substanting the work of Shrago et al. (1972).

THE INTRAORGANELLE DISTRIBUTION OF THE GLYOXYLATE BYPASS ENZYMES AND NADP-LINKED ISOCITRATE DEHYDROGENASE IN THE PEROXISOMES OF TETRAHYMENA PYRIFORMIS*

SUMMARY

Differential sedimentation of sonically treated peroxisomes shows that isocitrate dehydrogenase and malate synthase are located in the peroxisol[1] and that isocitrate lyase is bound to the peroxisomal membrane in Tetrahymena pyriformis. Isocitrate lyase activity decreases when the peroxisomes are treated with Triton X-100, Tween 40 and 80, sonication and phospholipase A. Because these disruptive agents have no effect on the activity of the soluble form of isocitrate lyase, the data implies that membrane-binding confers higher activity upon peroxisomal isocitrate lyase. Explanations are given as to how membrane-binding can confer activity upon isocitrate lyase. Isocitrate dehydrogenase, malate synthase and catalase activities increase 2-3 fold upon addition of Triton X-100 to, or upon sonication of partially purified peroxisomes. This latency is probably due to limitation of substrate access by the peroxisomal membrane.

*The data given in this paper were presented in part at the sixty-fifth annual meeting of the American Society of Biological Chemists in June, 1974.

[1]The peroxisol is the membrane enclosed portion of the peroxisome.

INTRODUCTION

Our interest in membranes and in organelles derives from a desire to find the mechanism controlling the glyoxylate cycle and, since the cycle participates in glyconeogenesis, the mechanisms for controlling gluconeogenesis. Hogg and Kornberg in 1963 showed that incorporation of the glyoxylate cycle enzymes into a discrete particle appeared to be necessary for glyconeogenesis to occur in Tetrahymena. They proposed that the new large granule was a special type of mitochondrion. In 1968 Muller, Hogg and de Duve using more sophisticated isolation procedures showed that only the bypass enzymes of the glyoxylate cycle were contained in a unique new organelle, the peroxisome. Because of the division of the glyoxylate cycle enzymes between two organelles, the necessity of a number of membrane transport steps for a functioning glyoxylate cycle became clear. We decided to study the point at which isocitrate can either enter the glyoxylate cycle via isocitrate lyase or, through isocitrate dehydrogenase by oxidatively decarboxylated to α-ketoglutarate and thus enter the TCA cycle. One mode of giving advantage to either the anabolic or catabolic pathways would involve the intraorganelle distribution of the enzymes. Thus, if one of the enzymes is membrane-bound (Hogg and Kornberg, 1963) and the other within the inner portion of the peroxisome (which we would like to call the peroxisol), the membrane-bound enzyme should have the advantage of prior access to the isocitrate. Results presented in this paper

support such an intraperoxisomal distribution. A subsequent report (Dorsey and Hogg, b) embodies kinetic studies which suggest that membrane binding greatly favors isocitrate lyase action under simulated physiological conditions.

METHODS AND MATERIALS

Tetrahymena pyriformis cultures were maintained and grown as described by Wu and Hogg (1952). The medium contains 10.0 gm proteose peptone (Difco), 1.0 gm sodium acetate, 1.0 gm glucose, 1.0 gm K_2HPO_4 and 0.1 gm yeast extract per liter , pH 7.2. The sterile medium was inoculated with a 1% volume of a growing culture of T. pyriformis, strain E which was allowed to multiply and reach the stationary phase (4-7 days) at 25° C. before harvesting.

The cells were harvested and washed in a modified plankton centrifuge (Conner et al. 1966), using a volume of Ryley's Ringer phosphate solution (Ryley, 1952) equal to the medium volume, followed by glass distilled water equal to 1/4 of the medium volume. The washed cell suspension was adjusted to 10% v/v with distilled water using a Constable protein tube for determining cell density of the suspension. The Constable tubes containing samples were centrifuged at 1,000 x g for 10 minutes in a swinging bucket rotor to pack the cells into the graduated tip. After adjusting the cell suspension density, the cells were either lyophilized in an all glass Kontes lyophilizer or broken by filtration to obtain homogenates for the separation of peroxisomes (Hogg

and Kornberg, 1963).

The cell suspension to be homogenized was added to an equal volume of cold 0.5 M mannitol or sucrose and immediately passed through a (British no. 3 pore size, 20-30 micron) fritted glass filter by suction, making sure that no foaming occurred. The homogenate was examined under a phase contrast microscope (470X) for assurance of complete cell breakage. Occasionally a second passage through the filter was necessary for complete breakage of cells.

The peroxisomal fraction was obtained according to the procedure of Hogg and Kornberg, 1963, except that 0.25 M sucrose was sometimes substituted for 0.25 M mannitol. If the volume of cold 0.25 M mannitol or sucrose used to suspend the peroxisomes was kept to a minimum (about 1/5 of homogenate volume) no loss in activity was detected for the enzymes studied so long as the fraction was kept on ice.

Extracts of lyophilized cells for comparison with the peroxisomal fractions were made in either 0.25 M mannitol or 0.25 M sucrose by suspending 25 mgs of lyophilized cells per ml of the appropriate sugar solution and grinding for 1 minute at 1,000 rpm in a precooled Brendler homogenizer, followed by centrifuging at 12,000 x g for 15 minutes at 5^{o} C in a Sorvall RC-2 centrifuge with an SS-34 Rotor.

The following assay methods were used except that the suspending medium was either 0.25 M sucrose or mannitol, as in the isolation medium in order to preserve the integrity of the organelles during the assay: isocitrate lyase and

(slightly modified version) and malate synthase, Dixon and Kornberg (1959); catalase, Luck (1963); and NADP-linked isocitrate dehydrogenase (slightly modified), Kornberg (1955). The enzyme assays were performed in a Gilford model 2400 spectrophotometer equipped with a Lauda K-2/R circulating bath to maintain cuvette temperature at 30° C. See Appendix for complete details.

A stock culture of Tetrahymena pyriformis strain E was obtained from A. M. Elliott, Department of Zoology, University of Michigan. Ultra-pure density gradient sucrose was purchased from Mann Chemical Co.. Potassium threo-D_s-isocitrate was generously donated by Dr. H. B. Vickery. Acetyl-CoA was prepared for coenzyme A (sigma) by the procedure of Stadtman (1957). Other biochemicals including bee vernom phospholipase A were brought from Sigma Chemical Co.. All inorganic chemicals were Baker analyzed grade purchased from the J. T. Baker Chemical Co..

RESULTS

After isolating the peroxisomal fraction in 0.25 M sucrose, it was gently sonicated at the lowest setting of the Branson Sonifier model W140D for a total of 70 seconds in 10 second intervals, with two minute intermediate cooling periods on ice. The sonication was meant to disrupt the membrane without solubilizing it and thus to release the peroxisol enzymes. After assaying the sonic homogenate, it was centrifuged at 30,000 x g for 30 minutes and the supernate

assayed. The first table shows the fraction of the different enzymes remaining in the supernatant solution. The data show an order of magnitude difference between isocitrate lyase and isocitrate dehydrogenase and malate synthase, the isocitrate lyase having sedimented with the membranes. All enzymes yielded 100% of the homogenate activity after resuspending the pellet. These data are consistent with the concept that isocitrate lyase is membrane-bound and isocitrate dehydrogenase and malate synthase are membrane-enclosed.

Table 2 shows the effect of Triton X-100 on the measurable activity of some peroxisomal enzymes when the detergent is added to intact peroxisomes. Catalase, malate synthase and isocitrate dehydrogenase all show 2 to 3 fold increase, thus indicating that their activity is limited by a membrane, i.e., latent enzymes. Isocitrate lyase, however, shows a reduction in activity. The Triton was added to the peroxisomes in the enzyme assay mixture and the blank was followed for 5 minutes before adding substrate to start the reaction.

Table 3 shows a similar reduction in the lyase activity using other detergents. The fact that there is no significant loss in activity at a 10-fold higher concentration of detergent argues that the original reduction in activity is not a direct chemical inhibition. This is confirmed by the addition of these same detergents to soluble extracts of isocitrate lyase (Table IV). There is no reduction in act-

FRACTION OF ISOCITRATE DEHYDROGENASE AND GLYOXYLATE BYPASS ENZYMES REMAINING IN THE SUPERNATE AFTER 30,000 x g CENTRIFUGATION OF SONICALLY TREATED PEROXISOMES

ENZYME	% OF TOTAL ACTIVITY OF SONIC HOMOGENATE REMAINING IN 30,000 x g SUPERNATE
ISOCITRATE LYASE	8%
ISOCITRATE DEHYDROGENASE	75%
MALATE SYNTHASE	66%

Peroxisomes were prepared as described in Methods. Spectrophotometric assays were performed in 0.25 M mannitol to protect the organelles from osmotic shock.

Table 1

THE EFFECT OF 0.1% TRITON X 100 ON THE ACTIVITY OF SOME PEROXISOMAL ENZYMES

ENZYME	$\frac{\text{ACTIVITY WITH TRITON}}{\text{ACTIVITY WITHOUT TRITON}} \times 100\%$
ISOCITRATE DEHYDROGENASE	297%
MALATE SYNTHASE	200%
CATALASE	231%
ISOCITRATE LYASE	70%

Peroxisomes were prepared as described in Methods from cells grown to stationary phase on 1.0% proteose-peptone, 0.1% glucose and 0.1% acetate. Spectrophotometric assays were used throughout.

Table 2

THE EFFECT OF DETERGENTS ON THE ISOCITRATE LYASE ACTIVITY OF PEROXISOMES

DETERGENT	CONCENTRATION	% REDUCTION OF ISOCITRATE LYASE ACTIVITY
TRITON X 100	0.1%	30%
	1.0%	33%
TWEEN 40	0.1%	43%
	1.0%	42%
TWEEN 80	0.1%	55%
	1.0%	64%

Peroxisomes were prepared as described in Methods. The detergent was allowed to react with the organelles for 5 minutes before the enzyme was assayed. Fractions contain 0.46 mg protein/ 0.2 ml.

Table 3

THE EFFECT OF DETERGENTS ON THE ISOCITRATE LYASE ACTIVITY IN EXTRACTS

DETERGENT	CONCENTRATION	% REDUCTION OF ISOCITRATE LYASE ACTIVITY
TRITON X 100	1.0%	0%
TWEEN 40	1.0%	0%
TWEEN 80	1.0%	7.0%

Extracts were prepared in glycerol as described in Methods. The detergent was allowed to react with the extracts for 5 minutes before assaying isocitrate lyase spectrophotometrically.

Table 4

ivity with Triton X-100 and Tween 40 and only a very slight reduction with Tween 80.

Another method of disrupting the peroxisomes also shows a similar reduction in activity for isocitrate lyase and latency for the other exzymes (Table V). Very mild sonication greatly increases the activity of isocitrate dehydrogenase, malate synthase and catalase but lowers the activity of isocitrate lyase to approximately one-half. Figure 1 compares the effects of sonication on the extracted isocitrate lyase with the membrane-bound enzyme. Activity of the membrane-bound enzyme falls to approximately one-half in 70 seconds and then levels off. In contrast, the same sonication treatment shows virtually no effect on the activity of the soluble isocitrate lyase.

A similar reduction in the activity of isocitrate lyase occurs when the peroxisomes are subjected to phospholipase A action for 20 minutes. Table 6 shows a 50% loss of activity in membrane-bound isocitrate lyase activity when peroxisomes are incubated with phospolipase A but practically no change in the control containing soluble enzyme. The incubation medium is exactly the same for both experiment and control except for the addition of phospholipase A.

