CATALYTIC PROPERTIES OF ENZYMES AT PHYSIOLOGICAL CONCENTRATIONS

By

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CATALYTIC PROPERTIES OF ENZYMES AT

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LIST OF NONSTANDARD SYMBOLS AND ABBREVIATIONS

ε - Greek epsilon, indicating absorptivity

ANS - 8-Anilino-1-naphthalene sulfonic acid

BSA - Bovine serum albumin

CS - Citrate synthase

DTE - Dithioerythritol

DTNB - 5,5'-Dithiobis-(2-nitrobenzoic acid)

F6P - D-Fructose-6-phosphate

FDP - D-Fructose-1,6-diphosphate

G6P - D-Glucose-6-phosphate

G6PD - Glucose 6-phosphate dehydrogenase

MDH - Malate dehydrogenase

OAA - Oxaloacetic acid

PC - Pyruvate carboxylase

PFK - Phosphofructokinase

PMSF - Phenylmethyl sulfonyl fluoride

S.A. - Specific activity, μmoles product formed/(min mg)

U - Unit, μmoles product formed/min

RATIONALE OF STUDIES

The kinetic methods usually used for enzymatic studies only permit measurements with enzyme concentrations less than 1 μg/ml, and very little enzyme kinetic data exist above this concentration. Enzyme concentrations in cells, however, are typically 1000 times more concentrated. At these high cellular enzyme concentrations, protein-protein interactions and enzyme polymerizations may occur, which might significantly alter the enzyme's properties from those found in the dilute assay solutions. Thus a major objective of our studies is to measure the catalytic properties of selected enzymes at concentrations comparable to their cellular levels. Enzymes which were suspected of undergoing polymerization upon increasing their concentrations from assay values (< 1 μg/ml) to cellular values (~ 1 mg/ml) were chosen for study. A fast kinetic (stopped-flow) instrument permitted the rapid kinetic measurements needed with high enzyme concentrations.

Another cause of altered kinetic properties at high enzyme concentrations exists for a few enzymes such as malate dehydrogenase and citrate synthase. With these enzymes, their concentrations in the cell equal or exceed their substrate concentrations, for example oxaloacetate and NADH. This may cause altered equilibrium and kinetic properties of these reactions.

In our project, we have also tried to avoid another deficiency

in most previous enzyme investigations, which generally studiously avoid physiological buffer components. Phosphate was avoided because it binds divalent metal ions and is thought to bind weakly to nucleotide binding sites of enzymes, and because earlier measurements, which the investigator wishes to use for comparison, were made with phosphate free buffers. But phosphate is the principal cellular buffer ion, so we have chosen to use this and other ion components at concentrations close to those that are estimated to exist in the cell. Studies in this laboratory (1) have shown, for example, that the catalytic properties of mitochondrial malate dehydrogenase are greatly altered by use of phosphate in place of the Tris buffer, which was used almost exclusively in previous investigations.

This thesis reports studies on four different enzymes or enzyme pairs: porcine liver phosphofructokinase (PFK); chicken liver pyruvate carboxylase (PC); mitochondrial malate dehydrogenase and citrate synthase coupled reactions (MDH-CS); and rat liver glucose 6-phosphate dehydrogenase (G6PD). PFK and G6PD are examples of enzymes that undergo polymerization; PC was originally thought to polymerize, and the MDH-CS enzyme system is an example of enzymes with concentrations nearly equal to substrate concentrations.

REFERENCE CITED

(1) McGurk, K. S. R. (1976) Ph.D. Dissertation, Oklahoma State University.

CHAPTER 1

PORCINE LIVER PHOSPHOFRUCTOKINASE

Introduction

Phosphofructokinase (ATP: D-fructose 6-P-1-phosphotransferase, EC 2.7.1.11) (PFK) catalyzes the following reaction:

D-fructose 6-phosphate + ATP $\longrightarrow$

fructose 1,6-diphosphate + ADP + H^+

It is cytosolic in its location although morphological changes occurring between sacrifice of animals and isolation of the enzyme cause it to be associated with particulate fractions (1,2,3).

The reaction catalyzed by PFK is the rate limiting step in glycolysis. Cori first suggested its importance as the rate limiting step in the pathway (4).

The control of the enzyme is extremely complex, including allosteric effectors, pH and concentration effects, and possibly a phosphorylation-dephosphorylation mechanism.

If the terminology of Passonneau and Lardy (5) is used, the effectors of PFK can be divided into three categories: inhibitors, activators, and deinhibitors. An activator is distinguished from a deinhibitor in that it increases the velocity of the reaction at concentrations of ATP that are noninhibitory while a deinhibitor increases the reaction velocity at ATP concentrations that are inhibitory. Using this, one may generalize and say that inhibitors of PFK include ATP, citrate, Mg^{2+}, Ca^{2+}, phosphocreatine, 3-phosphoglycerate, phosphoenolpyruvate, 2-phosphoglycerate, and 2,3-diphosphoglycerate. Activators of the enzyme are ammonium ion, potassium ion, inorganic phosphate, 5'-AMP, 3',5'-cyclic AMP, ADP, and fructose

1,6-diphosphate. Deinhibitors are fructose 1,6-diphosphate, 3',5'-cyclic AMP, 5'-AMP, ADP, fructose 6-phosphate, inorganic phosphate, and glucose 1,6-diphosphate (6). Skeletal muscle PFK is known to exist in different molecular weight forms depending upon the pH and the enzyme concentration (7,8,9,10,11). Acid pH favors dissociation as does dilution. Both of these appear to proceed by the same mechanism (7).

The enzymatic activity is dependent upon the state of aggregation of the enzyme (11,12). Forms smaller than the tetramer do not possess significant activity. Lad et al. (16) report that the specific activity of the rabbit muscle PFK decreases when it is diluted from a concentrated stock solution of greater than 10 mg/ml in a 0.1 M potassium phosphate, 1.0 mM EDTA, pH 8.0 buffer to a concentration of 0.15 mg/ml in a 0.1 M potassium phosphate, 1.0 mM EDTA, and 1.0 or 5.0 mM DTE, pH 7.0. They find that the final equilibrium specific activity and the rate at which the specific activity approaches equilibrium are dependent upon the final protein concentration. These changes in the specific activity reflect changes in the state of aggregation of the enzyme.

Effectors appear to modify the effects of acid pH and dilution which cause a dissociation of the enzyme (10,12,13,14,15). For example, fructose 1,6-diphosphate at a concentration of 5.0 mM causes reassociation of PFK previously diluted to a concentration of 0.15 mg/ml, and MgATP at a concentration of 5.0 mM (considered to be inhibitory) prevents dissociation of the enzyme upon dilution (16). Citrate, an inhibitor of PFK, appears to cause the higher polymeric forms of the enzyme to dissociate, and to stabilize the monomeric and

dimeric forms of the enzyme (16).

Viñuela et al. presented evidence for a phosphorylation-dephosphorylation mechanism of PFK from yeast (17). The PFK existed in two forms, one sensitive to inhibition by ATP and citrate and the other insensitive. The insensitive form was believed to be the phosphorylated enzyme, the phosphorylation catalyzed by a "desensitizing protein."

Further work by another group confirmed the existence of the sensitive and insensitive forms of PFK, but disagreed with the interpretation (18,19). They found that the conversion between the two forms of the enzyme is entirely mediated by effectors, and that the desensitizing protein did not catalyze a modification of the enzyme directly, but instead modified the assay system composition so that ATP, which prevents the transformation of a PFK form sensitive to ATP inhibition to one not sensitive, is converted to ADP which favors the conversion. The resulting enzyme is then more active. Other groups express the view that PFK exists in two conformational states (20,21). The conformation is believed to again be due to effectors. More recently, Brand and Söling have presented evidence for a phosphorylation-dephosphorylation mechanism for PFK from rat liver (22). This involves both an activating and an inactivating protein. The phosphate group appears to be attached to a serine residue in the protein. They show a decrease in enzyme activity concommitant with the release of P_i from the active protein. Hofer and Fürst have isolated a radioactive PFK from the skeletal muscle of mice previously injected with [^{32}P]orthophosphate (23). Through acid hydrolysis, they too find evidence for the presence of an

O-phospho-L-serine residue in the protein.

PFK from liver might differ from the same enzyme from skeletal muscle as liver is a gluconeogenic tissue while the skeletal muscle is a glycolytic tissue. In agreement with this expectation the liver and the muscle PFK's are actually different isoenzymes (24,25,26). The rabbit isoenzymes differ in several respects (27). These include electrophoretic mobility, stability, molecular weight, and kinetic properties. For instance, the liver enzyme at pH 7.0 is more inhibited by ATP and less sensitive to the deinhibiting action of AMP, ADP, and c-AMP. It is less inhibited by citrate, phosphoenolpyruvate, phosphocreatine, 2-phosphoglycerate and 3-phosphoglycerate, and more inhibited by 2,3-diphosphoglycerate. Because of the above differences, which suggest that the liver PFK is less suited to anaerobic energy production, and because it is reported to undergo extensive polymerization (27), it appears to be an excellent candidate for study.

Experimental

Materials

KH_2PO_4, NH_4Cl, K_2SO_4, and phenol were purchased from the Fisher Scientific Company; ultra pure EDTA and $(NH_4)_2SO_4$ were from Schwarz-Mann; and $MgCl_2$ came from Mallinckrodt. Tris base, 2-mercaptoethanol, tannic acid, gum arabic, ATP, ADP, F6P, FDP, and NADH came from the Sigma Chemical Company. The enzymes, aldolase, α-glycerol phosphate dehydrogenase, and triose phosphate isomerase were also from the Sigma Chemical Company. The α-glycerol phosphate dehydrogenase and triose phosphate isomerase were purchased as a mixture. Bovine

serum albumin came from Pentex, while the pig livers were obtained from Ralph's Packing Company, Perkins, Oklahoma immediately upon their removal from the animals.

Methods

Enzyme Purification. Immediately upon obtaining pig livers, they were placed in plastic freezer bags, closed, and frozen in powdered dry ice. They were then transported in this condition to the laboratory and stored at -25°C until needed. Livers were allowed to partially thaw in the cold room, 2-5°C, overnight before being used. The purification procedure used is that of Massey and Deal (28,29). The later reference is more informative and would be one's choice for instructions.

One should pay great attention to the time of the year in procuring livers for this isolation. Massey and Deal mention that livers from winter animals provide the best source for the enzyme. It appears that only abnormally cold Oklahoma winters provide suitable material for this procedure. The warmer winters provide livers which result in difficulties in the solubilization-precipitation steps as the greatest portion of the enzyme remains insoluble.

Determination of Protein Concentration. The protein concentration is determined by the method of Katzenellenbogen and Dobryszycka as modified by Massey and Deal (29). The method is turbidimetric, making use of bovine serum albumin as the protein standard, tannic acid, and gum arabic which is used to stabilize the turbidity. Other methods of protein determination are unsuitable due to the high

concentrations of 2-mercaptoethanol in this isolation procedure.

Reagent Preparations. Substrate solutions were made and the pH adjusted the same day they were used. $MgCl_2$ used in the enzyme isolation and in enzyme assays was purified using the method of Morrison and Uhr (30) in order to remove any heavy metals.