DISCUSSION

The differential sedimentation of sonically treated peroxisomes performed in this study strongly supports the concept that isocitrate lyase is membrane bound in the per-

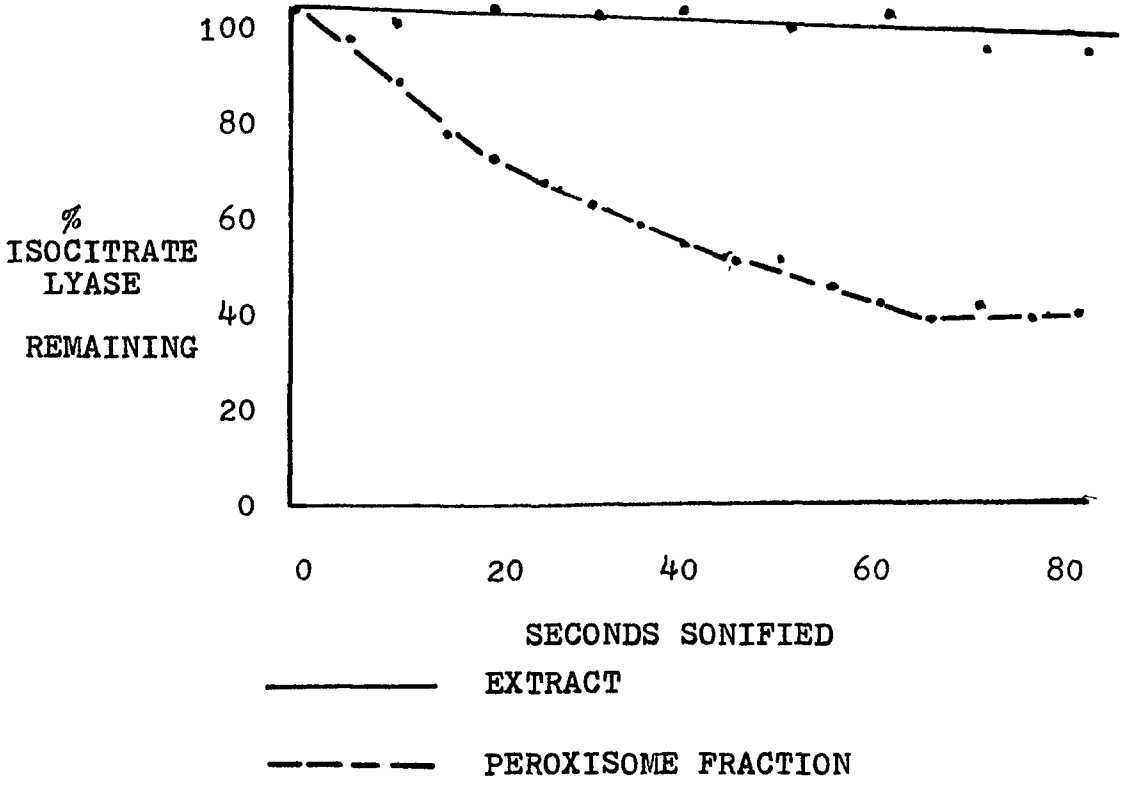
THE ACTIVITY OF SOME PEROXISOMAL ENZYMES AFTER A 70 SECOND ULTRASONIC TREATMENT

ENZYME	% OF CONTROL NOT SONICATED
ISOCITRATE DEHYDROGENASE	160%
MALATE SYNTHASE	190%
CATALASE	180%
ISOCITRATE LYASE	40%

See Methods section for preparation of peroxisomes and for details of sonic treatment. The enzymes were assayed spectrophotometrically. Peroxisomal fractions contain between 2.0-6.0 mg protein/ml.

Table 5

THE EFFECT OF SONICATION ON THE ISOCITRATE LYASE ACTIVITY OF THE PEROXISOME COMPARED TO THE EXTRACTED ENZYME BOTH IN 0.25 M SUCROSE



Peroxisomes were prepared in 0.25 M sucrose as described previously. The sonic probe and peroxisome suspension were cooled on ice for 2 minutes after each 5 second sonic treatment. The extract was prepared in the usual manner but in 0.25 M sucrose. Peroxisomal fractions contain between 2.0-6.0 mg protein/ml.

Figure 1

THE EFFECT OF PHOSPHOLIPASE A ON THE ISOCITRATE LYASE ACTIVITY OF PEROXISOMES

EXPERIMENT	% ISOCITRATE LYASE ACTIVITY REMAINING
WITHOUT PHOSPHOLIPASE A	94%
WITH PHOSPHOLIPASE A	50%

The phospholipase A incubation was run at 30° C. for 20 minutes and contained: 0.2 mgs phospholipase A, 1.2 mgs peroxisomal protein in 1.4 mls of 0.01 M "Tris" - HCl, pH 8.4 that was 0.001 M in Ca^{++}; 0.0001M in EDTA; 0.01% albumin and 0.25 M in sucrose. The peroxisomes were prepared as described in Methods. Isocitrate lyase activity was measured spectrophotometrically.

Table 6

oxisomes of T. pyriformis. The vast difference in the release of isocitrate lyase vs isocitrate dehydrogenase and malate synthase upon sonication, when all were originally particle bound, would be difficult to explain if isocitrate lyase were not membrane-bound. After sonication and sedimentation, isocitrate dehydrogenase and malate synthase are about equally present in the supernatant, 75% and 66% respectively, thus indicating that the two enzymes are from the peroxisol. The detergent and phospholipase A data for isocitrate lyase confirm that the lyase is membrane-bound. There is never any latency indicated for isocitrate lyase with any of the disruptive agents tried which would be very unusual if the lyase were enclosed by a membrane and its substrate had to be transported across the hydrophobic membrane barrier. In contrast, isocitrate dehydrogenase, malate synthase and catalase all show latency when peroxisomes are disrupted by sonication or detergent under the same conditions.

The question arises as to why the membrane-bound isocitrate lyase activity should decrease by approximately one-half by sonication, detergents and phospholipase A. There are several possibilities: one is that one-half of the enzyme is denatured in each case; a second is that there is a change in the tertiary sturcture of the protein that reduces the activity of all of the enzyme molecules to about one-half of their original activity; the third possibility is that there is a disruption of quaternary structure i.e. that the enzyme

exists in a more active multisubunit form in the membrane and that this form is partially or completely disrupted by the various perturbations employed. Conceivably, more than one of these possibilities can be responsible for the observed decrease in isocitrate lyase activity. The remarkable similarity of the magnitude of loss of activity from various treatments (freezing-thawing, deoxycholate and acetone extraction at -10^{0} C gave like results, Hogg, personal communication), coupled with their lack of effect on the soluble form of the enzyme would appear to exclude the possibility of denaturation and even perhaps alteration of tertiary structure. The results presented in this paper and in a subsequent one do support the concept of isocitrate lyase existing in an aggregated form in the peroxisomal membrane.

In 1963, Tomkins and Yielding showed that when steroids or detergents were added to a purified mitochondrial enzyme, glutamate dehydrogenase (GDH), the enzyme lost activity. They also showed that this loss of GDH activity coincided with dissociation of the enzyme into subunits, of approximately one-fourth the molecular weight of the original enzyme molecule. They ascribed this dissociation to a disruption of critical hydrophobic bonds in the enzyme aggregate by the steroids or detergents. Conversely, the alanine dehydrogenase activity of GDH was stimulated by the disaggregation of the enzyme subunits. Datta et al. (1964) showed, however, that homoserine dehydrogenase is aggregated

by threonine to an inactive form of the enzyme. In contrast, both activation and aggregation of acetyl-CoA carboxylase occurs by addition of citrate as shown by Vagelos et al. (1963). Thus disaggregation of subunits can either inhibit or stimulate protein catalytic activity. Conversely, association can do the same.

The possibility still exists that isocitrate lyase is first of all structurally rearranged when it is incorporated into the hydrophobic environment of the peroxisomal membrane and second, that the altered isocitrate lyase in the hydrophobic phase forms enzyme aggregates that have greater enzyme activity. It is conceivable that the enzyme aggregates might be in the form of "pores" as described by Singer (1974). These "pores" would be more susceptible to disruption which could explain why isocitrate lyase activity can be lowered by sonication while still remaining membrane-bound. The data obtained during this and a subsequent study seems to require a change in conformation with the membrane binding of isocitrate lyase.

We observe a lowering of activity for isocitrate lyase when the enzyme in the peroxisome is disrupted by the most gentle procedures. The lowest possible setting of the instrument was used during sonication. Although the enzyme was not removed from the membrane, its activity was lowered by about 50%. We have shown a consistent reduction of isocitrate lyase activity when the membrane is attacked or disturbed by several other procedures. The different detergents

tried, Tween 40, Tween 80, and Triton X-100, all lowered the membrane bound isocitrate lyase activity by one-third to one-half the original amount of enzyme. Phospholipase A digestion of the peroxisome reduces the isocitrate lyase activity by one-half. Yet, none of these disruptive agents have any significant effect on the soluble enzyme during the same time period and under identical conditions. Similar results are obtained even in different media such as sucrose and mannitol. We believe, therefore, that we are observing a true structural change in isocitrate lyase as the membrane is altered and the activity lowered. The detergents used completely solubilized the isocitrate lyase and also lowered its activity. These same detergents do not have any highly reactive groups in them which might be reacting chemically with the isocitrate lyase. In addition the detergents do not have an inhibiting effect, even at high concentration.

There is at least one other case in which similar disruptive agents were used to show a lowering of enzyme activity upon solubilization of a particle bound enzyme. Lynen et al. in 1971 showed a similar loss of activity for β hydroxymethylglutaryl Coenzyme A reductase upon disruption of purified rat liver microsomes by detergents and phospholipase A. It is conceivable that addition examples of loss of enzyme activity with solubilization of particle-bound enzymes will be found in the near future.

The fact that a membrane-bound enzyme such as isocitrate lyase can be dissolved in aqueous solution should not be un-

expected. Singer (1974) states that a membrane-bound enzyme even one which is an integral protein in a membrane, can still be soluble in aqueous solutions under some conditions. He also states that complete insolubility in aqueous solution should not be a prerequisite for a protein that traverses the thickness of a membrane. Although we do not know whether isocitrate lyase is an integral or a peripheral protein in the peroxisomal membrane, we do know that it is membrane-bound and that it can be solubilized, especially after the cellular structure has been obliterated by lyophilization.

The following paper on the kinetic studies of particle bound isocitrate lyase compared to soluble isocitrate lyase shows conclusively that the two forms of the enzyme differ distinctively. For peroxisomes from cells active in gluconeogenesis, the particle bound enzyme is much more active at low substrate concentrations than is the soluble enzyme. This leads to very interesting modes of metabolic regulation which will also be discussed in the next paper. But there seems to be no doubt from this study that isocitrate lyase is membrane bound in the peroxisome and that when it is solubilized or disrupted in the membrane its activity is lowered.

The work presented in this paper concerning the localization of the glyoxylate cycle enzymes in the peroxisomes of T. pyriformis is in direct contrast to the situation that Bieglmayer et al. (1973) and Huang and Beevers (1973) find

for the glyoxysomes (peroxisomes) of castor bean endosperm. Our studies show that isocitrate lyase is membrane-bound and that malate synthase is membrane-enclosed. The work with castor bean endosperm indicates to the contrary that isocitrate lyase is membrane enclosed and malate synthase is membrane-bound. NADP linked isocitrate dehydrogenase is in the peroxisol in T. pyriformis but is not found in caster bean glyoxysomes which however, do contain malate dehydrogenase. Both species would have to use the glyoxylate cycle, Kornberg and Krebs (1957) to form glucose from acetate but the glucose labeling patterns in glycogen and starch are different, as Hogg (1969) has already stated. This difference in labeling patterns could well result from the different localization of the glyoxylate bypass enzymes.

The two most important points to be drived from this study on the enzymes of the peroxisome are: (1) Isocitrate lyase activity is lowered if the membrane is disrupted indicating the existence of more than one form of the membrane bound enzyme; (2) The two peroxisomal enzymes competing for isocitrate have different intraperoxisomal localizations which results in an important metabolic consequence. Since the mitochondrion is the only known source for isocitrate in T. pyriformis, the membrane bound isocitrate lyase should have the important metabolic advantage of first access over the peroxisolic isocitrate dehydrogenase.

THE KINETICS OF ISOCITRATE LYASE AND ISOCITRATE DEHYDROGENASE IN THE PEROXISOMES OF TETRAHYMENA PYRIFORMIS*

SUMMARY

Isocitrate lyase, membrane-bound in intact peroxisomes from Tetrahymena pyriformis shows positive cooperativity if the organelles are isolated from gluconeogenic or glycogenolic cells and near linearity if the organelles are isolated from steady state glycogen cells. Membrane-bound isocitrate lyase in membrane fragments of sonicated peroxisomes isolated from cells capable of gluconeogenesis also shows positive cooperativity. The kinetics of isocitrate dehydrogenase (NADP) in the peroxisome indicate that the enzyme is membrane enclosed. Extracts of isocitrate lyase and of isocitrate dehydrogenase show linear Lineweaver-Burk plots, if assayed in potassium phosphate buffer pH 6.9 alone or with either 0.25 M mannitol or 0.25 M sucrose with the extracts of isocitrate dehydrogenase which shows negative cooperativity in 0.25 M sucrose. Nearing the 5 micromolar physiological substrate concentration as determined by enzymatic fluorometric methods, the organellar enzyme rates of isocitrate lyase and isocitrate dehydrogenase show large changes for peroxisomes isolated from cells in different

*The data given in this paper were presented in part at the sixty-fifth annual meeting of the American Society of Biological Chemists in June, 1974.

states of glycogen metabolism; these rate changes might help to explain the overall changes in glycogen metabolism in some cases.

INTRODUCTION

Stationary phase cells of Tetrahymena pyriformis can double their glycogen content in 2-4 hours if incubated without substrate in dilute Ringer phosphate solution and moderately aerated Wagner, C. (1956), Hogg and Wagner (1956). This is a major metabolic pathway when we consider that the glycogen content is already 5-10% of the dry weight of the organism. Log phase cells which appear to have a sufficient complement of the glyoxylate bypass enzymes necessary for gluconeogenesis are incapable of increasing glycogen under the same conditions Hogg and Kornberg (1963). Stationary phase cells in culture are unable to perform gluconeogenesis after a few hours of vigorous aeration even though there appear to be suffient glyoxylate bypass enzymes necessary for gluconeogenesis, Levy et al. (1964). The concentrations of the coenzymes used in the competing catabolic pathways could be regulating the ability of the organism to synthesize glycogen from lipids in some cases, but it is doubtful that the coenzyme concentrations could be regulating synthesis in all instances. Since Hogg and Kornberg (1963) have shown that incorporation of the glyoxylate bypass enzymes into an organelle structure is necessary for gluconeogenesis to occur, and we have determined that localization

of the two enzymes competing for isocitrate is quite different, the anaplerotic enzyme isocitrate lyase being membrane-bound and the catabolic enzyme isocitrate dehydrogenase being in the peroxisol, we decided to investigate the consequences of organellar structure on the kinetics of these opposed enzyme activities. This paper presents kinetic data for isocitrate lyase and isocitrate dehydrogenase obtained with intact peroxisomes.

METHODS AND MATERIALS

Tetrahymena pyriformis strain E. originally obtained from A. M. Elliott was maintained as described by Elliott and Hogg (1952); and Wu and Hogg (1952). The stock culture medium contained 1.0% w/v proteose-peptone (Difco), 0.1% w/v glucose, 0.1% sodium acetate, 0.1% K_2HPO_4 and 0.01% yeast extract. 800 ml batches of sterile medium in 2800 ml Fernbach flasks were inoculated with 8.0 mls of a rapidly growing log phase culture and incubated at 25° for 4 days (early stationary phase) at which time they were harvested in a modified plankton centrifuge, Conner et al. (1966), and washed with an equal volume of dilute Ringer phosphate solution (Ryley, 1952). The cell suspensions were made to 1% (v/v) by diluting the cells with dilute Ringer phosphate solution after determining the suspension density by packing samples of the cells at 1000 x g for 10 minutes in a Constable protein tube, Constable (1930).

The following conditions were responsible for providing

cells of different metabolic states: (1) highly aerated, well shaken cells with a steady state glycogen metabolism were obtained by placing 15-20 ml samples of 1% cell suspensions into 2800 ml Fernbach flasks rotating at 75 RPM in a Metabolite waterbath shaker (New Brunswick) at 30° for 4 hours. After this time they were collected in the Conner plankton centrifuge and washed with 100 mls of distilled water before breaking the cells to get the peroxisomal fraction; (2) moderately aerated cells with active gluconeogenesis were obtained when 50-100 ml portions of 1% cell suspension were placed in 1 liter conical flasks and swirled in the waterbath shaker at 75 RPM for 4 hours before collecting and washing as before; (3) oxygen deprived cells with active glycogenolysis were obtained when 200 ml portions of the 1% cell suspensions were placed in 250 ml conical flasks for 1-2 hours without shaking at which time they were collected and washed in the same fashion as the other cells. These cells are equally capable of gluconeogenesis if changed to the conditions of (2) above. Each of the experimental sets of cells were tested for glycogen content before and after incubation on the shaker. The viability and general appearance of the cells were checked after incubation by viewing samples of the protozoal suspensions under a phase contrast microscope. No cells showed signs of division.

The above suspensions of T. pyriformis at 10% v/v were each added to an equal volume of ice cold 0.5 M mannitol and

immediately passed through a British #3 pore size (20-30 micron) fritted-glass filter with the aid of low vacuum. Cell breakage was complete as determined by phase contrase microscopy. The peroxisomal fractions (P_1) used for all kinetic studies were obtained in about 30 minutes using the procedures of Hogg and Kornberg (1963). If the final peroxisomal suspension volume is kept to a minimum of about 1/5 of the homogenate volume, organelle integrity is maintained for at least 6-8 hours on ice during which time all of the kinetic enzyme data were obtained. None of the data are from peroxisome fractions kept for longer than this period of time.

The spectrophotometric enzyme assays were performed with a Gilford model 2400 spectrophotometer, with cuvette temperature maintained at 30° C by a Lauda circulating bath. Isocitrate lyase was measured by a modified procedure of Dixon and Kornberg (1959), except that the assay mixture contained 66 mM potassium phosphate buffer pH 7.5. Isocitrate dehydrogenase (NADP) was assayed by a modified procedure of Kornberg, A. (1955). Both assays included 0.25 M mannitol to preserve the integrity of the organelles. See Appendix for complete details of the enzyme assays.

When extracts were needed, 10% (v/v) cell suspensions (washed with 200 ml distilled water after the usual Ringer phosphate wash) were frozen in an alcohol-dry ice bath and dried in high vacuum in a Kontes lyophilizer. The lyophilized cell powders were kept at -20° C. Extracts were made

by grinding the cell powders (12.5 mg/ml) in 50% (v/v) glycerol, 0.01 M potassium pyrophosphate buffer pH 7.5 with a cold Brendler homogenizer and centrifuging the homogenate for 20 minutes at 20,000 x g in a refrigerated Sorvall RC-2 centrifuge. The small amounts of glycerol and pyrophosphate added with the extract did not interfere with the enzyme assays.

In order to determine the intracellular concentration of isocitrate during gluconeogenesis, suspensions of washed T. pyriformis cells that were performing gluconeogenesis were frozen rapidly (less than one minute) in Kontes lyophilizing flasks immersed in liquid nitrogen and immediately dried in high vacuum. The lyophilized cells were extracted with ethanolic perchloric acid according to the procedures of Williamson, J. R. and Corkey, B. E. (1969, p. 437-438). The extracts were then analyzed for isocitrate using the fluorometric enzymatic methods of Williamson, J. R. and Corkey, B. E. (1969, p. 453-455). The only change made was that 5 mg Darco G-60 charcoal was added per ml of extract to remove endogenous fluorescent materials. This amount of charcoal did not remove any isocitrate as determined by additions of 2 concentrations of threo-D_s-isocitrate within the range determined. The assays were performed with a Perkin-Elmer fluorescence spectrophotometer in assay medium that contained 0.2 ml of 1.0 M triethanolamine-HCl pH 7.4; 10 microliters of $MnSO_4$, 1.0 M; 1.0-2.0 mls of acid extract sample and water up to 3.0 ml. The reaction was started by

the addition of 5.0 microliters of NADP-linked isocitrate dehydrogenase (type X Sigma) of at least 1.8 U/ml. The intracellular concentration of isocitrate in T. pyriformis strain E is determined to be 5.1±0.3 micromolar in gluconeogenic cells. The basis of calculating concentration is based on the experimental finding of Scherbaum et al. (1959) that there is 0.172 mg dry weight/ml cell volume. This value should not be very far off since Scherbaum found that although the dry weight of the cell varies in synchronized cultures, so does the volume of the cell; thus the dry weight per unit volume remains constant.

Glycogen was determined by the anthrone method (Hogg, J. F., personal communication). The glycogen is extracted from fresh cells by homogenizing in 0.5 M perchloric acid with a Brendler homogenizer at 1,000 RPM. The tubes used for homogenization are then centrifuged at 1,000 x g for 10 minutes. The supernate is transferred to a fresh centrifuge tube. The residue is resuspended in 1.0 ml of H_2O, centrifuged and the second supernate combined with the first. The residue is then discarded. 2.5 volumes of 95% ethanol are added to the tube containing the combined supernates. The tube is placed in a 70° water bath for 20-30 minutes to induce flocculation of glycogen. The water level preferibly should be lower than the liquid level in the tube. After cooling, the tubes are centrifuged at 1,000 x g for 10 minutes and the supernate decanted and discarded. The glycogen is redissolved in 2.0 ml H_2O and the precipitation

by ethanol repeated. The second glycogen precipitate is dissolved in 1.0 ml H_2O and stored at 0°-5° C. until used. Aliquots of samples and standards containing from 10-100 micrograms of glucose were brought to 1.0 ml with water and then combined with 10 volumes of cold 90% (v/v) sulfuric acid containing 0.1% anthrone. The color developed by heating 10 minutes at 90° was read at 620 nm. in a Spectronic 20 colorimeter.

Protein was determined by the method of Lowry, O. H. et al. (1951) after the protein of the sample was precipitated from and washed once with 2% perchloric acid. Bovine serum albumin was used as the standard.