Enzyme Assay. A coupled spectrophotometric assay was used to determine the activity of the PFK. Unless otherwise specified, the assay reaction mixture contained 50 mM Tris-Cl, 10 mM KH_2PO_4, 5 mM $MgCl_2$, 50 mM 2-mercaptoethanol, 2 mM ATP, 4 mM F6P, 0.2 mM NADH, 50 µg/ml of the α-glycerol phosphate dehydrogenase and triose phosphate isomerase mixture, and 100 µg/ml aldolase all at pH 8.0. The decrease in absorbance was monitored at 340 nm and 25°C. The activity was calculated using a value of 6.22 mM^{-1} cm^{-1} for the extinction coefficient of NADH. Since two moles of NAD^+ are formed for every mole of F6P converted to FDP, one unit of enzyme is defined as the conversion of 2.0 µmoles of NADH to NAD^+ per minute.

Instrumentation. All measurements were performed on a Coleman 124 spectrophotometer. It is equipped with a No. 165 recorder, a No. 0319 thermostatted cell holder, a Haake model FS constant temperature circulator, and a No. 801 scale expander.

Results and Discussion

Enzyme Purity

Massey and Deal (28,29) have reported that pure porcine liver PFK has a specific activity of 100 units per milligram of enzyme protein.

All of the enzyme preparations used in this study had a specific activity of 100 upon completion of the purification.

Investigation of Lag Times

In the course of performing assays of the PFK a lag period was observed before a linear velocity was obtained. Increasing the concentration of the coupling enzymes did not reduce the lag. The lag has been reported previously, but the method used to deal with the lags was to read the velocity after a given period of time (27).

In a limited study, we tried various treatments to assess their effect on the assay. Results are summarized in Tables I, II, and III. The reference buffer for the study was 50 mM Tris-Cl, 5 mM $MgCl_2$, and 50 mM 2-mercaptoethanol. The enzyme was first diluted into an incubation mixture at 240 µg enzyme/ml and was then added to the assay cuvette to initiate the reaction at 1.2 µg enzyme/ml. Incubation times within conditions tested ranged between 1-20 minutes with no significant changes in the lag time as a function of the time of incubation. The addition of phosphate to the incubation and/or assay mixture increased lag times from about 33 to 45 seconds (Table I). Nevertheless, since phosphate is a physiological buffer, it was used in both incubation and assay solutions for the majority of tests. All combinations of a single substrate with a single product used in the incubation solutions increased lag times relative to the reference condition. Incubation of the enzyme with both substrates (the last row of Table I), however, decreased the lag time noticeably, although it remained appreciable. If all of the substrates present in this test condition were converted

TABLE I

STUDY OF LAG TIMES[a]

Additions to Incubation Mixture	PO_4^{3-} in Assay Medium	Time Before Linearity, seconds
-	Yes	45.5
-	No	33.0
10 mM PO_4^{3-}	Yes	43.8
4 mM F6P	Yes	39.3
4 mM F6P	No	30.6
4 mM F6P + 9 mM PO_4^{3-}	Yes	54.1
4 mM F6P + 9 mM PO_4^{3-}	No	44.3
4 mM F6P + 5 μM FDP + 9 mM PO_4^{3-}	Yes	61.7
4 mM F6P + 25 μM FDP + 9 mM PO_4^{3-}	Yes	65.7
2 mM ATP + 5 μM FDP + 9 mM PO_4^{3-}	Yes	51.0
2 mM ATP + 25 μM FDP + 9 mM PO_4^{3-}	Yes	65.2
4 mM F6P + 0.5 mM ADP + 9 mM PO_4^{3-}	Yes	49.7
4 mM F6P + 1.5 mM ADP + 9 mM PO_4^{3-}	Yes	47.3
2 mM ATP + 0.5 mM ADP + 9 mM PO_4^{3-}	Yes	47.5
2 mM ATP + 1.5 mM ADP + 9 mM PO_4^{3-}	Yes	57.3
2 mM ATP + 9 mM PO_4^{3-}	Yes	40.3
2 mM ATP + 4 mM F6P + 9 mM PO_4^{3-}	Yes	28.2

[a]Concentrations of PFK in the incubation mixture and the assay mixture were 0.24 mg/ml and 0.012 mg/ml respectively. The incubation mixture was 50 mM Tris-Cl, 5 mM $MgCl_2$, 50 mM 2-mercaptoethanol, pH 8.0 plus the specified additions. The assay was performed as described under "Methods".

TABLE II

EVALUATION OF THE INCUBATION OF SUBSTRATE OR SUBSTRATE AND PRODUCT ON THE SPECIFIC ACTIVITY[a]

4 mM F6P[b] without PO_4^{3-}		4 mM F6P		4 mM F6P 5 μM FDP		4 mM F6P 25 μM FDP		2 mM ATP 5 μM FDP		2 mM ATP 25 μM FDP		4 mM F6P 0.5 mM ADP		4 mM ADP 1.5 mM ADP		2 mM ATP 0.5 mM ADP		2 mM ATP 1.5 mM ADP		2 mM ATP	
min	S.A.	min	S.A.	min	S.A.	min	S.A.	min	S.A.	min	S.A.	min	S.A.	min	S.A.	min	S.A.	min	S.A.	min	S.A.
0.83	91.9	7.17	98.7	3.10	70.6	3.07	76.6	2.0	68.8	2.67	68.8	1.67	69.4	3.32	79.6	2.95	73.9	2.50	67.4	1.78	69.0
4.17	90.6	10.08	101.0	8.00	93.0	7.50	82.5	8.0	78.0	8.12	78.9	7.50	93.9	8.25	90.6	8.18	83.1	7.58	91.2	6.12	85.2
32.75	121.0	35.58	107.9	16.08	98.6	16.17	91.0	16.0	89.7	17.70	58.9[c]	18.20	93.0	16.50	99.8	17.42	93.0	12.43	95.7	13.28	97.1
		38.50	113.3																		
30.05	110.0	65.75	130.0																		
60.03	127.6	68.05	122.0																		
63.00	123.4	97.40	113.5																		
90.03	116.0																				
92.50	129.7																				

[a] The enzyme was diluted from a stock solution of 4.45 mg/ml in 50 mM Tris-Cl, 50 mM 2-mercaptoethanol, 110 mM $(NH_4)_2SO_4$, 5 mM $MgCl_2$, 0.1 mM ATP, and 0.1 mM FDP at pH 8.0 into the incubation buffer described in Table I. Assays were performed as described under "Methods".

[b] Two separate experiments were run, as indicated by the space in the column.

[c] This value is lower than expected and is probably in error.

TABLE III

THE EFFECT OF PHOSPHATE ON THE SPECIFIC ACTIVITY[a]

Incubation Times	S.A. without Phosphate	S.A. with Phosphate
~ 1 minute	103	92.4
~ 6 minutes	103	88.7
~ 15 minutes	97.5	88.7

[a]The incubation mixture contained 0.238 mg PFK/ml of a 50 mM Tris-Cl, 5 mM $MgCl_2$, 50 mM 2-mercaptoethanol, pH 8.0 buffer either with or without 10 mM potassium phosphate as indicated. The assay was performed as described under "Methods" with 1.19 µg PFK/ml.

to products, the resulting 200-fold dilution into the assay mixture would decrease ADP to 1 μM and FDP to 2 μM, concentrations too low to affect the maximal velocity determination.

Specific Activity Dependence on Time of Incubation and Enzyme Concentration

The lag time studies showed some interesting trends. The enzyme, incubated at a concentration of 0.22 mg/ml with either one substrate or one substrate and one product shows an increase in specific activity (Table II). This increase is at times as great as 40 percent in less than 15 minutes of incubation. Incubation of the enzyme at a concentration of 0.24 mg/ml without substrate or product results in no increase in the specific activity (Table III).

Because the lag was least pronounced when starting the assay with PFK incubated with both substrates it was decided to initiate the assay in this manner. The standard assay composition described in the "Methods" section was used throughout the studies. Varying concentrations of the PFK were incubated with F6P and ATP as indicated in Tables IV-VII. Aliquots were taken at specified times and assayed. The incubation mixture was diluted two hundred fold into the assay cuvette. As can be seen, the enzyme incubated at a concentration of 0.153 mg/ml shows a specified activity of about 110 units/mg and does not change with the time of incubation. Enzyme incubated at a concentration of 0.096 mg/ml shows an increased specific activity (about 180 units/mg), but again does not appear to change significantly with the incubation time. Dramatic results begin to appear at a concentration of 15 μg of PFK/ml of the incubation mixture (Table VI).

TABLE IV

INCUBATION AND ASSAY OF 0.153 mg PFK/ml, SPECIFIC ACTIVITIES[a]

Incubation Time, Minutes and Seconds	Trial Number			Average
	1	2	3	
1' 50"	100.8	104.8	125.8	110.5
5' 20"	98.0	105.9	120.3	108.1
12' 00"	109.0	114.0	121.0	114.7
23' 00"	-	-	112.0	-

[a]The enzyme was incubated with 8 mM ATP and 16 mM F6P in the assay buffer described in the "Methods" section. The assay is described in the "Methods" section.

TABLE V

INCUBATION AND ASSAY OF 0.0959 mg PFK/ml, SPECIFIC ACTIVITIES[a]

Incubation Time, Minutes and Seconds	Trial Number			Average
	1	2	3	
1' 50"	148	191.8	180.8	173.5
5' 20"	183.5	177.8	176.7	179.3
12' 00"	191.8	191.2	181.1	188.0
23' 00"	188.5	176.1	186.5	183.7

[a]The enzyme was incubated with 10 mM ATP and 20 mM F6P in the assay buffer described in the "Methods" section. The assay is also described in the "Methods" section.

TABLE VI

INCUBATION AND ASSAY OF 0.0153 mg PFK/ml, SPECIFIC ACTIVITIES[a]

Incubation Time, Minutes and Seconds	Trial Number			Average
	1	2	3	
1' 50"	366.8	270.4	310.2	315.8
5' 20"	807	622.5	643.5	691.0
12' 00"	926.5	855.2	763	848.2
23' 00"	815	962.1	813.3	863.5

[a]Incubation and assay of the enzyme are as in Table IV.

TABLE VII

INCUBATION AND ASSAY OF 9.59 μg PFK/ml[a]

Incubation Time Minutes and Seconds	Specific Activity
1' 50"	224.7[b]
5' 20"	583.6[b]
12' 00"	1227
23' 00"	1563
35' 00"	1570
45' 00"	1667
61' 20"	1650
81' 05"	1593

[a]The incubation and assay of the enzyme are as in Table V.

[b]These values are lower than expected and may be in error.

Here the initial specific activity is increased almost three fold over the enzyme incubated at a concentration ten times higher. A time effect is also present, with the enzyme increasing in specific activity with increasing incubation time. Again, startling results are obtained when the enzyme is incubated at 9.59 μg/ml. The specific activity increases to a value greater than 1600 units/mg upon incubation for 45 minutes (Table VII).

The time dependence for these cases is pictured in Figure 1 where the more dilute enzyme is seen to increase in activity to a far greater extent than the more concentrated enzyme and the time of activation is longer. The dependence of the "infinite time" specific activity on the enzyme concentration is depicted in Figure 2 and Table VIII. Again the increase in specific activity with decreasing enzyme concentration is seen.