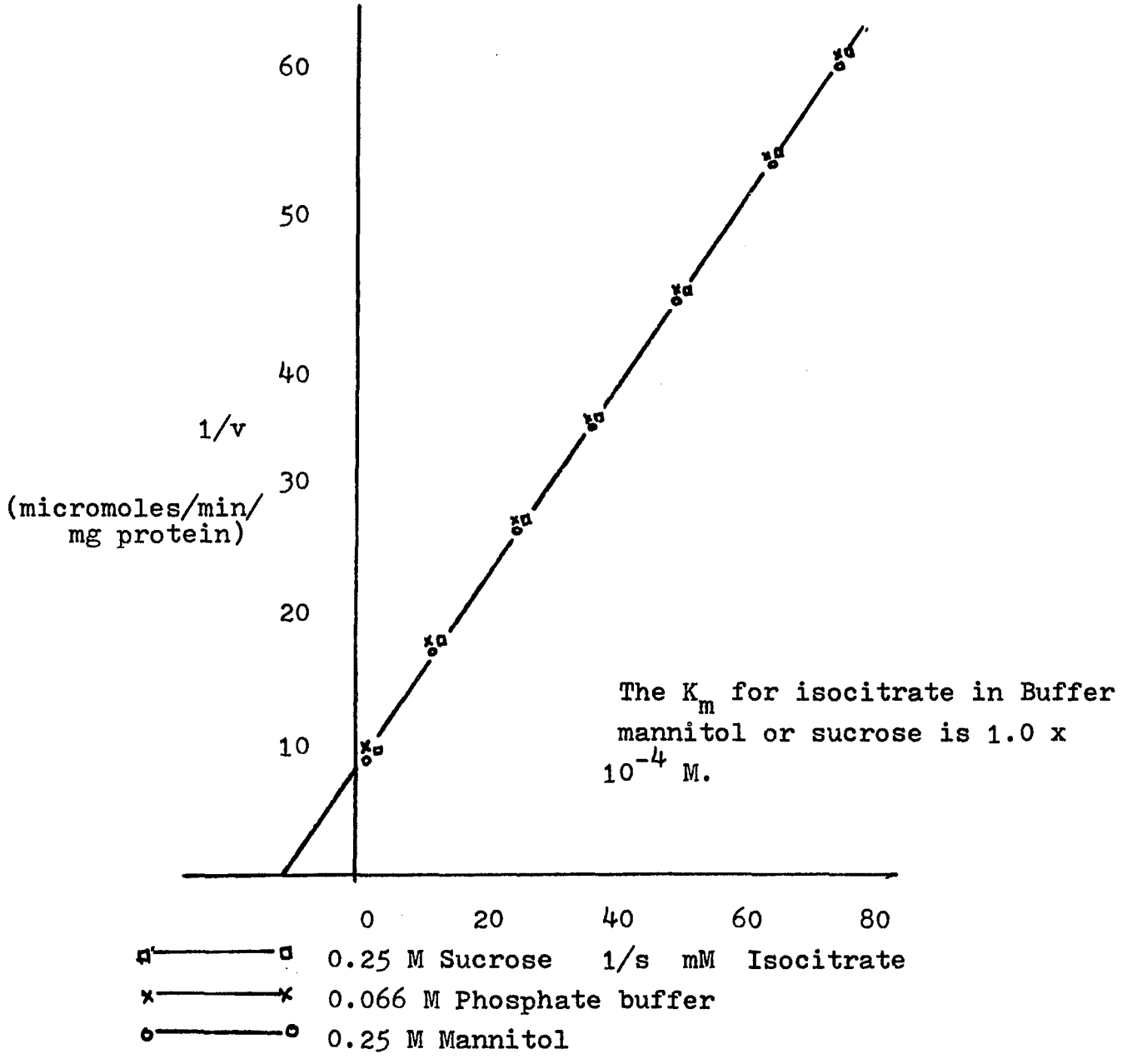
The chemicals were obtained from the following sources: proteose-peptone, Difco Laboratories; potassium threo-D_s isocitrate was a gift from Dr. H. B. Vickery; anthrone tetra-potassium pyrophosphate and all other biochemicals, Sigma Chemical Co.; sucrose and all inorganic chemicals were Baker Analyzed grade from the J. T. Baker Chemical Co..

RESULTS

Lineweaver-Burk plots for isocitrate lyase assayed in extracts from T. pyriformis in 66 mM potassium phosphate buffer pH 7.5, buffer plus 0.25 M mannitol and buffer plus 0.25 M sucrose are essentially the same as is shown in figure 1. The plot shows normal linear Lineweaver-Burk plots in each of the media.

Lineweaver-Burk plots for isocitrate lyase in the per-

LINEWEAVER-BURK PLOTS FOR ISOCITRATE LYASE EXTRACTS
RUN IN BUFFER, 0.25 M MANNITOL AND 0.25 M SUCROSE pH 7.5



All assays contained 0.066 M potassium phosphate buffer pH 7.5. The glycerol extracts were prepared as described in Methods.

Figure 1

oxisomes, protected from osmotic shock during assay by 0.25 M mannitol, show near linearity for the enzyme in fully oxygenated cells in steady state glycogen metabolism and positive cooperativity for the enzyme in cells active in gluconeogenesis or glycogenolysis (both low in O_2) as seen in figure 2. In the two states actively metabolizing glycogen, isocitrate lyase in the peroxisomes is more active at lower substrate concentrations than it is in the soluble form and much more active than in peroxisomes isolated from cells having steady state glycogen metabolism. The maximum isocitrate lyase activity at saturating substrate concentrations also changes in the three metabolic states studied. The peroxisomes from fully oxygenated cells have a specific activity of about 0.5 micromol/hr/mg protein which is about half the value usually obtained with isolated peroxisomes from gluconeogenic cells. The peroxisomes from glycogenolic cells have the highest maximum specific activity which is often about 3.0 micromol/hr/mg protein or three times the specific activity of peroxisomes isolated from gluconeogenic cells.

The plot for isocitrate lyase, membrane bound in isolated peroxisomes from cells capable of gluconeogenesis, is confirmed in figure 3 using the colorimetric assay of McFadden and Howes (1960) which is absolutely specific for the reaction product, glyoxylate. The assays were run in 0.25 M mannitol as before. In order to get the sensitivity required at low substrate concentrations, absorbance was

LEGEND FOR FIGURE 2

The glycogen content of the gluconeogenic cells went from 735 micrograms glucose/ml 1.0% cell suspension to 1250, an increase of 515 micrograms glucose/ml 1.0% cell suspension in 4 hours at 25° C. The glycogen content of glycogenolic cells went from 680 micrograms glucose/ml 1.0% cell suspension in 2 hours at 25° C. to 75 micrograms glucose/ml 1.0% cell suspension. There was no change in the glycogen content of the cells in steady state glycogen metabolism. The peroxisomes were prepared and the assays run as described in Methods.

LINEWEAVER-BURK PLOTS FOR MEMBRANE-BOUND ISOCITRATE LYASE IN PEROXISOMES ISOLATED FROM TETRAHYMENA CELLS DURING GLUCONEOGENESIS, GLYCOGENOLYSIS AND STEADY STATE GLYCOGEN METABOLISM

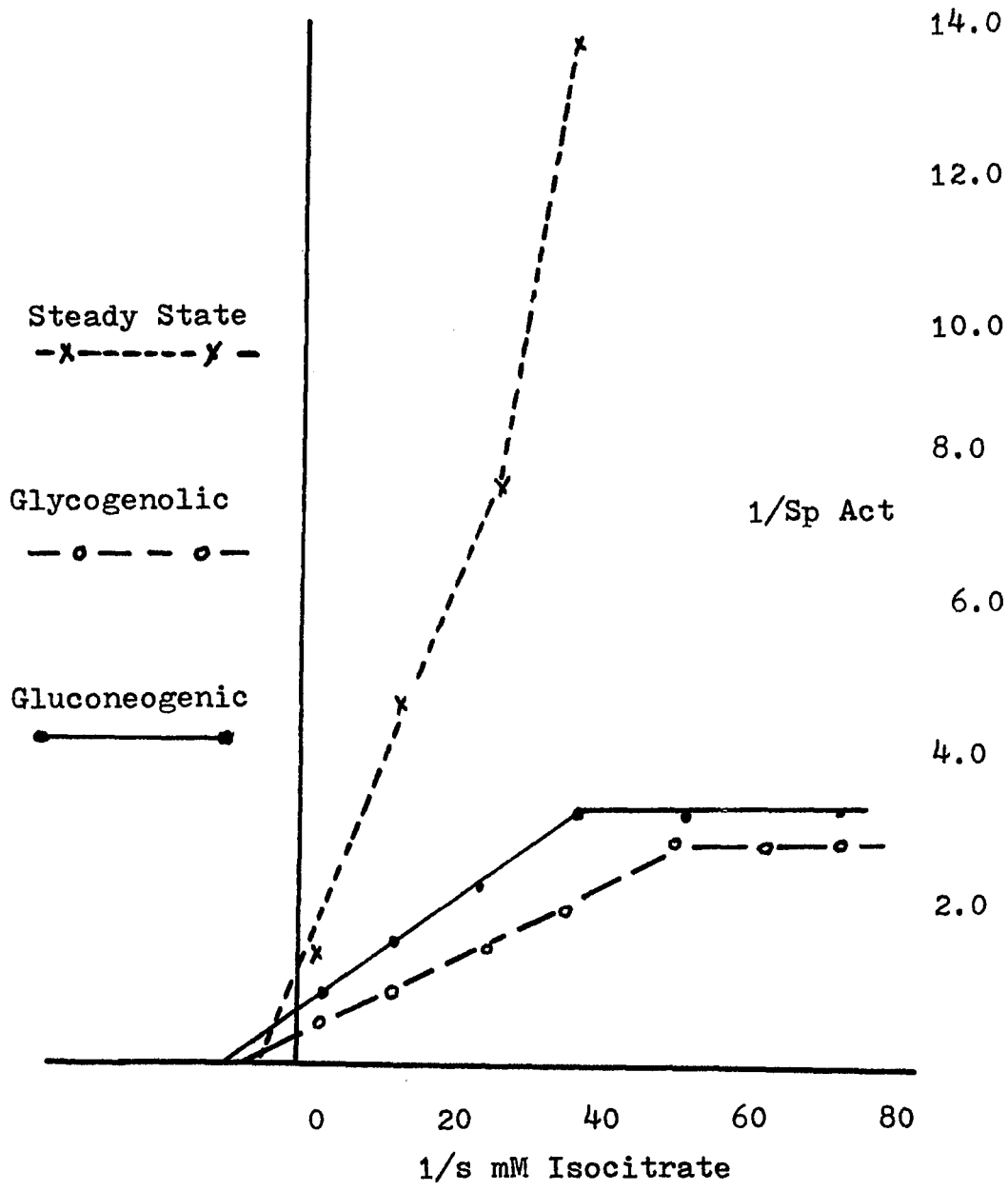
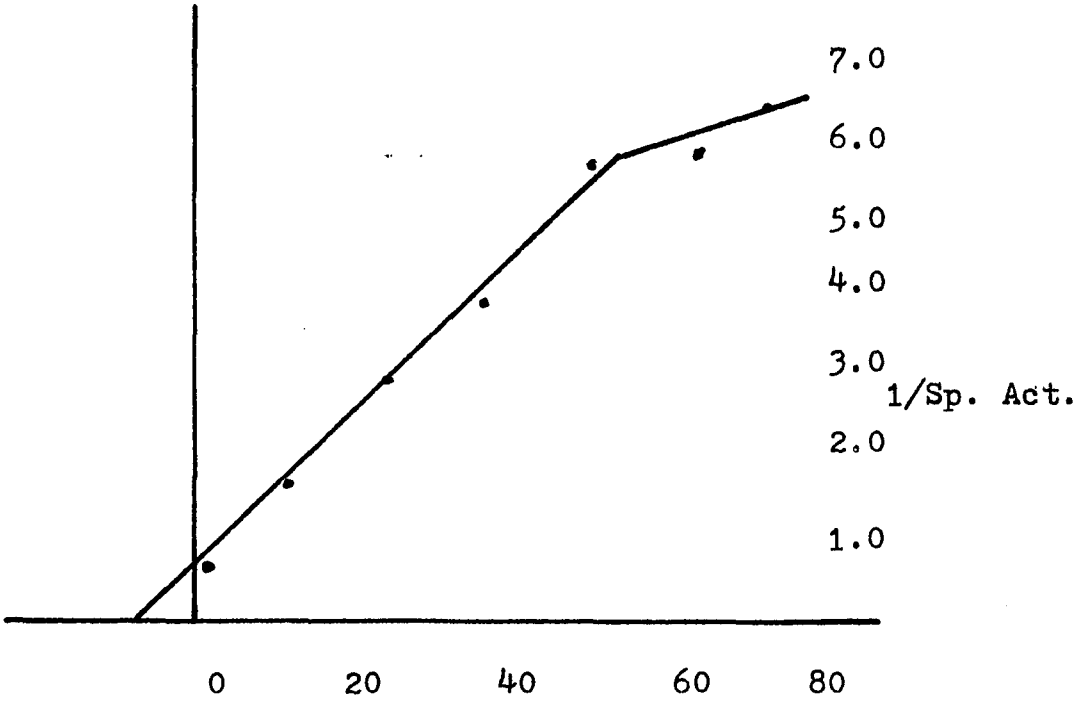


Figure 2

LINEWEAVER-BURK PLOT FOR MEMBRANE-BOUND ISOCITRATE LYASE IN PEROXISOMES RUN IN 0.25 M MANNITOL pH 7.5 USING THE SPECIFIC COLORIMETRIC ASSAY



See Methods for the preparation of the peroxisomes and for the description of the colorimetric assay.