The dramatic increase in the specific activity appears to require an enzyme dissociation-association equilibrium with more active subunits. Since the increased activity with decreasing enzyme concentration does not occur in the absence of substrate (Table III), the dissociation equilibrium appears to be coupled to the preferential binding of the substrates to the dissociated enzyme, as in scheme 1,

$$\begin{array}{ccccccccc} E_4 & + & 4S & \overset{K_1}{\rightleftharpoons} & E_4S_4 & \xrightarrow{V_1} & E_4 & + & 4P \\ \Big\Vert & & & & & & & & \\ 4E & + & 4S & \underset{K_2}{\rightleftharpoons} & 4ES & \xrightarrow{V_2} & 4E & + & 4P \end{array} \qquad (1)$$

where E represents PFK. This necessitates that the binding constant $K_2 > K_1$, and the maximal velocity $V_2 > V_1$. In view of the evidence

Figure 1. The Dependence of PFK Specific Activity in the Enzyme Concentration and Time During Incubation. Incubation and assay of the enzyme are as in Tables VI and VII. Enzyme concentrations are: 9.59 µg/ml, 0—0; 15.3 µg/ml, □—□. The values plotted are averages of three experiments at 15.3 µg PFK/ml, but a single experiment at 9.6 µg PFK/ml.

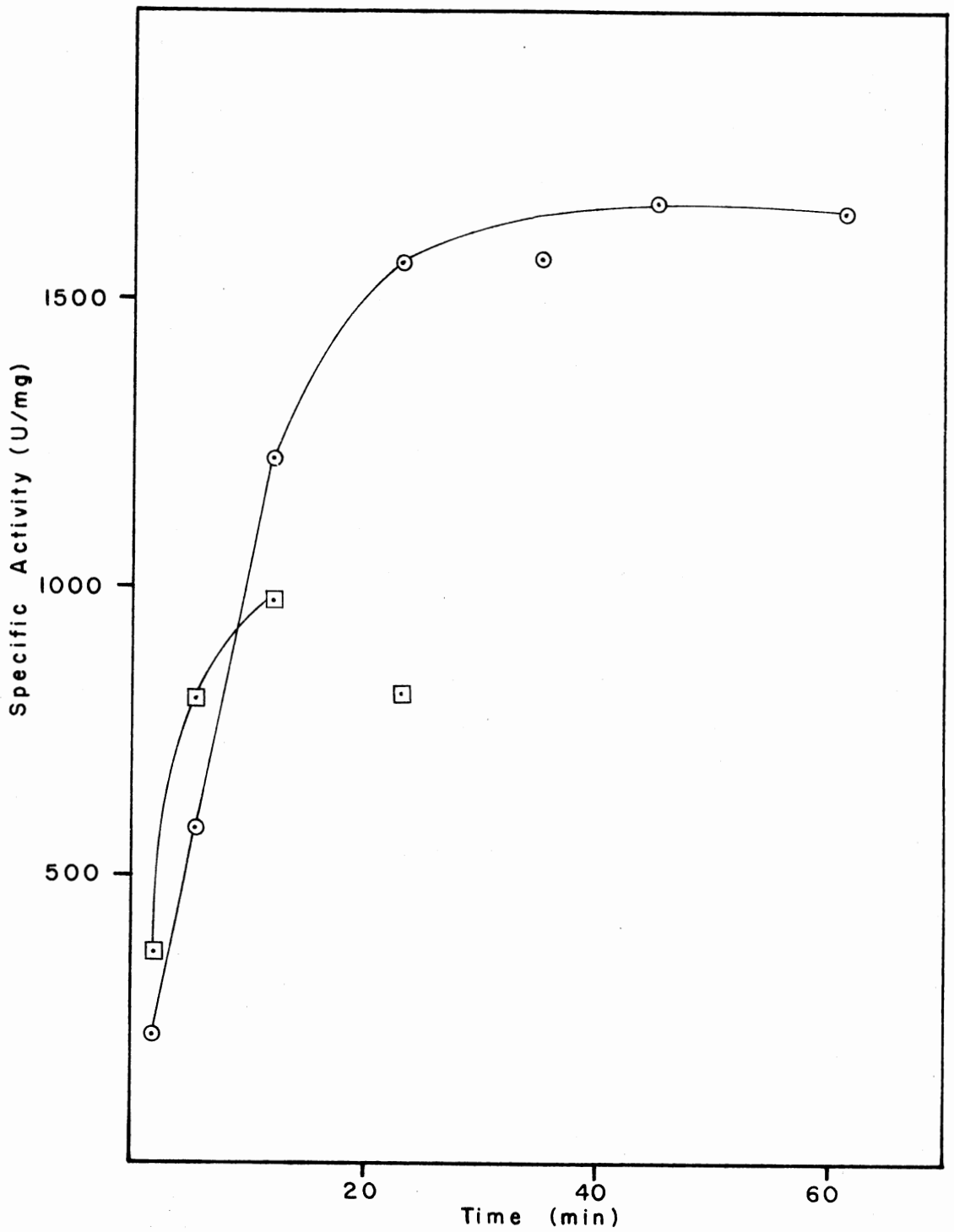

Specific Activity (U/mg)
1500
1000
500
20
40
60
Time (min)

Figure 2. The Dependence of PFK Specific Activity on the PFK Concentration After "Infinite" Time Incubation. Incubation and assay of the enzyme were performed as in Tables IV-VII. The value plotted for 9.59 μg/ml is from one experimental determination, the rest are averages of three determinations.

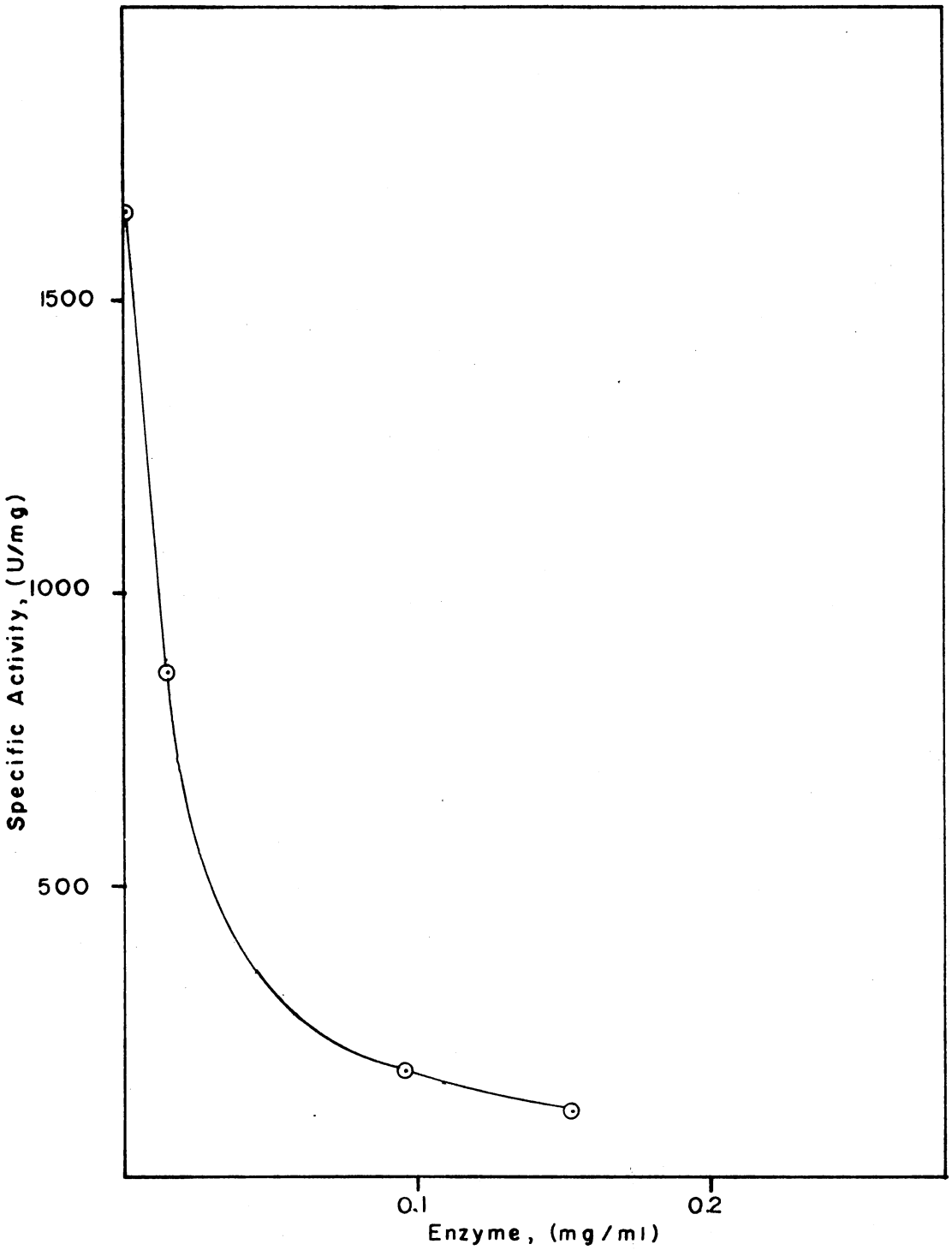

1500
1000
500
Specific Activity, (U/mg)
0.1
0.2
Enzyme, (mg/ml)

TABLE VIII

COMPARISON OF ENZYME CONCENTRATION, INCUBATION TIMES, AND SPECIFIC ACTIVITIES[a]

Enzyme Concentration mg/ml	Incubation		
	1' 50"	23' 00"	Equilibrium Value
0.153	110.5	112.0	112.0
0.0959	173.5	183.7	183.7
0.0153	315.8	863.5	863.5
0.00959	224.7[b]	1563	1650

[a]The incubation and assay of the enzyme are as in the previous tables.

[b]This value is lower than expected and is probably in error.

for enzymes modifying PFK (22) however, the possibility that a PFK kinase impurity exists with preferential activity toward dissociated enzyme in the presence of substrates should also be considered.

Though further experiments would greatly clarify the mechanism, we have been unable to pursue them, since repeated attempts to purify more enzyme have failed. Eight purifications were attempted with the inability in all of them to solubilize the enzyme after having precipitated it. The effects described above, however, were seen with two different preparations.

Summary and Conclusion

Incubation and assay of porcine liver PFK in a buffer containing 10 mM potassium phosphate lead to increased lag times over the enzyme assayed in the same buffer without phosphate. Incubating the enzyme with both of its substrates reduced this lag. Incubation of the enzyme with one substrate, or with one substrate and one product resulted in an increasing specific activity with time. When the PFK was incubated with both of its substrates, an enzyme concentration dependent increase in specific activity occurred. The increase in specific activity is greatest with an enzyme concentration of 9.6 µg/ml PFK where a specific activity of 1650 U/mg is observed (100 U/mg is the previously reported maximum). An enzyme dissociation-association equilibrium with preferential binding of the substrates to the dissociated enzyme is proposed, although the possibility of a PFK kinase impurity with preferential activity toward dissociated enzyme in the presence of substrates is not eliminated yet.

REFERENCES CITED

(1) Mansour, T. E., Wakid, N., and Sprouse, H. M. (1966) J. Biol. Chem., 241, 1512-1521.

(2) Mansour, T. E. (1970) in Advan. Enzyme Regul. (Weber, G., ed.) Vol. 8, pp. 37-51.

(3) Mansour, T. E. (1963) J. Biol. Chem., 238, 2285-2292.

(4) Cori, C. F. (1942) in A Symposium on Respiratory Enzymes, p. 175, University of Wisconsin Press, Madison.

(5) Passonneau, J. V. and Lowry, O. H. (1964) in Advan. Enzyme Regul. (Weber, G., ed.) Vol. 2, pp. 265-274.

(6) Ramiah, A. (1974) in Current Topics in Cellular Regulation (Horecker, B. L. and Stadtman, E. R., ed.) Vol. 8, pp. 297-345.

(7) Hofer, V. H. W. and Pette, D. (1968) Hoppe-Seyler's Z. Physiol. Chem., 349, 1105-1114.

(8) Layzer, R. B., Rowland, L. P., and Bank, W. J. (1969) J. Biol. Chem., 244, 3823-3831.

(9) Parmeggiani, A., Luft, J. H., Love, D. S., and Krebs, E. G. (1966) J. Biol. Chem., 241, 4625-4637.

(10) Leonard, K. R. and Walker, I. O. (1972) Eur. J. Biochem., 26, 442-448.

(11) Pavelich, M. J. and Hammes, G. G. (1973) Biochemistry, 12, 1408-1414.

(12) Paetkau, V. and Lardy, H. A. (1967) J. Biol. Chem., 242, 2035-2042.

(13) Hofer, H. W. (1971) Hoppe-Seyler's Z. Physiol. Chem., 352, 997-1004.

(14) Emerk, K. and Frieden, C. (1975) Arch. Biochem. Biophys., 168, 210-218.

(15) Hulme, E. C. and Tipton, K. F. (1971) FEBS Lett., 12, 197-200.

(16) Lad, P. M., Hill, D. E., and Hammes, G. G. (1973) Biochemistry, 12, 4303-4309.

(17) Viñuela, E., Salas, M. L., Salas, M., and Sols, A. (1964) Biochem. Biophys. Res. Commun., 15, 243-249.

(18) Afting, E.-G., Ruppert, D., Hagmaier, V., and Holzer, H. (1971) Arch. Biochem. Biophys., 143, 587-592.

(19) Lynen, A., Afting, E.-G., and Holzer, H. (1973) FEBS Lett., 30, 71-73.

(20) Alpers, J. B., Paulus, H., and Bazylewicz, G. A. (1971) Proc. Nat. Acad. Sci., U.S.A., 68, 2937-2940.

(21) Ramaiah, A. and Gopi, A. (1973) Eur. J. Biochem., 39, 183-192.

(22) Brand, I. A. and Söling, H. D. (1975) FEBS Lett., 57, 163-168.

(23) Hofer, H. W. and Fürst, M. (1976) FEBS Lett., 62, 118-122.

(24) Gonzáles, F., Tsai, M. F., and Kemp, R. G. (1975) Comp. Biochem. Physiol., 52B, 315-319.

(25) Tsai, M. Y. and Kemp, R. G. (1972) Arch. Biochem. Biophys., 150, 407-411.

(26) Kirby, W. and Taylor, C. B. (1974) Int. J. Biochem., 5, 89-93.

(27) Kemp, R. G. (1971) J. Biol. Chem., 246, 245-252.

(28) Massey, T. H. and Deal, W. C., Jr. (1973) J. Biol. Chem., 248, 56-62.

(29) Massey, T. H. and Deal, W. C., Jr. (1975) in Methods in Enzymology (Wood, W. A., ed.) Vol. 42, pp. 99-110, Academic Press, New York.

(30) Morrison, J. F. and Uhr, M. L. (1966) Biochim. Biophys. Acta, 122, 57-74.

CHAPTER II

CHICKEN LIVER PYRUVATE CARBOXYLASE

Introduction

Pyruvate carboxylase (pyruvate: CO_2 ligase (ADP); EC 6.4.1.1) catalyzes the following reaction:

$$\text{pyruvate} + \text{MgATP}^{2-} + \text{HCO}_3^- \xrightleftharpoons[\text{Me}^+,\ \text{Me}^{2+}]{\text{acetyl-CoA}} \text{oxaloacetate} + \text{MgADP}^- + \text{P}_i^{\,2}$$

where Me^+ is a univalent cation and Me^{2+} is a divalent cation.

The chicken liver enzyme is converted from an essentially inactive form to the active form by the binding of acetyl-CoA. This conversion of the enzyme from an inactive to an active form is apparently due to a conformational transition (1,2). Previous measurements indicated a large increase in the enzyme's acetyl-CoA activation constant (the concentration of acetyl-CoA required to give half of the enzyme maximal activity) when the concentration of the pyruvate carboxylase was increased above 10 U/mg (0.7 mg/ml) (3). Enzyme association from the normal tetrameric form to octamers at high enzyme concentrations has been reported (1,4). The effective cellular concentration of pyruvate carboxylase has been estimated to be greater than 20 mg/ml (1), so that altered properties at high enzyme concentrations relative to the usual low assay concentrations (~ 0.002 mg/ml) are relevant to its physiological properties.

McGurk (2), using ANS-fluorescence studies, confirmed conformational changes in pyruvate carboxylase with the addition of acetyl-CoA to the enzyme, and also with the addition of MgATP. The fluorescence quenching accompanying the conformational change occurred

within 4 ms of mixing the enzyme with the respective ligand, indicating that the half times of activation are less than 4 ms, when acetyl-CoA was added or already present.

In this study we attempt to determine whether there is a correlation between the rate of catalytic activation of pyruvate carboxylase by acetyl-CoA and MgATP and the rate of enzyme conformational transitions induced by these ligands and reported by McGurk. We also reexamine the reports of Hill (3) that the acetyl-CoA activation constant increases as the enzyme concentration is increased, and that the specific activity of the enzyme decreases as the enzyme concentration is increased.

Experimental

Materials

Tris base, PMSF, DTNB, DTE, ATP, NADH, LiCoA, sucrose, Sephadex A-50, and Sephadex G-50 were purchased from the Sigma Chemical Company; ultrapure EDTA and ammonium sulfate were from Schwarz-Mann; and oxaloacetate was from Calbiochem. Pyruvic acid was supplied by the Aldrich Chemical Company; $MgCl_2$ was from Mallinckrodt; KH_2PO_4, acetic anhydride, and $KHCO_3$ came from Fisher Scientific. Porcine heart malate dehydrogenase was purchased from Sigma, citrate synthetase from Boehringer, and BSA was from Pentex. Cornish-White Rock cross chicks were purchased from the Stillwater Hatchery.

Methods

Reagent Preparations. $MgCl_2$ was further purified by the method

of Morrison and Uhr (5). Pyruvic acid was vacuum distilled twice and stored frozen as a 1 M solution. The acetyl-CoA was prepared from LiCoA and acetic anhydride as suggested by Frey and Utter (6). The LiCoA was dissolved in ice cold 0.1 M $KHCO_3$ and acetic anhydride at approximately 134% the molar concentration of the LiCoA was added (this is the amount of acetic anhydride necessary to give a negligible amount of unreacted LiCoA as determined with DTNB). The reaction mixture was kept cold at all times. The pH of the acetyl-CoA was adjusted just prior to use to the value desired in the measurements. The acetyl-CoA was assayed with citrate synthetase, oxaloacetate, and DTNB (7). ATP stock concentrations were calculated from spectrophotometric data assuming ε = 15,400 M^{-1} cm^{-1} at 259 nm (8).

Coupling Enzyme Preparation. The ammonium sulfate precipitate of malate dehydrogenase was collected by centrifugation and then dialyzed 4-5 times against 100 volumes of 10 mM potassium phosphate buffer, 1 mM EDTA, 8.58 mM $MgCl_2$, 2 mM DTE, pH 7.6.

Pyruvate Carboxylase Purification. The method of Scutton and Fung (9) was used in the purification of the pyruvate carboxylase. The livers of 9-10 week old Cornish-White Rock chickens (starved 36 hours prior to sacrifice) were used for the purification. Purified enzyme was stored at pH 7.2 in 1.5 M sucrose, 0.025 M KPO_4, 0.05 M $(NH_4)_2SO_4$, 1 mM EDTA, 2 mM DTE, and 2 µM PMSF at 5°C. The Sephadex G-25 column used in the purification was replaced with a 1.5 hour dialysis against 3 liters of 0.025 M potassium phosphate buffer, 2 mM EDTA, 5 mM 2-mercaptoethanol, 2 µM PMSF, pH 7.2. Prior to use,

pyruvate carboxylase was passed over a Sephadex G-50 (medium) column at room temperature to remove sulfate.

Enzyme Assays. Malate dehydrogenase was assayed at 340 nm and 25°C. The assay mixture was 10 mM potassium phosphate, 1 mM EDTA, 8.58 mM $MgCl_2$, 2 mM DTE, 0.1 mM oxaloacetate, 0.2 mM NADH, all at pH 7.6. One unit of malate dehydrogenase was defined as the conversion of 1 μmole of NADH to NAD^+ per minute. An absorptivity of 6.22 mM^{-1} cm^{-1} was used for NADH. Pyruvate carboxylase was assayed spectrophotometrically at 340 nm and 25°C by coupling the reaction with malate dehydrogenase. The functional definition of one unit of enzyme is again the conversion of 1 μmole of NADH to NAD^+ per minute. The assay mixture consisted of 10 mM potassium phosphate, 1 mM EDTA, 8.58 mM $MgCl_2$, 2 mM DTE, 10 mM pyruvate, 2 mM ATP, 15 mM $KHCO_3$, 0.1 mM acetyl-CoA, 0.2 mM NADH, and about 5 U/ml malate dehydrogenase, all at pH 7.6. The reaction was initiated by the addition of pyruvate carboxylase.

The protein concentration was determined by the method of Kalckar (10) and increased by a factor of 2.0 (11). Thus these specific activities are two fold lower than those reported before this correction was determined.

Instrumentation. Assays were performed at 25°C in a Coleman 124 spectrophotometer and stopped-flow apparatus as described in the glucose 6-phosphate dehydrogenase studies.

Results and Discussion

Rates of Enzymatic Reactions Following Addition of Acetyl-CoA or MgATP

The rates of pyruvate carboxylation were measured at high enzyme concentrations to correlate rates (or lower limits of rates) of enzyme activation by acetyl-CoA or MgATP with the rates of induced conformational changes seen by McGurk (2), and to test for changes in the acetyl-CoA activation constant, Ka, with increased enzyme concentration as observed by Spivey and Hill (12). A high pyruvate carboxylase concentration was chosen so that its activity could be calculated from a segment of the progress curve (~ 2 μM extent of reaction) within 2 milliseconds after the flow was stopped, assuming the enzyme would develop maximum activity during the 4 milliseconds of flow dead-time. Malate dehydrogenase concentrations were chosen so that the time to achieve 90 percent of the steady-state velocity in the coupled reaction was about 1.0 millisecond (13). Using the convention, [A] + [B], to represent rapid mixing of reactants A, stored initially in one drive syringe, with reactants B stored initially in the second syringe, the experiments were made with [acetyl-CoA + pyruvate] + [enzymes + MgATP + HCO_3^- + $MgCl_2$ + NADH] to measure enzyme activation times by acetyl-CoA, and a similar distribution except for interchange of acetyl-CoA and MgATP to measure duration of the presteady-state response of enzyme to MgATP addition.