Figure 3

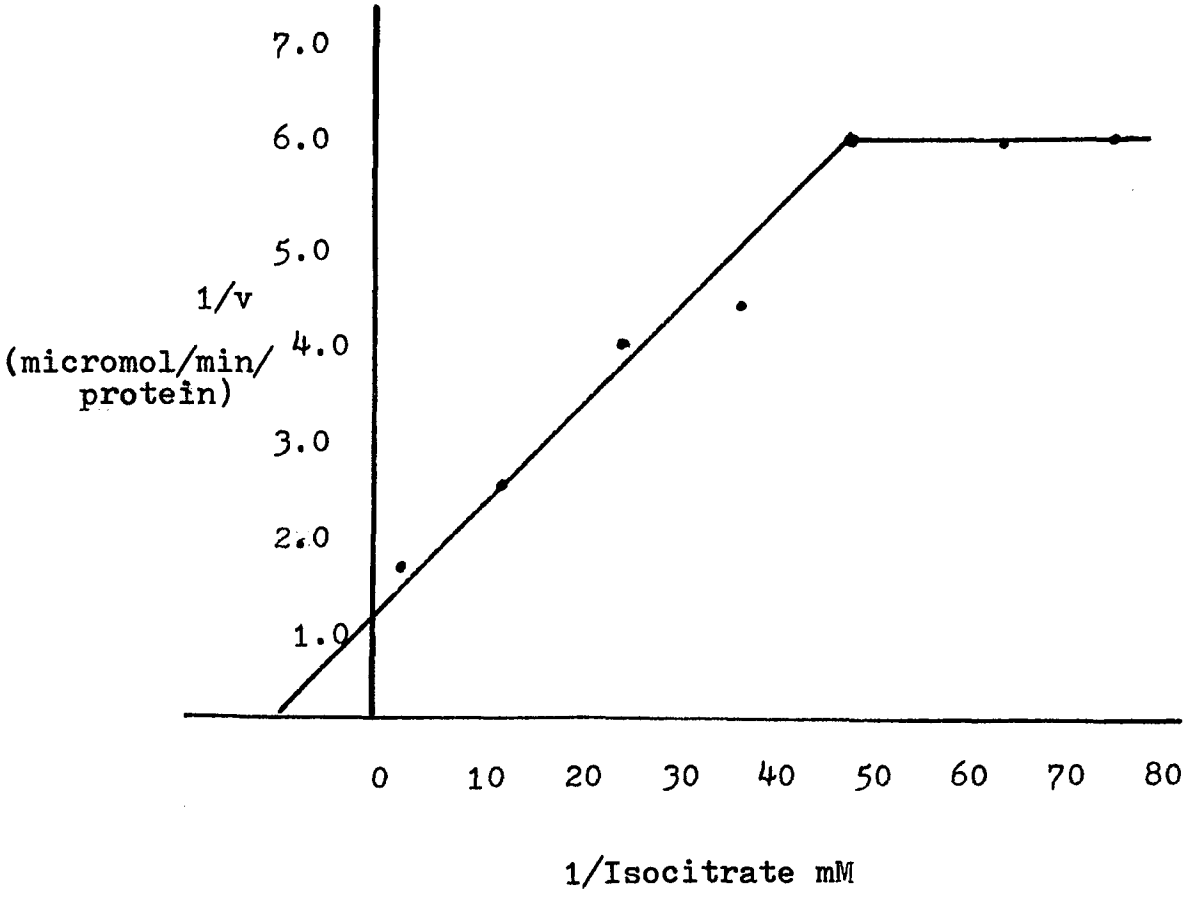
measured in ten cm. spectrophotometer cells in a Cary 14 spectrophotometer.

When the enzymic response to isocitrate was repeated on the centrifugal pellet obtained after sonication of peroxisomes from gluconeogenic cells (membrane fragments or vesicles), essentially the same Lineweaver-Burk plot was obtained as for the peroxisomes (figure 4). As shown in the previous chapters, however, the specific activity of isocitrate lyase in the sonic homogenate is only one-half that in the peroxisomes; thus the unique kinetic characteristic of the membrane-bound lyase has been retained after sonication sufficient to release the major part of the enzymes in the peroxisol.

Because of the radically different kinetic plots for isocitrate lyase in peroxisomes isolated during gluconeogenesis or steady state glycogen metabolism, we decided to determine if the lyase moves from the membrane into the peroxisol in cells during steady state glycogen metabolism. The same procedure for determing whether a peroxisomal enzyme is membrane bound or in the peroxisol was followed as previously described in the methods section of the intraperoxisomal enzyme distribution paper. As table 1 shows, the isocitrate lyase is membrane bound in both types of cells and the isocitrate dehydrogenase is in the peroxisol in both cases.

Figure 5 gives the Lineweaver-Burk plots for assays of extracted isocitrate dehydrogenase that were run in pH 7.4

LINEWEAVER-BURK PLOT FOR MEMBRANE-BOUND ISOCITRATE LYASE IN THE MEMBRANE FRACTION FROM SONICATED PEROXISOMES



A physiological concentration of 10 - 5 micromolar would give reciprocal numbers of 100 - 200 on this chart.

Figure 4

SEDIMENTABILITY OF ISOCITRATE LYASE AND ISOCITRATE DEHYDROGENASE IN SONICATES OF PEROXISOMES FROM CELLS WITH AND WITHOUT ACTIVE GLUCONEOGENESIS

ENZYME	THE TABLE GIVES THE FRACTION (%) OF EACH ENZYME REMAINING IN THE SUPERNATE AFTER CENTRIFUGATION AT 30,000 x g FOR 30 MINUTES	
	ACTIVE GLUCONEOGENESIS	STEADY STATE GLYCOGEN METABOLISM
ISOCITRATE LYASE	8%	11%
ISOCITRATE DEHYDROGENASE	75%	81%

Table 1

LINEWEAVER-BURK PLOTS FOR ISOCITRATE DEHYDROGENASE EXTRACTS RUN IN PHOSPHATE BUFFER, 0.25 M MANNITOL AND 0.25 M SUCROSE ALL AT pH 7.5

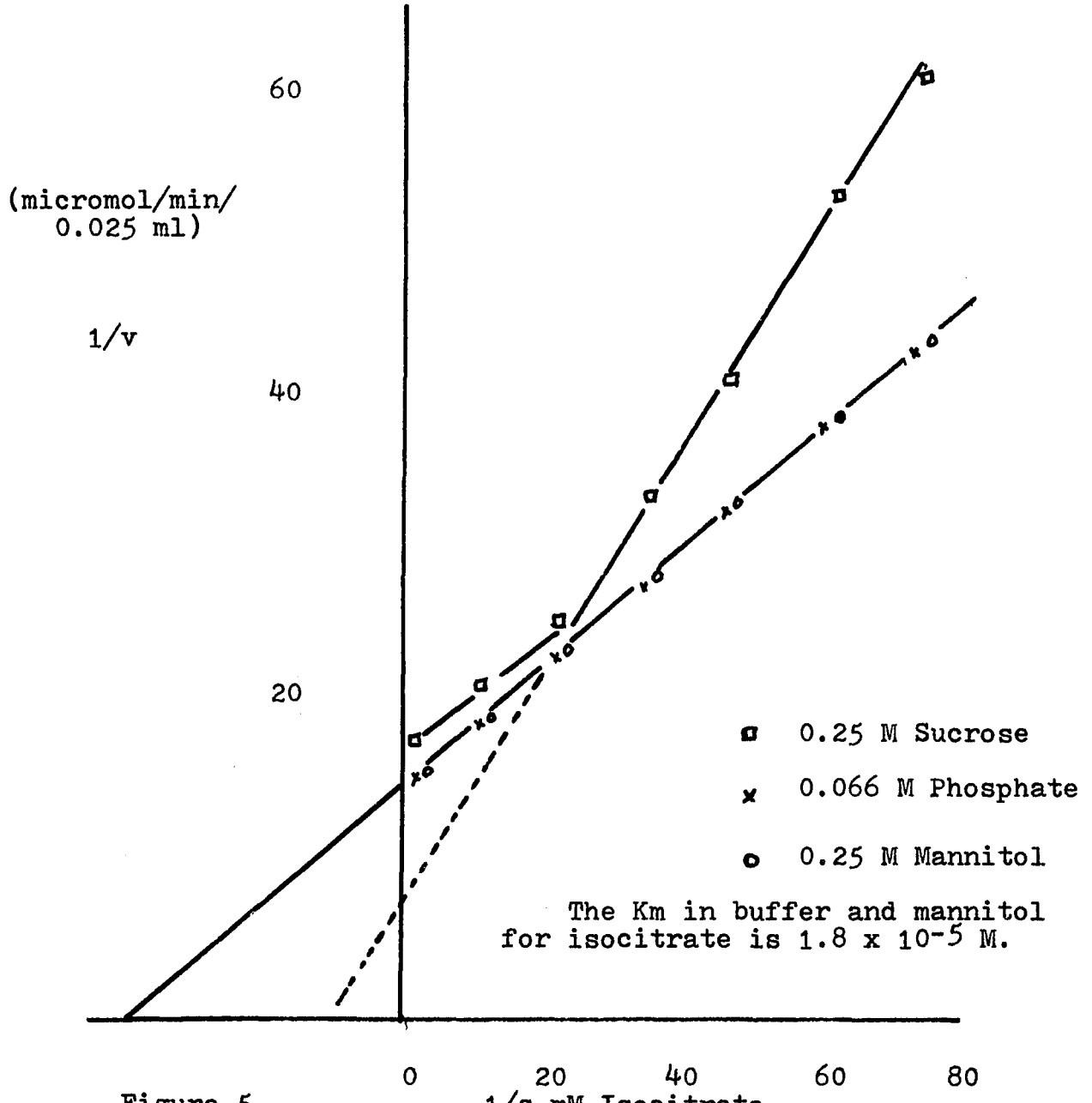


Figure 5

All assays were 0.066 M in potassium phosphate buffer pH 7.5.

buffer, containing 0.25 M mannitol, or 0.25 M sucrose. The enzyme shows negative cooperativity when assayed in buffered 0.25 M sucrose and linear Michaelis-Menten kinetics in buffered 0.25 M mannitol. The negative cooperativity found in 0.25 M sucrose apparently is a sucrose effect directly on the enzyme rather than a complexing with the substrate, since a similar lowering of activity is not found with the Lineweaver-Burk plots for isocitrate lyase under the same conditions.

Mannitol (0.25 M), therefore, was used as a suspending medium to investigate the organelle kinetics of isocitrate dehydrogenase since this medium did not alter the kinetics of the enzyme in solution. Figure 6 shows that the activity of isocitrate dehydrogenase enclosed within the peroxisome has lowered at low substrate concentrations whether the organelles are obtained from gluconeogenic, glycogenolic or steady state glycogen cells. Notice that there is virtually no enzyme activity detectible below 50 micromolar isocitrate in peroxisomes isolated from cells having steady state glycogen metabolism for either isocitrate (Figure 2) or isocitrate dehydrogenase.

The ratio of activities, isocitrate lyase to isocitrate dehydrogenase, for peroxisomes is plotted against the reciprocal of (isocitrate) in Figure 7. This ratio increases rapidly as isocitrate concentration falls below 0.05 mM, thus indicating that isocitrate lyase action is highly favored at the physiological concentration of 0.005 mM. The

LINEWEAVER-BURK PLOTS FOR MEMBRANE ENCLOSED ISOCITRATE DEHYDROGENASE IN PEROXISOMES ISOLATED FROM TETRAHYMENA CELLS DURING GLUCONEOGENESIS, GLYCOGENOLYSIS AND STEADY STATE GLYCOGEN METABOLISM

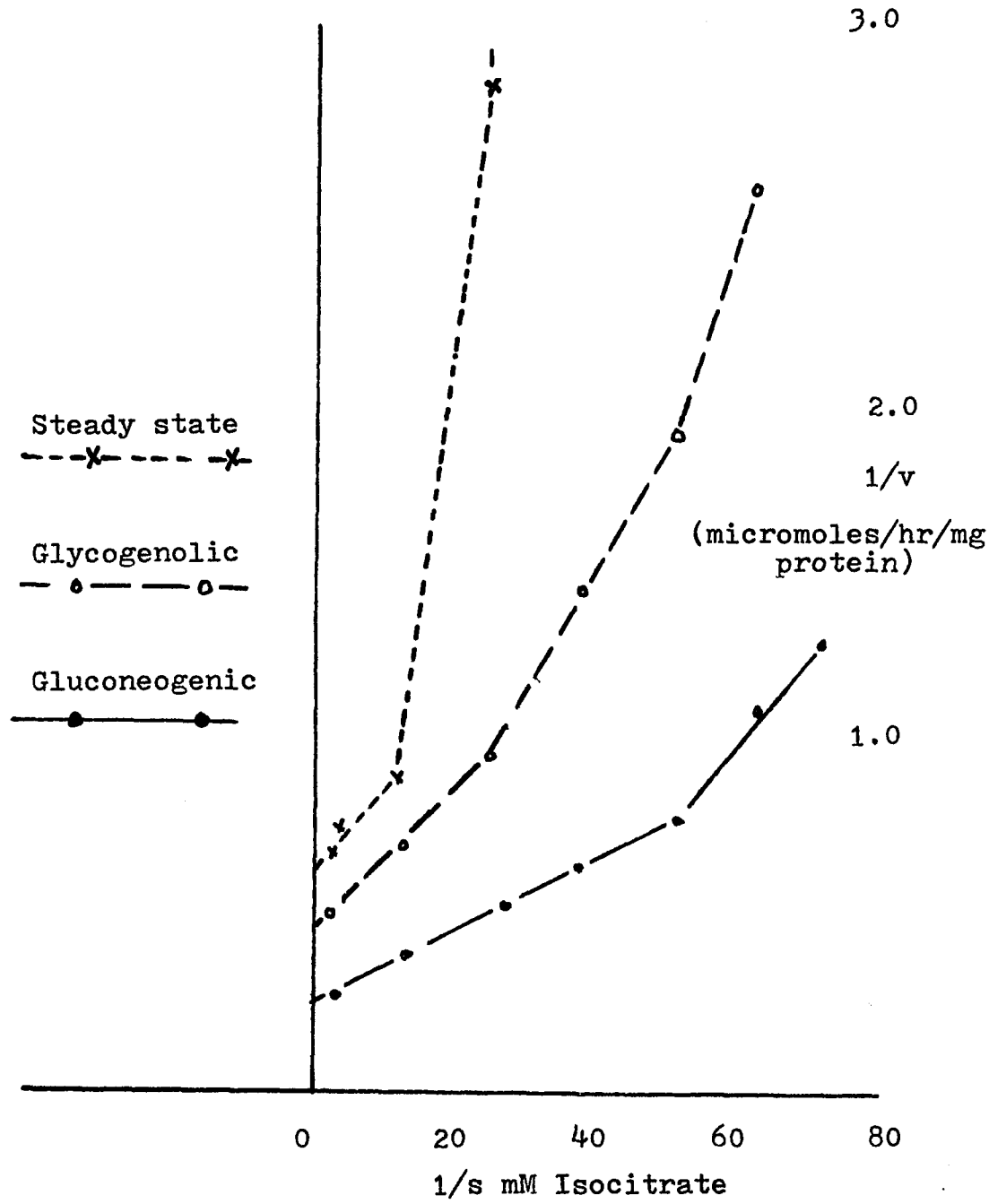


Figure 6 See legend for figure 2 for experimental details.

PLOT OF $\frac{\text{(IL rate)}}{\text{(ICDH rate)}}$ versus 1/ISOCITRATE USING ORGANELLE RATES OBTAINED IN 0.25 M MANNITOL

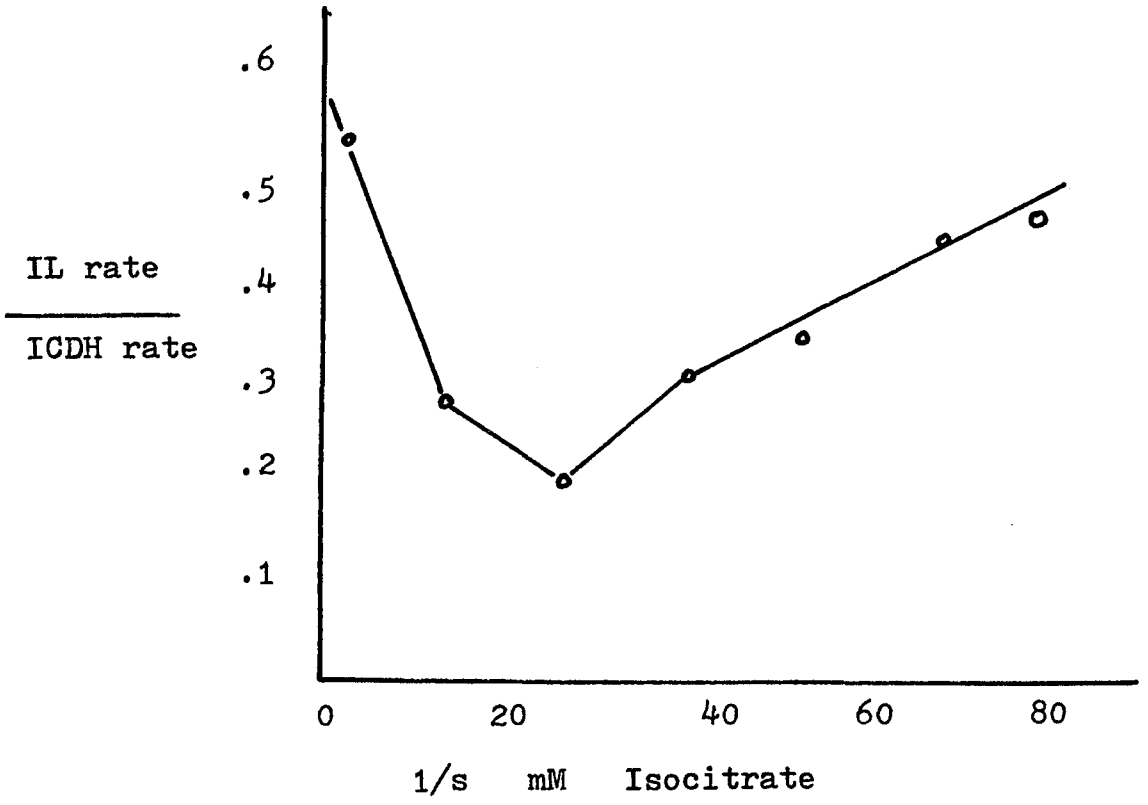


Figure 7

favoring of isocitrate lyase in gluconeogenic cells results from the kinetic properties that the enzyme obtains by virtue of incorporation into the peroxisomal membrane. This is important because it is in direct contrast to what would be expected of the individual enzymes free in solution.

The Lineweaver-Burk plots of NADP for isocitrate dehydrogenase extracts run in pH 7.4 buffer, 0.25 M mannitol and 0.25 M sucrose, are given in Figure 8. These plots indicate that 0.25 M sucrose inhibits the enzyme uncompetitively. Thus the Lineweaver-Burk plot for both substrate and coenzyme of isocitrate dehydrogenase show an interaction of sucrose with the enzyme. These plots were performed using the spectrophotometic assay (Kornberg, A. 1955) and corroborated by means of fluorometric assay.

The Lineweaver-Burk plot of NADP for isocitrate dehydrogenase enclosed in the peroxisome is given in Figure 9. No change is found in the shape of the curve for the coenzyme plot when comparing the extract and organelle. The concentrations of NADP used are higher than would be expected under physiological conditions, so that this comparison may be misleading. The Lineweaver-Burk plots for isocitrate were brought much closer to the physiological concentration range.

DISCUSSION

The most important conclusion to come from this study is that an enzyme should be studied as close to physiolog-