Maximum enzyme specific activities were found at the earliest times measurable (4 milliseconds dead-time + 2 milliseconds subsequent reaction time) in both types of experiments, showing that the half

lives of enzyme activation were less than 2 milliseconds. The specific activities at these high enzyme concentrations (14.3 U/mg at 1.27 mg/ml) and 0.1 mM acetyl-CoA were the same as with dilute enzyme (0.0023 mg/ml) within experimental error. These results contrast with previous measurements (3,12) where greatly reduced enzyme specific activities (due to apparent increased acetyl-CoA activation constant) and relatively slow (hysteretic) responses to addition of acetyl-CoA ($t_{\frac{1}{2}}$ ~ 0.2 second) or MgATP ($t_{\frac{1}{2}}$ ~ 0.25 second) were observed as enzyme concentrations were increased above about 0.7 mg/ml.

The conformational changes in pyruvate carboxylase monitored by McGurk (2) through ANS fluorescence occurred faster than the 4 millisecond dead-time of the stopped-flow spectrophotometer. Similarly, the activation times for acetyl-CoA and MgATP, as determined in this study, were within the 4 millisecond dead-time of the apparatus, suggesting a correlation between the activation times and the conformational change observed in the enzyme.

Possible Reasons for Differences Between Current and Past Results

In the previous study by Hill (3), reactions were started by rapid addition of pyruvate to enzyme incubated with acetyl-CoA and all other substrates. Thus no hysteretic transitions were observed at any of the enzyme concentrations examined. Sufficiently small increments in enzyme concentration were used for each experiment to give extensively overlapping times of the initial velocity periods between experimental records. Thus a hysteretic transition was rigorously excluded as the cause of the reduced enzymic activities observed by Spivey and Hill

(3,12). Rates of acetyl-CoA hydrolysis were far too slow to account for the reduced activity; nor was the enzyme concentration (0.5 - 1.0 mg/ml) sufficient to bind enough of the total acetyl-CoA (100 μM) to explain the results. It does not appear reasonable to blame the results on a low molecular weight impurity, since the Sephadex G-50 column decreased the 50 mM $(NH_4)_2SO_4$ used in the enzyme storage buffer to below detection limits by $BaCl_2$ or Nessler's reagents prior to using the enzyme.

Therefore the most reasonable possibilities explaining the difference between previous and current results are: 1) different buffers; the more recent experiments were done with enzyme in phosphate buffer due to instances of extreme enzyme lability in the Tris buffer previously used, and 2) the higher enzyme purity (by about 2-fold) in current experiments.

Recent measurements of the enzyme-acetyl-CoA dissociation constant at higher enzyme concentrations (14) support our more recent observations, since the dissociation constant so determined agrees very well with the acetyl-CoA activation constant with dilute enzyme, and is far too low (13.9 μM) to explain the reduced activity observed by Hill (3) and Spivey and Hill (12) with 100 μM acetyl-CoA. Furthermore the conditions causing enzyme polymerization are not understood, and subsequent attempts to achieve appreciable enzyme polymerization have failed (15). The majority of the evidence then indicates that the enzyme's specific activity is independent of enzyme concentration. This suggests that other reasons must be sought to explain acetyl-CoA regulation of the enzyme <u>in vivo</u>. Further

stopped-flow measurements would be desired, however, if extensive enzyme polymerization is achieved in the future.

Regulation of Pyruvate Carboxylase

Recent extensive, but as yet unpublished, work by Barritt, Zander, and Utter (16) has studied the question of whether, and how acetyl-CoA regulates the enzyme's activity *in vivo*. They calculate that a large fraction of the total cellular acetyl-CoA in the mitochondrial matrix (100 to 1500 μM) could be bound to matrix proteins, which would raise the apparent activation constant to about 145 μM acetyl-CoA. Perhaps, then an increase in the acetyl-CoA activation constant as a result of enzyme polymerization is unnecessary to provide enzyme regulation. Using isolated chicken liver mitochondria, they also find a correlation between the rate of pyruvate carboxylation and the mitochondrial acetyl-CoA concentration generated by exogenous acetylcarnitine in the presence of arsenite. These calculations and results suggest that acetyl-CoA variations *in vivo* can regulate pyruvate carboxylase activity.

Summary and Conclusion

The times of activation of pyruvate carboxylase by acetyl-CoA and MgATP are less than the 4 millisecond dead-time of the stopped-flow apparatus. These times correspond to those reported by McGurk (2) for the conformational change induced in the enzyme by these ligands. The activation is apparently due to the conformational change of the enzyme. These measurements suggest that there is no change in specific activity of pyruvate carboxylase with increasing enzyme concentration,

leading to the conclusion that this is not a means for cellular control of the enzyme. Recent measurements and calculations from Utter's lab (16) suggest that regulation of pyruvate carboxylase by varying acetyl-CoA concentrations *in vivo* does occur, however, perhaps due to the high concentration of protein binding sites for acetyl-CoA in mitochondrial matrix.

REFERENCES CITED

(1) Utter, M. F., Barden, R. E., and Taylor, B. L. (1975) in Advances in Enzymology, (Meister, A., ed.) Vol. 42, pp. 1-72. John Wiley and Sons, New York.

(2) McGurk, K. S. R. (1976) Ph.D. Dissertation, Oklahoma State University.

(3) Hill, D. E. (1972) Ph.D. Dissertation, Oklahoma State University.

(4) Taylor, B. L., Barden, R. E., and Utter, M. F. (1971) Fed. Proc., 30, 1057.

(5) Morrison, J. F., and Uhr, M. L. (1966) Biochim. Biophys. Acta, 122, 57-74.

(6) Frey, W. H., and Utter, M. F., personal communication.

(7) Srere, P. A. (1969) in Methods in Enzymology, (Lowenstein, J. M., ed.) Vol. 13, pp. 3-11. Academic Press, New York.

(8) Bock, R., Ling, N., Morell, S., and Lipton, S. (1956) Arch. Biochem. Biophys., 62, 253-264.

(9) Scrutton, M. C., and Fung, C. H. (1972) Arch. Biochem. Biophys., 150, 636-647.

(10) Kalckar, H. M. (1947) J. Biol. Chem., 167, 461-475.

(11) Taylor, B. L., Barden, R. E., and Utter, M. F. (1972) J. Biol. Chem., 247, 7383-7390.

(12) Spivey, H. O., and Hill, D. E. (1973) Fed. Proc., 32, 2516.

(13) McClure, W. R. (1969) Biochemistry, 8, 2782-2786.

(14) Frey, W. H., and Utter, M. F. (1977) J. Biol. Chem., 252, 51-56.

(15) Utter, M. F., personal communication.

(16) Frey, W. H. (1975) Ph.D. Thesis, Case Western Reserve University.

CHAPTER III

COUPLED EQUILIBRIA OF PORCINE HEART MITOCHONDRIAL MALATE DEHYDROGENASE AND CITRATE SYNTHASE

Introduction

Srere (1) aptly pointed out that enzymological research has as its goal the explanation and the prediction of the metabolic behavior of cells and tissues. The study of purified enzymes is used to further this goal. This study looks at the coupled equilibria of the porcine heart mitochondrial malate dehydrogenase (L-malate:NAD oxidoreductase, EC 1.1.1.37) (MDH) and citrate synthase (citrate oxaloacetate lyase (CoA-acetylating), EC 4.1.3.7) (CS).

The mitochondrial concentrations of MDH and CS substrate binding sites (30-50 μM in enzyme subunits) are of the same magnitude as mitochondrial oxaloacetate and NADH concentrations (2). These enzyme bound substrate concentrations may exceed those of free substrates. This situation can cause significantly different kinetic and equilibrium properties of the catalyzed reactions from those determined with dilute enzymes, where enzyme concentrations are about 1 nM. Calculation of these effects, though straightforward in principle, is not yet practical since the enzyme binding constants determined experimentally are with greatly different and simpler conditions from those *in vivo*. The experimental NADH and NAD^+ binding constants to MDH, for example, may be greatly modified by the presence of oxaloacetate (3). Srere has found too that the calculated activity of CS differs from the observed *in vivo* rate (1).

A more reliable estimate of the effects of high enzyme concentrations on this system is obtained by experimental measurement. This study measures the extent of the MDH and CS reactions with combinations of dilute and concentrated enzymes.

Experimental

Materials

NAD^+, Tris-base, LiCoA, and DTNB were purchased from the Sigma Chemical Company; malate and oxaloacetate were from Calbiochem; ultrapure EDTA was from Schwarz-Mann; and acetic anhydride came from the Fisher Scientific Company. Citrate synthase came from Boehringer Mannheim GmbH, as did alcohol dehydrogenase. Malate dehydrogenase was purchased from the Sigma Chemical Company.

Methods

Substrate Preparations. Acetyl-CoA was prepared in 0.1 M Tris by the method of Srere (4). The concentration of the acetyl-CoA was determined by enzymatic assay with citrate synthase by monitoring the release of CoA (4).

For a short while, NAD^+ was further purified on the day it was used as described by Holbrook and Wolfe (3). There was no difference in the results obtained with either the commercial NAD^+ or the further purified NAD^+, so the purification was discontinued. NAD^+ was assayed enzymatically with alcohol dehydrogenase by monitoring the production of NADH at 340 nm, the absorptivity of which was 6.22 mM^{-1} cm^{-1}. Assays were performed in a 0.1 M Tris-Cl buffer, 1 mM EDTA, pH 8.1 containing 0.34 M ethanol, 2.4×10^{-3} mg/ml alcohol dehydrogenase, and 0.2-0.3 mM NAD^+. All other substrates were prepared, and the pH adjusted the day of the measurement.

Enzyme Preparations and Assay. Citrate synthase and malate

dehydrogenase were both prepared by collecting their ammonium sulfate precipitates by centrifugation. The enzyme precipitates were dissolved in 0.1 M Tris-Cl, 1 mM EDTA, pH 8.1 and dialyzed against four changes of 100 volumes of the same buffer, allowing two to three hours between changes. Citrate synthase was assayed at 25°C in the same buffer containing 0.5 mM DTNB, 0.05 mM acetyl-CoA, 0.125 mM oxaloacetate, and about 5 x 10^{-5} mg/ml CS at 412 nm. An absorptivity of 13.6 mM^{-1} cm^{-1} was used.

Malate dehydrogenase was assayed at 25°C, also in the same buffer. The assay included 3.0 mM NAD^{+}, 2.5 mM malate, and approximately 2.5 x 10^{-5} mg/ml MDH. Assays were performed at 340 nm and the production of NADH monitored.

Instrumentation. Equilibrium measurements, enzyme assays, and substrate assays were performed on a Coleman 124 spectrophotometer equipped as described in the phosphofructokinase methods and stopped-flow spectrophotometer as described in the glucose 6-phosphate dehydrogenase methods.

Results and Discussion

Equilibrium Effects.