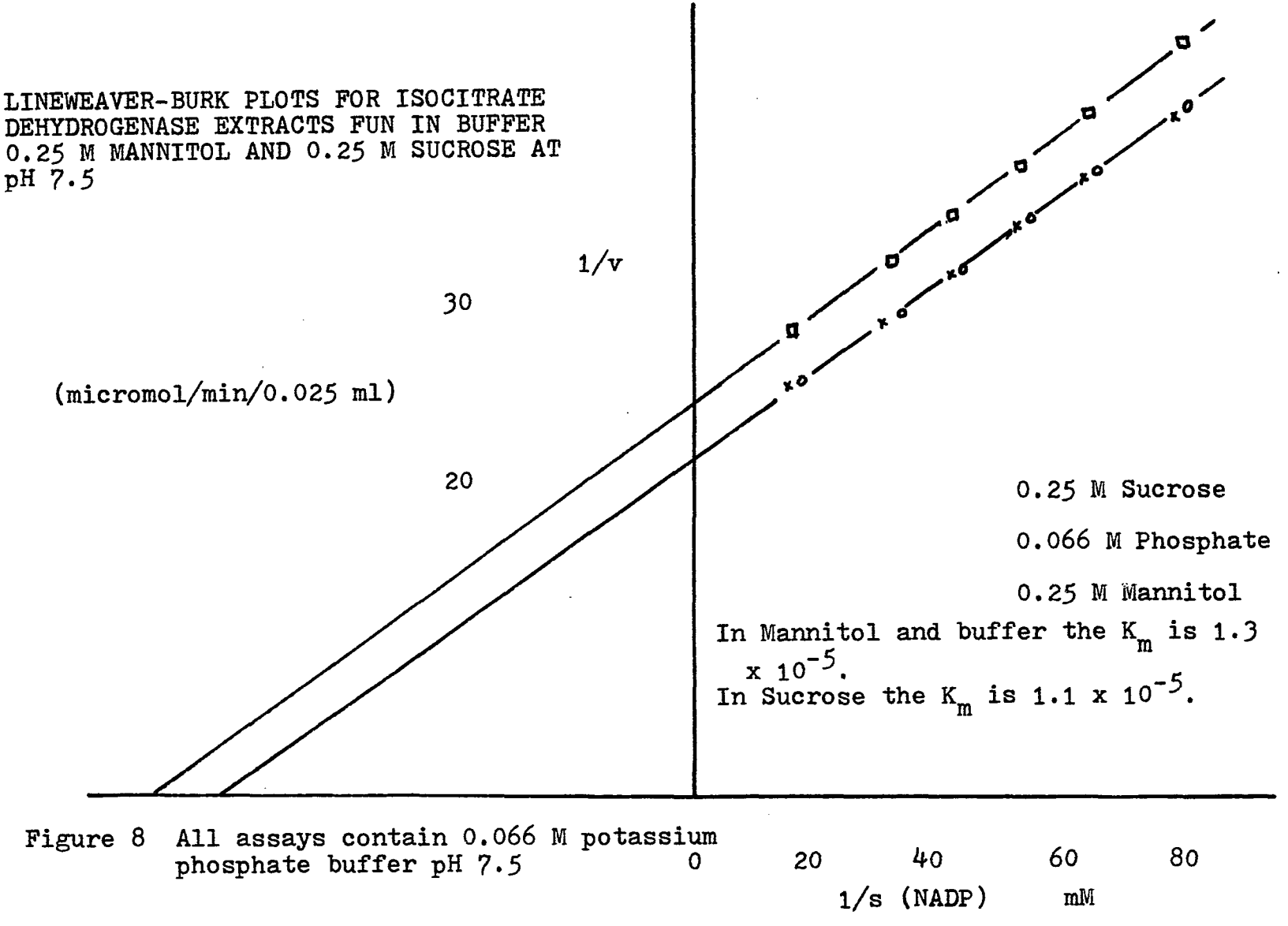


Figure 8 All assays contain 0.066 M potassium phosphate buffer pH 7.5

LINEWEAVER-BURK PLOT FOR ISOCITRATE DEHYDROGENASE ENCLOSED IN THE PEROXISOME ASSAYED IN 0.25M MANNITOL AT pH 7.5

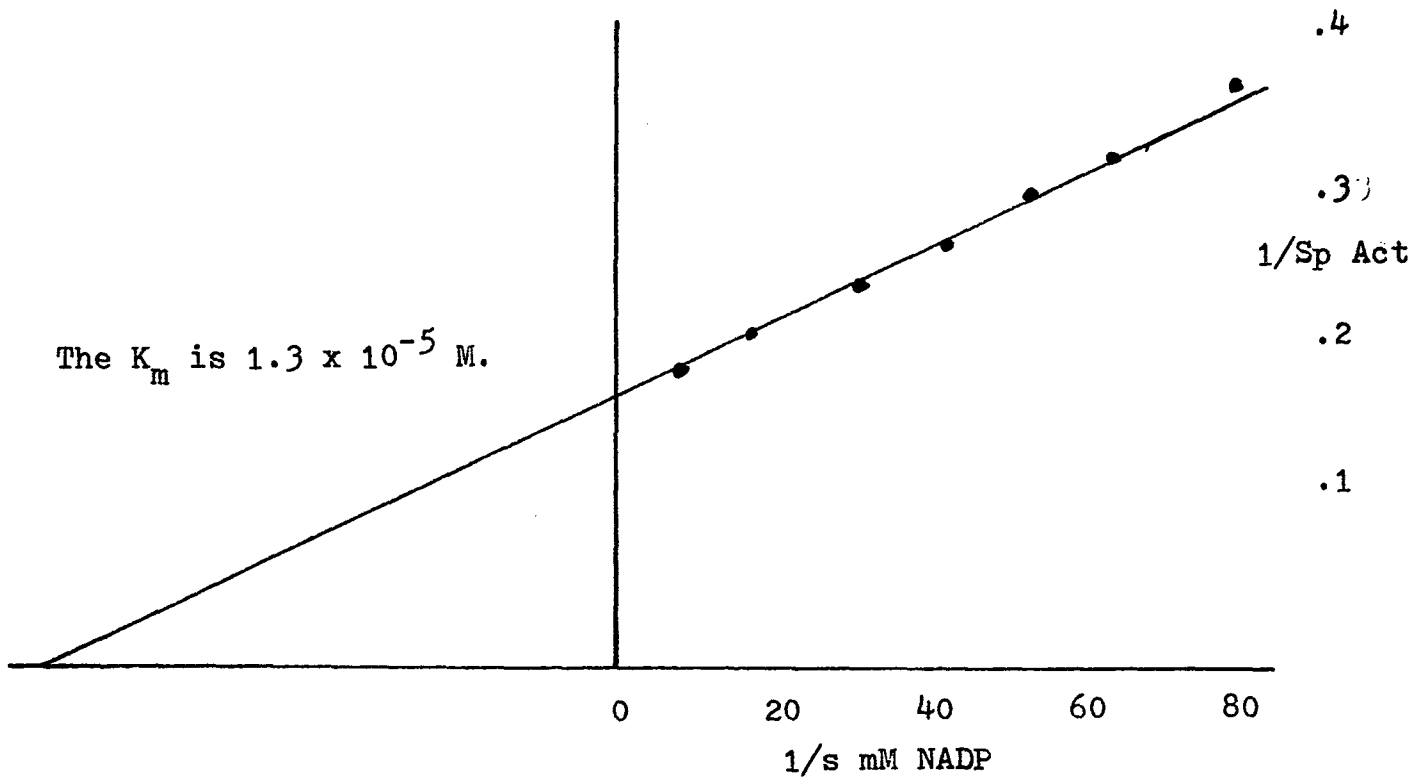


Figure 9

ical conditions as possible in order to derive any physiological meaning from its activity. Although this statement seems obvious, there are many examples in present day literature where pysiological conditions are disregarded in an effort to obtain more precise numerical data. We have not achieved physiological conditions in this study either, for we assay organellar enzymes in a quarter molar polyalcohol solution. What we have done is to assay as nearly as possible to physiological substrate concentrations and to measure enzyme activity in the organelle rather than in solution. Our data show that the organellar enzyme activity can vary significantly near physiological substrate concentration, depending upon the overall metabolic state of the cells from which the organelles are obtained.

In trying to determine the reason for the variations in isocitrate lyase activity in organelles isolated during different metabolic states, the problem should be separated into two categories. First, we should consider reasons for differences in activity between the soluble and organellar form of the enzyme. Second, we must look at the reasons for changes in activity when the organellar form of the enzyme is isolated from cells in different metabolic states.

There are at least two possible reasons for activity differences in the soluble and particulate (organellar) form of isocitrate lyase which deal strictly with the protein structure itself. These are discussed in greater detail

in a previous paper, Dorsey and Hogg (c). The first reason is that there may be a change in tertiary structure necessitated by the change in environment from a polar aqueous environment to the hydrophobic environment of the membrane. The second reason is that there may be subunit aggregation in either or both phase which causes a change in enzymic activity. Either of these explanations could help account for the change in shape of the Lineweaver-Burk plots for membrane-bound isocitrate lyase when comparing it to the soluble enzyme.

There are at least three explanations as to how organellar enzyme activities of membrane-bound enxymes such as isocitrate lyase can change for cells in different metabolic state. One way is for the membrane-bound enzyme to become more internalized into the hydrophobic environment of the membrane. This means that the enzyme changes from a peripheral to an integral protein, thus blocking at least partially the active or the control site of the enzyme. Another method would be for the organellar enzyme to shift from the membrane to the peroxisol. The third possibility is that a regulator molecule, either protein or non-protein in nature blocks the active or regulator site of the membrane-bound enzyme.

We have excluded the second possibility in our study of isocitrate lyase in gluconeogenic and steady state cells. The enzyme has not shifted from the membrane into the peroxisol of the peroxisome in steady state cells, according to

the data on sedimentability after sonication of peroxisomes. The isocitrate lyase activity sediments with the membranes in peroxisomes from both gluconeogenic and steady state cells; therefore the difference in the Lineweaver-Burk plots for isocitrate is not due to a shift of isocitrate lyase into the membrane-enclosed volume of theperoxisome in T. pyriformis cells during steady state glycogen metabolism. The third explanation appears implausable because both the membrane-bound isocitrate lyase and the peroxisolic isocitrate dehydrogenase were equally affected by change of metabolic state of the cells.

One aspect of regulation for membrane-bound enzymes which should be investigated is what effect changes in surface conditions such as gross changes in structure i.e. wrinkling of the surface, or changes in surface tension have on the Lineweaver-Burk plots of the membrane-bound enzymes. The effect that surface conditions have on the rates of reactions in inorganic and in colloid chemistry are well documented but little is known about how surface conditions can effect enzyme rates. Different conformational states have been proposed for the mitochondrial inner membrane corresponding to different energy states, Hackenbrock, C. (1967, 1968); Packer, L. (1967) and Green, D. E. (1968). Reversible ultrastructural changes with changing metabolic state have also been reported for chloroplasts under differing conditions of light intensity, R. A. Dilley and L. P. Vernon, (1965); G. Hind and A. T.

Jagendorf, (1965). Unfortunately, the kinetics of the bound enzymes were not included in those previous studies on changing metabolic state; only ultrastructural changes were shown. We have not investigated to see if any ultrastructural change occurs in peroxisomes during changes in metabolic state, but we do find a change in the kinetics of the bound enzymes from organelles isolated during different metabolic states. It would be interesting to know if ultrastructural change occurs in the peroxisomes of cells in different metabolic states.

The differences in the Lineweaver-Burk plots of membrane-enclosed NADP linked isocitrate dehydrogenase from cells in different metabolic states is easiest to understand in terms of changes in the rate of substrate transport across the peroxisomal membrane. The membrane appears to be much less permeable to isocitrate in cells during steady state glycogen metabolism than in gluconeogenic or glycogenolic cells. The shape of the isocitrate Lineweaver-Burk plots for isocitrate dehydrogenase probably does not denote negative cooperativity but rather shows that the substrate at lower concentrations, is used by the membrane-bound isocitrate lyase before it reaches the dehydrogenase.

An interesting experimental point was uncovered when we investigated the effect of different media on extracted isocitrate dehydrogenase. The dehydrogenase shows definite negative cooperativity if the enzyme is assayed in 0.25 M sucrose whereas it does not in buffer or in 0.25 M mannitol.

Since 0.25 M sucrose is a commonly used suspension medium, a problem could result if an investigator wanted to use this medium for incubating peroxisomes, especially if metabolic studies with tracers were attempted.

Our kinetic work on isocitrate lyase and isocitrate dehydrogenase studied in intact peroxisomes shows that organellar enzyme rates can help to explain some but not all the basic changes in metabolism. In gluconeogenic and steady state cells the organelle enzyme rates are consistent with metabolism. The two peroxisomal enzymes, isocitrate lyase and isocitrate dehydrogenase, actively compete for substrate in gluconeogenic cells with isocitrate lyase increasing its relative rate with decreasing isocitrate concentrations. Thus the organelle enzyme rates are favoring glycogen production in gluconeogenic cells. In steady state glycogen metabolism cells, there is absolutely no peroxisomal enzyme activity detectible below 50 micromolar isocitrate. The organellar enzyme rates do not help to explain metabolism in glycogenolic cells since isocitrate lyase, the enzyme required for glycogen buildup, is most active in the peroxisomes of these cells even at very low isocitrate concentrations. The concentration of oxidized NADP undoubtedly must participate in regulating the overall metabolism in these cells. It is interesting to note that the glycogenolic cells are capable of gluconeogenesis at rates greater than regular gluconeogenic cells if the oxygen conditions are changed to those of moderate aeration

Hogg, J. F. (1962). We can conclude that the activities of isocitrate lyase and isocitrate dehydrogenase in the organelles do aid in understanding the basic metabolic picture in T. pyriformis in several cases studied.

Appendix

Enzyme Assay:

1) For all enzyme assays the initial linear portion of the reaction rate plot is recorded and used for the enzyme activity calculation.

2) If enzyme rates are to be compared, as with extract and organelle preparations, the initial maximun rates are chosen to be nearly equal.

3) With the exception of zero enzyme activity most all enzyme rates recorded in this thesis had a minumum absorbance change of 0.010 absorbance units per minute at the lowest substrate concentration employed. The Gilford spectrophotometer used had an expanded scale of 0.050 total absorbance.

4) The isocitrate lyase spectrophotometric assay system used in chapter one contains:

a) 66 mM potassium phosphate buffer pH 6.9
b) 5 mM magnesium chloride
c) 33 mM phenylhydrazine-HCl (recrystallized)
d) 50 micromolar EDTA

The increase in absorbance is measured at 324 nm after the addition of 3.3 mM threo-D_s-isocitrate.

5) The colorimetric oxidized phenylhydrazone assay mixture for isocitrate lyase contains: 1.00 ml 0.20 M buffer potassium phosphate buffer, pH 7.5; 0.20 ml 0.0010 M EDTA; 0.15 ml 0.10 M $MgCl_2$; 0.10 M potassium threo-D_s-isocitrate; up to 1.00 ml enzyme preparation and H_2O to 3.00 ml. Add

all reagents except enzyme to tubes. Place tubes in water bath at 30° C. After 5 min., begin adding extract to the tubes on a regular time schedule (eg. each 30 sec.) for up to 4 minutes. After exactly 5 minutes incubation, add 1.5 ml of 8.75% perchloric acid to the tubes on the same time schedule. Mix well and set the tubes in ice for 30 min. to remove $KClO_4$. Centrifuge down the protein precipitate and transfer two 2.00 ml aliquots of the solution to colorimeter tubes for colorimetric analysis.

Several tubes without enzyme should be carried through the procedure to obtain a solvent for the glyoxylate standards. Each extract should be incubated without isocitrate (highest level only) to check for endogenous reaction (enzyme blank). Ordinarily, however, this is negligible because of the very high specificity of the color reaction.

Colorimetric Analysis:

Reagents

0.1% phenylhydrazine-HCl in 0.3 N HCl
4 N HCl (1:2). Chill in ice.
5% Potassium ferricyanide, freshly prepared.

Standard

Crystalline glyoxylic phenylhydrazone should beprepared according to the procedured of El Hawary and Thompson (Biochem. J., 53, 344 (1953)).

Stock - 10 micromoles/ml in 95% alcohol (1.64 mg/ml)

Working - 0.50 micromoles/ml. Dilute stock in H_2O, 1:20.