We have measured the extent of the MDH and CS reactions with combinations of dilute and concentrated enzymes. The combinations (Table IX) were used to reveal how much of the altered equilibria could be attributed to each enzyme. Reaction equilibria were measured spectrophotometrically by NADH absorbances, assuming enzyme bound- and free NADH-absorptivities are equal (5). Total NADH concentrations

TABLE IX

EQUILIBRIA OF THE MDH AND CS ENZYME CATALYZED REACTIONS AT 25°C[a]

	(MDH)/(CS)			
	D/D	C/D	D/C	C/C
10^4 App K_{eq}, M	0.88	5.6 - 6.1	0.9 - 1.0	7.7
Total (NADH) in Step 1, μM	9.4	24.0 -25.0	9.5 -10.0	28.0
Δ(NADH) with CS, μM	0	0	10.2 -10.3	12.0
Δ(NADH)/(AcCoA)	0.59	0.34- 0.38	0.81- 0.85	0.70

[a]Measurements were performed in 0.1 M Tris-Cl, 1 mM EDTA, pH 8.1 using dilute enzymes (D = 0.0007 and 0.00029 mg/ml of MDH or CS, respectively) or concentrated enzymes (C = 2.4 and 1.0 mg/ml of MDH or CS, respectively. This corresponds to 69 μM binding sites of MDH and 23 μM binding sites of CS.) The app $K_{eq} = \frac{(NADH)^2}{(malate)(NAD^+)}$, while Δ(NADH) with CS is the increase in NADH total concentration when concentrated CS is added to the cuvette (Step 2). The Δ(NADH)/(AcCoA) is the change in NADH concentration (Step 3-Step 2) divided by the 10 μM acetyl-CoA added in Step 3.

were thus calculated from the three steps of each experiment in the double-beam spectrophotometer as follows, where + indicates additions to the solution of the previous step. The volume increments in steps 2 and 3 were less than or equal to 10 percent of the volume in step 1.

Step	Sample Beam Cuvette	Reference Beam Cuvette
1	MDH + NAD^+ + Malate at Reaction Equilibrium	MDH
2	+ CS	+ CS
3	+ 10 μM Acetyl-CoA	+ 10 μM Acetyl-CoA

These measurements were made for each of the combinations of concentrated (C) and dilute (D) enzyme concentrations summarized in Table IX. Initial substrate concentrations were 3.0 mM NAD^+ and 0.5 mM malate (physiological levels) in all experiments, and enzyme concentrations were (mg/ml): concentrated enzymes, 2.4 (MDH) and 1.0 (CS), and dilute enzymes, 0.0007 (MDH) and 0.00029 (CS). This enzyme ratio of 2 units MDH/unit CS corresponds closely to the cellular ratios of mitochondrial activities as measured in Srere's lab on several mammalian tissues. MDH units in this context refer to the malate oxidation direction.

Table IX indicates that concentrated MDH alone alters the extent of the reaction extensively (7-fold increase in the apparent K_{eq} and a 2.6-fold increase in NADH), presumably by binding NADH and oxaloacetate. The relative amounts of these two bound products are unknown. Thus, the free concentrations are also unknown, although the product of the latter [(NADH)(oxaloacetate)] is known. Although the competitive binding of NAD^+ and malate substrates reduces the extent to which the

products NADH and oxaloacetate bind, only the products are effective in altering the reaction equilibrium, since the substrate concentrations are so high relative to enzyme concentrations. Addition of concentrated CS increases the NADH concentration 10-12 μM whether with dilute or concentrated MDH, presumably by binding oxaloacetate. Except with the combination C-MDH/D-CS, 10 μM acetyl-CoA causes about 6-8 μM production of NADH. Binding of oxaloacetate to C-MDH would explain the low yield of the exception.

Kinetic Effects

Alterations in the equilibrium concentrations of NADH and oxaloacetate will extensively alter the kinetic response of the above system to the addition of acetyl-CoA (Step 3 in the above experiments). This was shown experimentally with dilute enzymes by simply changing the substrate (NAD^+ and malate) concentrations. Increased substrate concentrations give increased product concentrations at equilibrium. The latter cause the MDH reaction to respond slower to acetyl-CoA addition, although the CS reaction is initially faster. The slower response of the MDH reaction is a consequence of the opposing forward and reverse reactions. With dilute products, 10 μM acetyl-CoA and the CS reaction cause rapid decrease of oxaloacetate to near zero and the MDH reaction responds with near maximal (initial velocity) rates. With high product concentrations, the CS reaction does not disequilibrate the MDH reaction as extensively, and the latter responds closer to equilibrium (zero) net rates. In a more continuous situation, the rate of oxaloacetate regeneration will ultimately limit the CS reaction rate.

The binding of NADH and oxaloacetate to high MDH and CS concentrations could also alter the kinetic properties of the reactions relative to those with dilute enzymes. For example, if oxaloacetate were rapidly and extensively bound to CS, the reverse MDH reaction would be small, and oxaloacetate would be more rapidly regenerated. Stopped-flow experiments with combinations of dilute and concentrated enzymes (as with the above equilibrium measurements) were planned to characterize these kinetic properties. Previous attempts to obtain these data (along with ATP effects) utilized a distribution of reactants in the two drive syringes as follows, where [A] + [B] means the mixing of solution A in syringe 1 against solution B in syringe 2, and OAA is oxaloacetate.

[MDH + Malate + NAD^+ + (equilibrium OAA + NADH)] + [CS ± acetyl-CoA]

The two fold dilution of the syringe components upon mixing, however, altered the binding equilibria to MDH causing a MDH reaction in the absence of acetyl-CoA. (This does not occur with dilute enzymes.) Technical limitations (variations in dead-time from one mixing shot to another, and the rapid rates involved) made the simple subtraction of the reaction without acetyl-CoA from the reaction with acetyl-CoA inaccurate. Thus immediately following the equilibrium measurements, we attempted the following measurements hoping that the equal concentration of enzymes before and after mixing would reduce, if not eliminate, the MDH reaction in the absence of acetyl-CoA.

[MDH + Malate + NAD^+ + CS + (equilibrium OAA and NADH)] +

[MDH + CS ± acetyl CoA]

The reaction without acetyl-CoA with this distribution was much greater than with the previous distribution, however. Quantitative calculations of the binding equilibria before and after mixing, assuming simple binding of NADH with known binding constant, showed that this could occur. In the first arrangement, the effects on the binding equilibria of a reduction in product concentrations were partially cancelled by the effects of a reduction in enzyme concentrations. The equal reduction in substrate and product concentrations prevents a MDH reaction with dilute enzymes where enzyme bound reactants are negligible.

Since a high volume ratio mixing capability is needed for further measurements on this system, further investigation has been postponed.

Summary and Conclusion

Using physiological levels of malate and NAD^+ (0.5 mM and 3.0 mM respectively) along with both concentrated and dilute solutions of both MDH and CS in the ratio of 2/1 which closely corresponds to the ratio found within the cell, we find that concentrated MDH causes a 7-fold increase in the apparent K_{eq} and a 2.6-fold increase in NADH, we presume through the binding of NADH and OAA to the MDH enzyme. The addition of concentrated CS increases the concentration of NADH by 10-12 μM, also presumably through binding, this time of oxaloacetate. Thus we find that the concentrated enzymes alter equilibria through the binding of larger amounts of metabolites.

Increased concentrations of substrates cause a decrease in the response rate of the MDH to acetyl-CoA addition although the initial response of the CS is more rapid. In the continuous situation, the

rate of OAA regeneration will, however, ultimately limit the CS reaction. When the enzymes are present at high concentrations, the OAA is more extensively bound to the CS and OAA is more rapidly regenerated thus leading to a more tightly coupled system. This has implications for the TCA cycle in that the binding of the metabolites changes the kinetics and observations with the dilute enzyme system.

REFERENCES CITED

(1) Srere, P. A. (1976) in Gluconeogenesis, (Hanson, R. W. and Mehlman, M. A., eds.) pp. 153-161. John Wiley and Sons, New York.

(2) Srere, P. A. (1967) Science, 158, 936-937.

(3) Holbrook, J. J. and Wolfe, R. G. (1972) Biochemistry, 11, 2499-2502.

(4) Srere, P. A. (1969) in Methods of Enzymology, (Lowenstein, J. M., ed.) pp. 3-11. Academic Press, New York.

(5) Fisher, H. F., Adija, D. L., and Cross, D. G. (1969) Biochemistry, 8, 4424-4431.

(6) Srere, P. A., personal communication.

CHAPTER IV

RAT LIVER GLUCOSE 6-PHOSPHATE DEHYDROGENASE

Introduction

The enzyme glucose 6-phosphate dehydrogenase (D-glucose 6-phosphate: $NADP^+$ oxidoreductase, EC 1.1.1.49), G6PD, catalyzes the following conversion:

$$\beta\text{-D-glucopyranose 6-P} + NADP^+ \rightleftharpoons \text{D-gluconolactone 6-P} + NADPH + H^+$$

This reaction represents the first step in the pentose phosphate pathway, functions of which are the production of pentose phosphate sugars for the synthesis of nucleotides and for the production of NADPH for various reductive processes, especially fatty acid biosynthesis, and in erythrocytes for the regeneration of hemoglobin from methemoglobin (1).

The activity of the enzyme in the liver increases significantly upon feeding a high carbohydrate diet following a period of fasting (eg. 2-7). Subsequent feeding of fats causes the G6PD activity to decrease (8,9,10), whether by a decrease in appetite and thus food intake as suggested by Gozukara *et al*. (8) or by some other method than reduction in appetite as suggested by the results of Nace and Szepesi (9).

There are two interpretations of the nutritionally induced increased G6PD activity in liver. First, is that the increase in activity is due to increased synthesis of the enzyme with a concomitant increase in the amount of G6PD protein, and a decrease in activity is accompanied by a decrease in the amount of protein (3,8); second, that new protein is synthesized thus increasing the enzymatic activity, with a constant amount of protein regardless of the enzymatic activity

(11,12,13). They claim that feeding of fats causes a decrease in the activity of G6PD by converting enzymatically active G6PD protein to an inactive form, while total G6PD protein measured by specific titration with G6PD antibody remains constant.

Eggleston and Krebs (1) confirmed the inhibition of G6PD by NADPH and showed that it is competitive with respect to $NADP^+$. The inhibition of the G6PD depends on the ratio of the NADPH concentration to the $NADP^+$ concentration, such that at cellular concentrations of these nucleotides, Eggleston and Krebs believe that the enzyme would be completely inhibited, although Thompson et al. calculate significant activity even if the enzyme is 98 percent inhibited (14). Eggleston and Krebs found that GSSG added to rat liver supernatants at normal tissue concentrations reversed this inhibition. The supernatant when dialyzed against water did not lose activity, but lost the ability to reverse the inhibition due to NADPH when GSSG was added. Thus they believe there is some dialyzable cofactor that relieves NADPH inhibition of the enzyme under intracellular conditions.

G6PD from yeast is a tetramer containing four moles of $NADP^+$ (15) and is dissociable to dimers having a molecular weight of 102,000 (16). The dimer specie is catalytically active. The erythrocyte enzyme is similar to the yeast enzyme in that it exists as tetramers and dimers both of which are active (17). The molecular weights are similar with the monomeric subunit having a molecular weight of about 50,000 and no catalytic activity. The mammary gland G6PD also exists as a dissociable tetramer (18). Holten found rat liver G6PD existing as active dimers, tetramers and hexamers with molecular weights of 130,000, 280,000, and 370,000 respectively (19).

Monomers of 64,000 are without activity.

The erythrocyte enzyme shows concentration dependent properties with only twenty-five percent of maximal activity in the range of 0.0007 to 0.3 mg G6PD/ml. Maximal activity was expressed only with enzyme concentrations greater than 2 mg/ml (20). The mammary gland G6PD requires a minimum concentration of 0.4 mg/ml in order to form the tetrameric form of the enzyme (18).

Yugari and Matsuda reported inhibition of purified rat liver G6PD by free fatty acids (10). The inhibition is competitive with respect to $NADP^+$, but once the enzyme is inhibited, the inhibition is reported to be irreversible. Taketa and Pogell reported inhibition of purified yeast G6PD and G6PD in rat liver supernatants by palmitoyl-CoA (21). They found that the extent of inhibition was dependent upon the concentration of the enzyme, and that the inhibition was prevented but not reversed by the addition of BSA. There was a fast inactivation of the yeast G6PD by palmitoyl-CoA during the first five minutes of incubation together, followed by a slow inactivation during the second five minutes. They concluded that the inhibition was nonspecific in that it affected several enzymes, that it was just a detergent effect, and of little physiological significance.

Kawaguchi and Block, in studying the yeast G6PD, found that palmitoyl-CoA dissociates the tetrameric G6PD to dimers (15). When SDS was used to dissociate the enzyme to monomers, there was no difference in the affinity of the monomer or the dimer for palmitoyl-CoA. It was possible to induce reaggregation of the dimeric enzyme after removal of the palmitoyl-CoA, although the reaggregated tetramer was only one-third as active, and contained no $NADP^+$.

Current literature data on liver G6PD are deficient in several areas. The liver enzyme extensively associates at concentrations near cellular values, yet the catalytic properties of these oligomers have not been studied. Varying enzyme concentrations at even lower values greatly modifies its inhibition by palmitoyl-CoA. Current available data, however, are too limited to quantitatively evaluate palmitoyl-CoA inhibition of G6PD at various enzyme concentrations. Although palmitoyl-CoA appears to inhibit several enzymes, this need not preclude its significant effects. Palmitoyl-CoA is present in the cell at high concentrations, thus it is desirable to understand to what extent and how it affects G6PD. The rat liver enzyme used for previous studies has generally been from crude dialyzed extracts. Since there are several enzymes with high specificity for degrading G6PD (22) (and probably palmitoyl-CoA also), experiments are needed with a more purified system. Also most previous studies of G6PD have used Tris buffers.

Our overall goals, therefore, are to evaluate the enzyme's catalytic properties using a more purified enzyme and more physiological conditions. This thesis project, however, deals principally with the palmitoyl-CoA inhibition of G6PD.

Experimental

Materials

KH_2PO_4 and zinc acetate were purchased from the Fisher Scientific Company; KCl, acetic acid, and ammonium hydroxide were from the J. T. Baker Chemical Company; and $MgCl_2$ was from Mallinckrodt. Ultrapure

EDTA and $(NH_4)_2SO_4$ were purchased from Schwarz-Mann; absolute ethanol came from the U. S. Chemical Company. G6P, $NADP^+$, palmitoyl-CoA, BSA, Tris base, 2-mercaptoethanol, and fluorescein isothiocyanate were supplied by Sigma; CM cellulose was purchased from Reeve Angel while DEAE cellulose was purchased from both Reeve Angel and Sigma.

Rats were obtained from Holtzman and were kept in the Biochemistry Animal Facility until needed. High carbohydrate-low fat diet was purchased from Teklad Test Diets of Madison, Wisconsin and consisted of 300 g/kg vitamin free casein, 600 g/kg sucrose, 55 g/kg cellulose, 35 g/kg Williams-Briggs modified mineral mix, and 10 g/kg Teklad vitamin mix.

Methods

Enzyme Purification. The procedure followed in the purification of the G6PD from rat liver is like that of Matsuda and Yugari (12) and Holten (18,23) with a few modifications. If after decanting the supernatant from the ultracentrifugation step, fat was carried over into the supernatant, the solution was strained through several layers of cheese cloth to remove the fat. The overnight dialysis of the enzyme against glass distilled water was changed to two 30-45 minute dialyses against two liters of glass distilled water containing 10^{-4} M $NADP^+$. The carboxymethyl-cellulose column was eluted with a linear gradient of 750 ml of 20 mM ammonium acetate, 0.1 mM EDTA, 10^{-4} M $NADP^+$, 5 mM 2-mercaptoethanol, pH 5.5 and 750 ml 50 mM potassium phosphate, 0.1 mM EDTA, 10^{-4} M $NADP^+$, 5 mM 2-mercaptoethanol, pH 7.0.

Assay. Standard conditions for determining maximal activity of G6PD are 0.1 M Tris-Cl, 0.1 mM EDTA, 0.8 mM G6P, 0.2 mM $NADP^+$, 2.8 mM $MgCl_2$, pH 8.0. The enzyme activity is determined spectrophotometrically at 340 nm and 25°C. The activity was calculated using a value of 6.22 mM^{-1} cm^{-1} for the absorptivity of NADPH. One unit of enzyme is defined as the conversion of one μmole of $NADP^+$ to NADPH per minute.

Protein Determination. Protein concentrations were determined by the Lowry method (24). BSA was used as the protein standard.

Reagent Preparations. The substrate solutions were made and the pH adjusted the same day they were used. $MgCl_2$ used in assays was purified by the method of Morrison and Uhr (25).

Instrumentation. A Coleman 124 spectrophotometer equipped as described in the phosphofructokinase section was used to perform standard and dilute enzyme assays. Measurements using more concentrated enzyme or requiring fast mixing, were made with a Durrum-Gibson stopped-flow spectrophotometer with either a tungsten light source or a mercury-xenon light source. Temperature control was accomplished using a Forma model 2095-2 bath and circulator. Data were recorded on a Tektronix storage oscilloscope. Fluorescence measurements were made in a thermostatted Aminco-Bowman spectrophotofluorometer with a mercury-xenon light source and with 3 mm slit widths. Fluorescence depolarization measurements were performed with the Durrum-Gibson stopped-flow spectrophotometer equipped with a fluorescence accessory. The light source used was an argon ion laser. Sedimentation velocity

measurements were made with a Beckman Model E ultracentrifuge.

Stopped-Flow Measurements. Stopped-flow measurements are performed using two drive syringes, portions of the reaction components being placed in each syringe. When a pneumatic plunger is activated, the contents of the two syringes are mixed, with a reaction dead-time of about three milliseconds.

Fluorescent Labelling. Rat liver G6PD was labelled with fluorescein isothiocyanate by the method of Shore and Chakrabarti (26). After labeling, the enzyme was exchanged into a 0.01 M potassium phosphate, 0.01 M NaCl, 0.12 M KCl, 0.1 mM EDTA, pH 7.4 buffer using a Sephadex G-25 column. The degree of labelling was determined by the absorbance at 490 nm, using an absorptivity of 3.4×10^4 M^{-1} cm^{-1} as described by Shore and Chakrabarti (26). The extent of labelling calculated from our data was 0.5 molecule of fluorescein per equivalent of 64,000 molecular weight G6PD monomer.

Results and Discussion

The studies of both Taketa and Pogell (21) and Kawaguchi and Bloch (15) concerning the palmitoyl-CoA inhibition of G6PD were done in a Tris-HCl buffer system, which may or may not have affected their results. We preferred instead to use a potassium phosphate buffer system in that it more closely approximates intracellular conditions. After a few preliminary measurements, we increased the ionic strength of the buffer by the addition of KCl, and NaCl in the amounts that one would expect to find in the cell. Substrate concentrations were adjusted to conform with those estimated to exist in the cell by

Greenbaum et al. (27). Thus the studies presented here should be more significant because of the more nearly physiological conditions under which they were performed.

Both groups previously mentioned concerned themselves with yeast enzyme and Taketa and Pogell also considered rat liver G6PD. The enzyme however came from rat liver supernatants prepared by centrifugation followed by overnight dialysis. Since there are three enzymes specific for the inactivation of G6PD (22), the results obtained with the rat liver supernatant are subject to question.

G6PD-Specific Activity Dependence on Assay Conditions

Comparison of various assay compositions on the specific activity is shown in Table X. As would be expected, the activity in the Tris-Cl buffer, pH 8.0 is greatest since it is at the pH optimum of the enzyme and saturating substrate concentrations. Assay of the enzyme in a 10 mM potassium phosphate buffer with a pH of 7.4 (that expected in the cytoplasm) and substrate concentrations expected within the cell gives a specific activity that is 67 percent of that with the Tris system at pH 8.0. $MgCl_2$, an accepted activator of G6PD, appears to cause a decrease in the specific activity (Table XI) when assayed in a 10 mM potassium phosphate, 0.12 M KCl, 10 mM NaCl, 0.1 mM EDTA, pH 7.4 buffer. This leads one to question whether the $MgCl_2$ activation of G6PD observed in other assay systems has physiological significance. BSA in amounts as small as 2.5 µg/ml is found to lead to increased activity over enzyme incubated overnight with $NADP^+$ but no BSA (Table XII).

TABLE X

ENZYME-SPECIFIC ACTIVITY VARIATION WITH ASSAY CONDITIONS[a]

Assay	Buffer	Substrates	$MgCl_2$	Salt	pH	Specific Activity
A	0.1 M Tris-Cl	0.80 mM G6P 0.39 mM $NADP^+$	2.8 mM	-	8.0	71.6
B	0.01 M potassium phosphate	0.80 mM G6P 0.39 mM $NADP^+$	2.8 mM	0.12 M KCl 0.06 M NaCl	7.4	59.6
C	0.01 M potassium phosphate	0.80 mM G6P 0.39 mM $NADP^+$	-	0.12 M KCl 0.01 M NaCl	7.4	63.4
D	0.434 M potassium phosphate	0.434 mM G6P 0.01 mM $NADP^+$	-	0.12 M KCl 0.01 M NaCl	7.4	48.0

[a]The G6PD concentration in the assays was 0.485 µg/ml. All assays were performed at 25°C, and were started by the addition of G6P to the assay cuvette. 0.1 mM EDTA was added to all assays.

TABLE XI

THE EFFECT OF $MgCl_2$ ON G6PD SPECIFIC ACTIVITY IN A HIGH IONIC STRENGTH PHOSPHATE BUFFER[a]

	$MgCl_2$, mM									
	0	0.005	0.01	0.05	0.10	0.20	1.0	3.0	5.0	10.0
S.A.	32.7	25.9	26.2	26.2	25.4	27.7	25.7	24.9	20.0	22.8

[a]The G6PD concentration in the assays was 0.62 µg/ml. The assays were performed at 25°C and were started by the addition of G6P to the assay cuvette. The assay system contained 0.01 M potassium phosphate, 0.12 M KCl, 0.01 M NaCl, 0.1 mM EDTA, 0.434 mM G6P, 0.01 mM $NADP^+$, pH 7.4.

TABLE XII

THE BSA EFFECT ON SPECIFIC ACTIVITY UPON OVERNIGHT INCUBATION[a]

	BSA, µg/ml				
	0	2.5	25.0	250.0	1000.0
S.A.	24	32.2	32.5	35.3	34.1

[a]The G6PD concentration was 0.97 µg/ml. The incubations and assays were performed at 25°C with 0.01 M potassium phosphate, 0.12 M KCl, 0.01 M NaCl, 0.1 mM EDTA, 0.01 mM $NADP^+$, pH 7.4. The reaction was started by adding G6P to make the mixture 0.434 mM, after overnight (10-12 hr) incubation.

Palmitoyl-CoA Inhibition

Palmitoyl-CoA inhibits the G6PD when incubated with the enzyme and $NADP^+$ as shown in Table XIII. With 0.9 µg enzyme/ml, 2 µM palmitoyl-CoA reduces the activity of the enzyme to about 54 percent of the activity of the control (the assay without palmitoyl-CoA) while 10 µM palmitoyl-CoA reduces the activity to about 6 percent. While palmitoyl-CoA inhibits G6PD, we found an apparent enhancement of the activity of G6PD by 1 µM palmitoyl-CoA (Table XIV) when compared to the control (the first entry in Table XII).