Measure duplicate 0.100 and 0.200 ml portions of the working standard (0.050 and 0.100 micromoles glyoxylate)

into colorimeter tubes and dilute to 2.00 ml with the acidified enzyme assay medium.

Procedure

To 2.00 ml portions of the samples in colorimeter tubes, add 1.00 ml phenylhydrazine. Heat exactly 2 min. in a boiling water bath and transfer rapidly to an ice water bath. after chilling, add 7.00 ml of iced 4N HCl. Mix well, remove tubes from ice bath, add 0.20 ml of ferricyanide to each tube and mix again. Let stand in air for 20 min. before reading the absorbance at 524 nm. Repeat the absorbance readings at 30 min. (from ferricyanide). The set of readings giving the higher absorbance should be taken for calculations.

6) The spectrophotometric isocitrate lyase assay used with organelle preparations contains: 66 mM potassium phosphate buffer, pH 7.5, 5.0 mM magnesium chloride, 3.3 mM phenylhydrazine-HCl, 50 micromolar EDTA, 0.25 M mannitol or sucrose and 3.3 mM threo-D_s-isocitrate in a 3.00 ml cuvette.

7) The spectrophotometric isocitrate dehydrogenase assay used with organelle preparations contains: 66 mM potassium phosphate buffer, pH 7.5, 5.0 mM magnesium chloride, 100 micromolar NADP, 50 micromolar EDTA, 0.25 M mannitol or sucrose and 3.3 mM threo-D_s-isocitrate in a 3.00 ml cuvette.

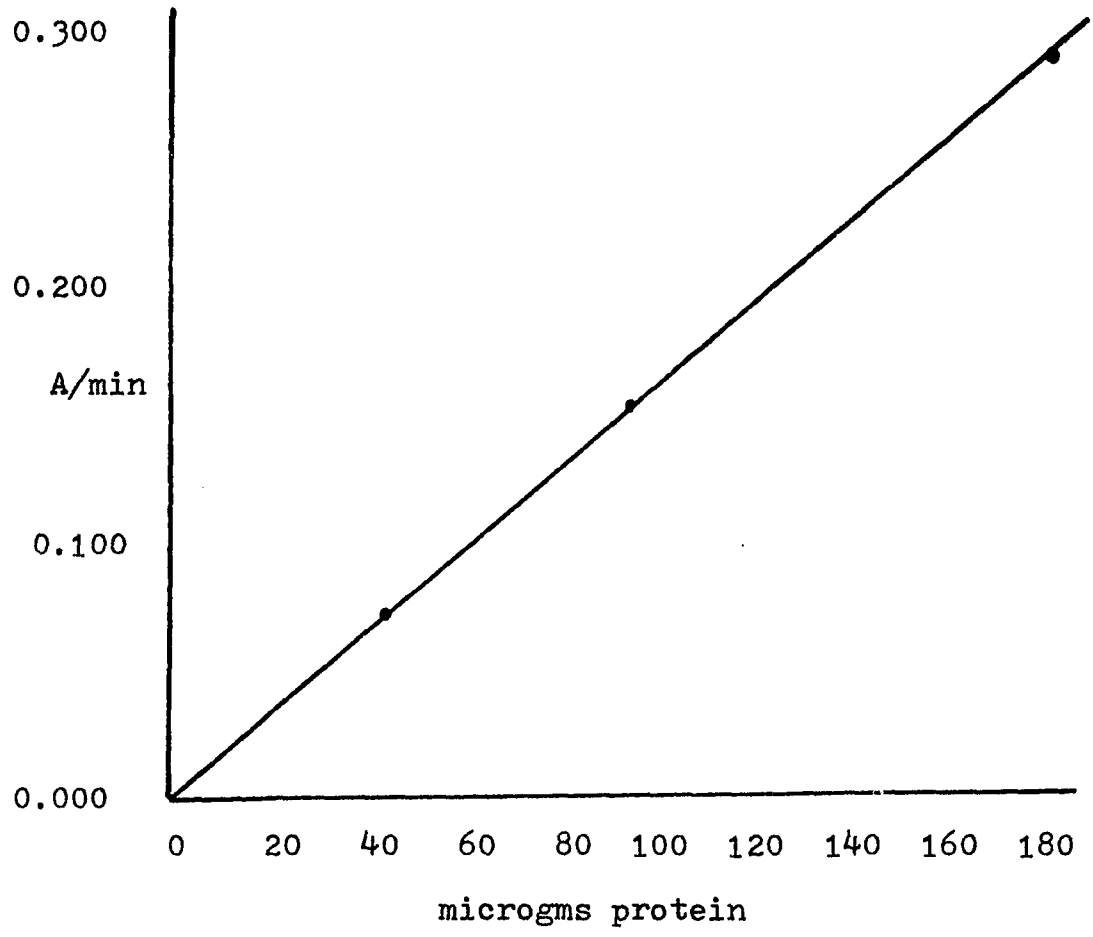
PYRUVATE KINASE ASSAY SHOWING THE CHANGE IN ABSORBANCE WITH TIME USING THE PHENYLHYDRAZINE ASSAY SYSTEM

TIME (MIN)	ABSORBANCE
0.0	0.00
1.0	0.06
2.0	0.17
3.0	0.29
4.0	0.42
5.0	0.55
6.0	0.68
7.0	0.81
8.0	0.94
9.0	1.07
10.0	1.20

The absorbance at 324 nm is measured after 3.3 mM ADP is added to a 3.00 ml cuvette containing; 66 mM potassium phosphate, pH 6.9, 5.0 mM $MgCl_2$, 6.6 mM phenylhydrazine-HCl, 1.6 mM potassium phosphoenolpyruvate and 0.0125 ml enzyme extract.

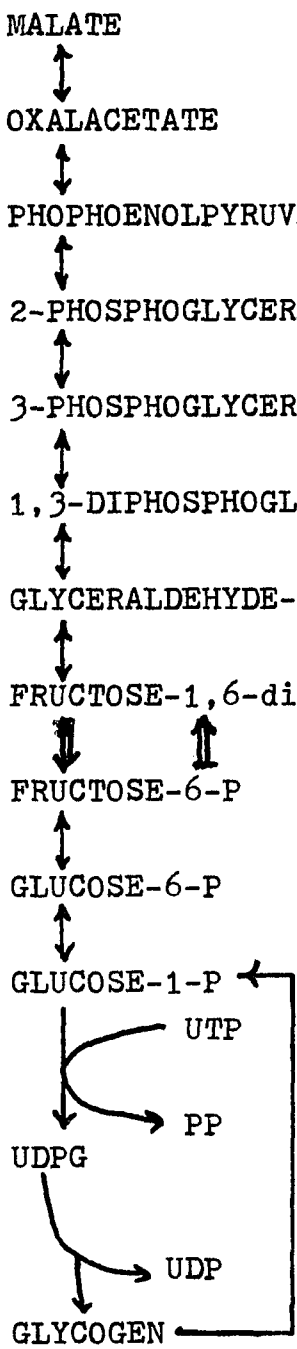
PYRUVATE KINASE GRAPH OF RATE VS

ENZYME CONCENTRATION USING THE PHENYLHYDRAZINE ASSAY



The assay system contains: 66 mM potassium phosphate pH 6.9, 5.0 m M $MgCl_2$, 6.6 m M phenylhydrazine-HCl, 1.6 m M potassium phosphoenolpyruvate plus 0.0125-0.500 ml enzyme solution in a three ml cuvette. The reaction was started by the addition of 3.3 m M ADP.

GLUCONEOGENESIS



Malate dehydrogenase

Phosphoenolpyruvate carboxylase

Enolase

Phosphoglycerate mutase

3-Phosphoglycerate kinase

3-Phosphoglyceraldehyde dehydrogenase

Aldolase

Fructose diP phosphatase (forward)
Phosphofructokinase (backward)

Phosphohexose isomerase

Phosphoglucomutase

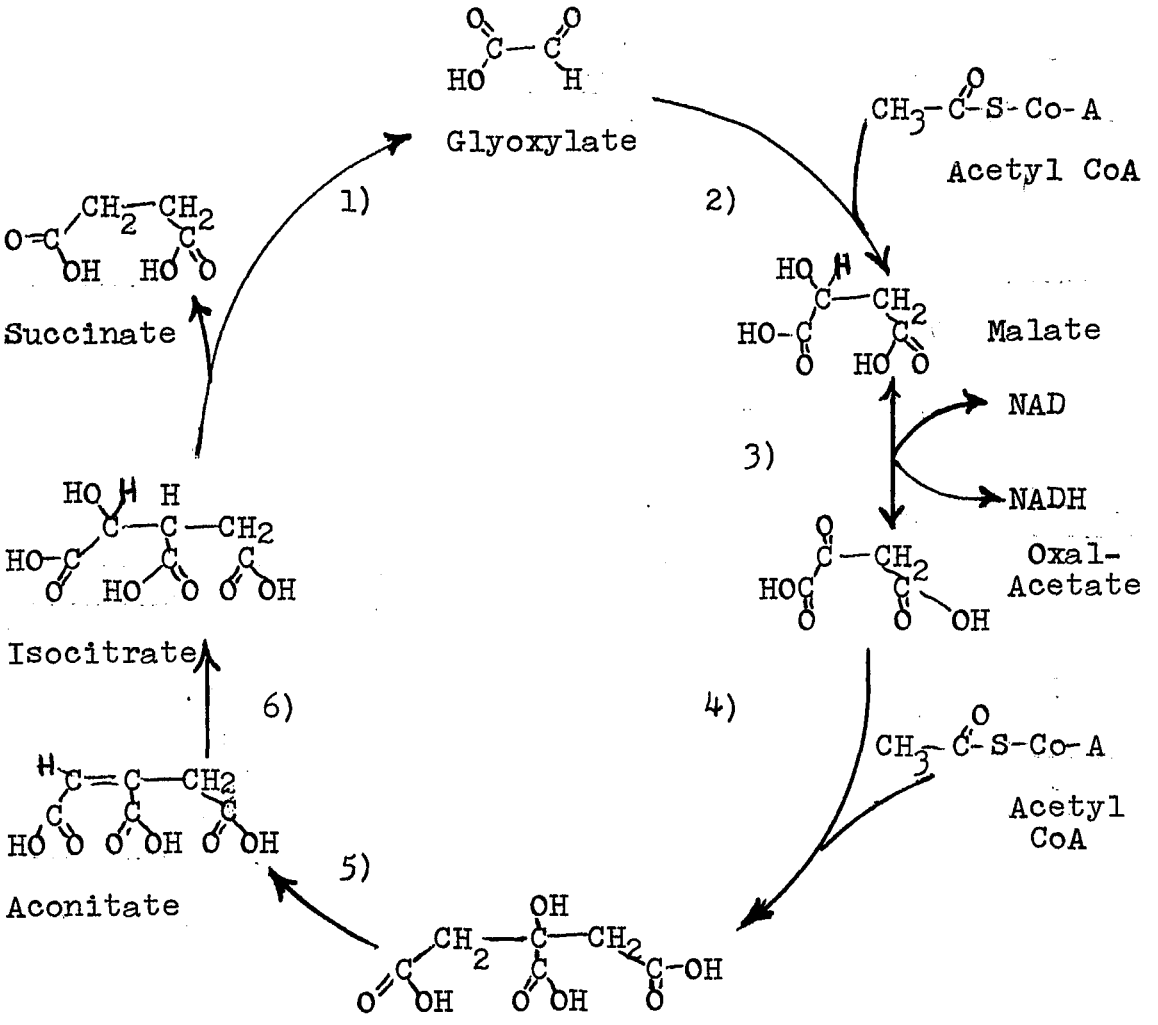
UDP-Glucose pyrophosphorylase

Glycogen synthase (forward)

Phosphorylase (backward)

* Glyceraldehyde-3-P reacts reversibly with triose phosphate isomerase to yield dihydroxyacetone-P.

GLYOXYLATE CYCLE



1) Isocitrate lyase
2) Malate synthase
3) Malate dehydrogenase
4) Citrate synthase
5) Aconitase
6) Aconitase

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