$MgCl_2$ Reversal of Palmitoyl-CoA Inhibition

The palmitoyl-CoA inhibition of G6PD activity was reversed by 3 mM $MgCl_2$ (Table XIII) in dilute enzyme assays presumably by precipitating the palmitoyl-CoA. In overnight incubations of the enzyme and palmitoyl-CoA with $MgCl_2$, 1.0 mM $MgCl_2$ appeared to remove the inhibition by the palmitoyl-CoA (Tables XIV and XV). This was disturbing, as intracellular concentrations of Mg^{2+} are about 1 mM (28) and the significance of the palmitoyl-CoA inhibition is questionable if this concentration of $MgCl_2$ abolishes the inhibition. Overnight incubation of the enzyme with palmitoyl-CoA, 0.01 mM $NADP^+$, and 1.0 mM $MgCl_2$ at 37°C shows that inhibition by palmitoyl-CoA at concentrations greater than or equal to 4 µM is not relieved by the $MgCl_2$ (Table XVI).

Protection from Palmitoyl-CoA Inhibition by BSA

We found that BSA at concentrations as low as 2.5 µg/ml appears to lead to an increased G6PD activity compared to the control when

TABLE XIII

PERCENT ACTIVITY OF ENZYME AS EFFECTED BY $MgCl_2$ AND PALMITOYL-CoA[a]

$MgCl_2$ mM	Palmitoyl-CoA, μM						
	0	1.0	2.0	3.0	4.0	5.0	10.0
0	100	98.1	53.7	-	-	36.8	5.8
3.0	100	104.7	99.4	97.0	101.4	-	105.8

[a]The G6PD concentration in the assays was 0.9 μg/ml. The assay mixture containing 0.01 M potassium phosphate, 0.1 mM EDTA, 0.078 mM $NADP^+$, enzyme, and $MgCl_2$ and palmitoyl-CoA as indicated was incubated at 25°C for 5 minutes before adding G6P to make the mixture 0.434 mM in G6P to start the reaction.

TABLE XIV

PALMITOYL-CoA AND $MgCl_2$ EFFECT ON ENZYME-SPECIFIC ACTIVITY UPON OVERNIGHT INCUBATION[a]

Palmitoyl-CoA μM	$MgCl_2$, mM		
	0	0.1	1.0
1	33.9	33.6	30.2
10	-	0	22.8

[a]The G6PD concentration in the incubations and assays was 0.97 μg/ml. The incubation and assay mixture contained 0.01 M potassium phosphate, 0.12 M KCl, 0.01 M NaCl, 0.1 mM EDTA, 0.01 mM $NADP^+$, pH 7.4 plus enzyme and indicated conditions, and was kept in 25°C overnight (10-12 hr). The reaction was initiated by making the mixture 0.434 mM in G6P.

TABLE XV

THE EFFECT OF $MgCl_2$ AND BSA ON THE PALMITOYL-CoA INHIBITION OF G6PD UPON OVERNIGHT INCUBATION AT 25°C[a]

$MgCl_2$ mM	BSA, μg/ml				
	0	2.5	25.0	250	1000
0	-	0.22	7.4	33.0	34.1
0.1	0	0.3	6.6	34.2	35.8
1.0	22.8	21.8	25.6	34.4	32.8

[a]The G6PD concentration in the incubation assay mixture was 0.97 μm/ml. The mixture contained 0.01 M potassium phosphate, 0.12 M KCl, 0.01 M NaCl, 0.1 mM EDTA, 0.01 mM $NADP^+$, 10 μM palmitoyl-CoA, plus indicated additions and enzyme at pH 7.4. The mixture was incubated overnight (10-12 hr) at 25°C and the reaction was started by making the mixture 0.434 mM in G6P. Specific activities are reported.

TABLE XVI

SPECIFIC ACTIVITY OF G6PD UPON OVERNIGHT INCUBATION AT 37°C WITH PALMITOYL-CoA AND $MgCl_2$[a]

Palmitoyl-CoA, μM								
0	1.0	2.0	3.0	4.0	5.0	6.0	7.0	8.0
37.7	43.9	39.0	23.2	8.1	3.2	2.0	1.4	1.3

[a]The G6PD concentration used was 0.97 μg/ml. The incubation-assay mixture contained 0.01 M potassium phosphate, 0.12 M KCl. 0.01 M NaCl, 0.1 mM EDTA, 1.0 mM $MgCl_2$, 0.01 mM $NADP^+$, enzyme, and palmitoyl-CoA as indicated, all at pH 7.4. The reactions were started after overnight incubation at 37°C by making the mixture 0.434 mM in G6P. All concentrations of palmitoyl-CoA greater than 8 μM gave zero specific activities.

incubated overnight at 25°C (Table XII). With 10 μM palmitoyl-CoA present in the overnight incubation mixture, however, 250 μg BSA/ml was required to provide the same activity that was observed with no palmitoyl-CoA and 2.5 μg BSA/ml (Table XV). It appears that the BSA may adsorb the palmitoyl-CoA and that higher concentrations of BSA are required to effect this.

The Effects of Palmitoyl-CoA Concentration, Time, and Enzyme Concentration

The inhibition of G6PD by palmitoyl-CoA is dependent upon the concentration of the palmitoyl-CoA incubated with the enzyme and upon the time of incubation (Figure 3). Increasing the palmitoyl-CoA concentration causes an increase in the rate with which inhibition develops. When the G6PD concentration is increased 10-fold, the expression of the palmitoyl-CoA inhibition is at a rate slower than that with the more dilute enzyme (Figure 4). Thus the inhibition of G6PD by palmitoyl-CoA is dependent upon the palmitoyl-CoA concentration, the time, and the concentration of the enzyme.

Incubation of Substrates with G6PD

The incubation of one of the substrates with the G6PD followed by the initiation of the reaction with palmitoyl-CoA and the other substrate gives activities that do not change with time of incubation, but instead depend upon the substrate with which the enzyme was incubated and the concentration of the palmitoyl-CoA. Figure 5 shows results from stopped-flow measurements of this nature, which provide a measure of the fast phase of inhibition. Initial velocities are obtained

Figure 3. The Dependence of G6PD Activity on Time and on Palmitoyl-CoA Concentration with 0.7 μg Enzyme/ml. Syringe I contained G6PD, 0.078 mM $NADP^+$, and palmitoyl-CoA as indicated. Syringe II contained 0.434 mM G6P, 0.010 mM $NADP^+$, and palmitoyl-CoA as indicated. The buffer in both syringes was 10 mM potassium phosphate, 0.12 M KCl, 10 mM NaCl, 0.1 mM EDTA, pH 7.4. The incubation time is the time that the G6PD was incubated with the contents of Syringe I before the contents of the two syringes are mixed to start the reaction. The incubation and reaction are carried out at 25°C. Palmitoyl-CoA concentrations were: 0 μM, 0—0; 10 μM, □—□; 20 μM, Δ—Δ; 30 μM, ●—●; and 50 μM, ■—■.

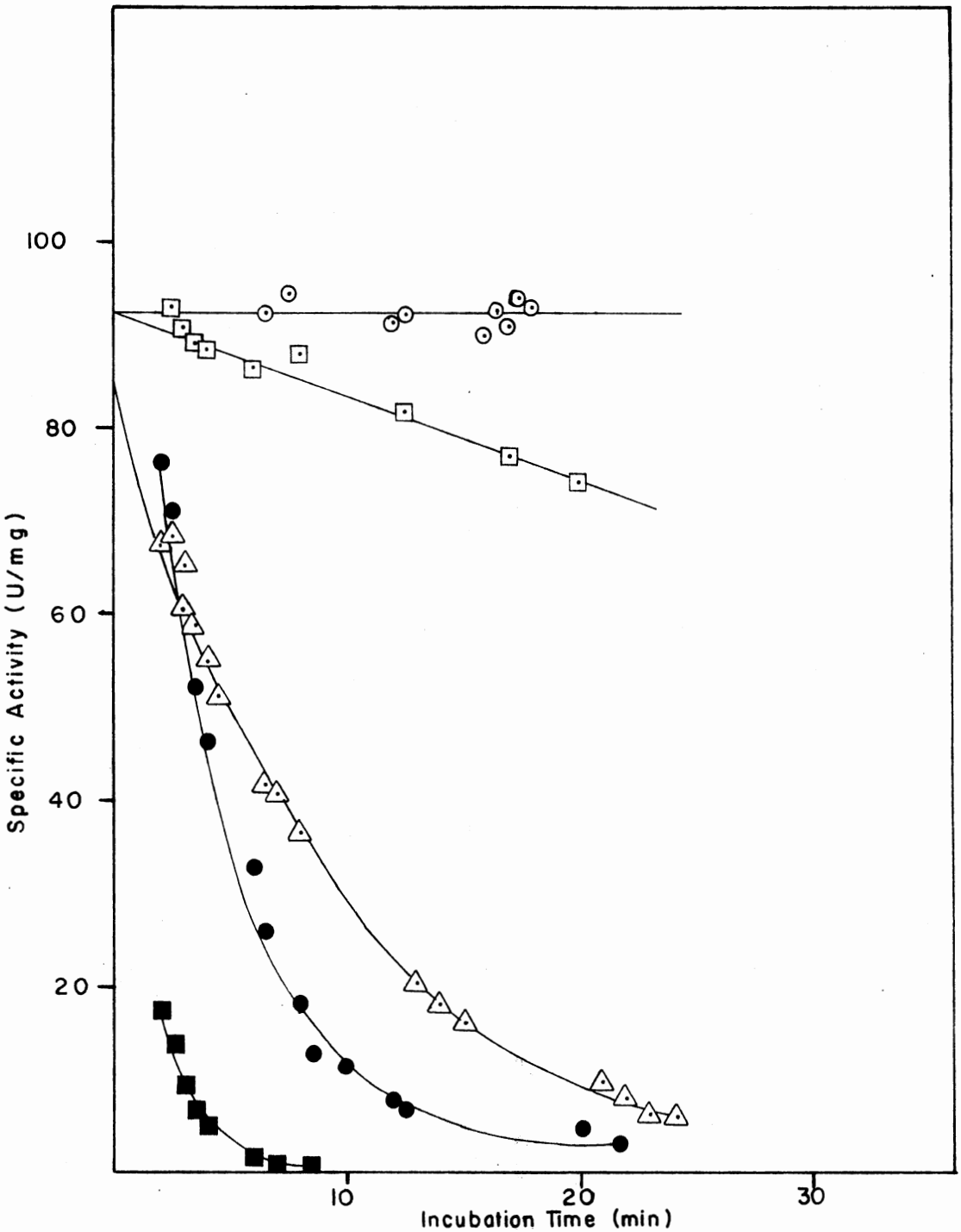

100
80
60
40
20
Specific Activity (U/mg)
10
20
30
Incubation Time (min)

Figure 4. The Dependence of G6PD Activity on Time and Palmitoyl-CoA Concentration with 7 μg Eznyme/ml. Conditions are identical to those of Figure 3. Palmitoyl-CoA concentrations are: 0 μM, 0—0; 10 μM, □—□ ; 30 μM, ●—●; and 50 μM, ■—■ .

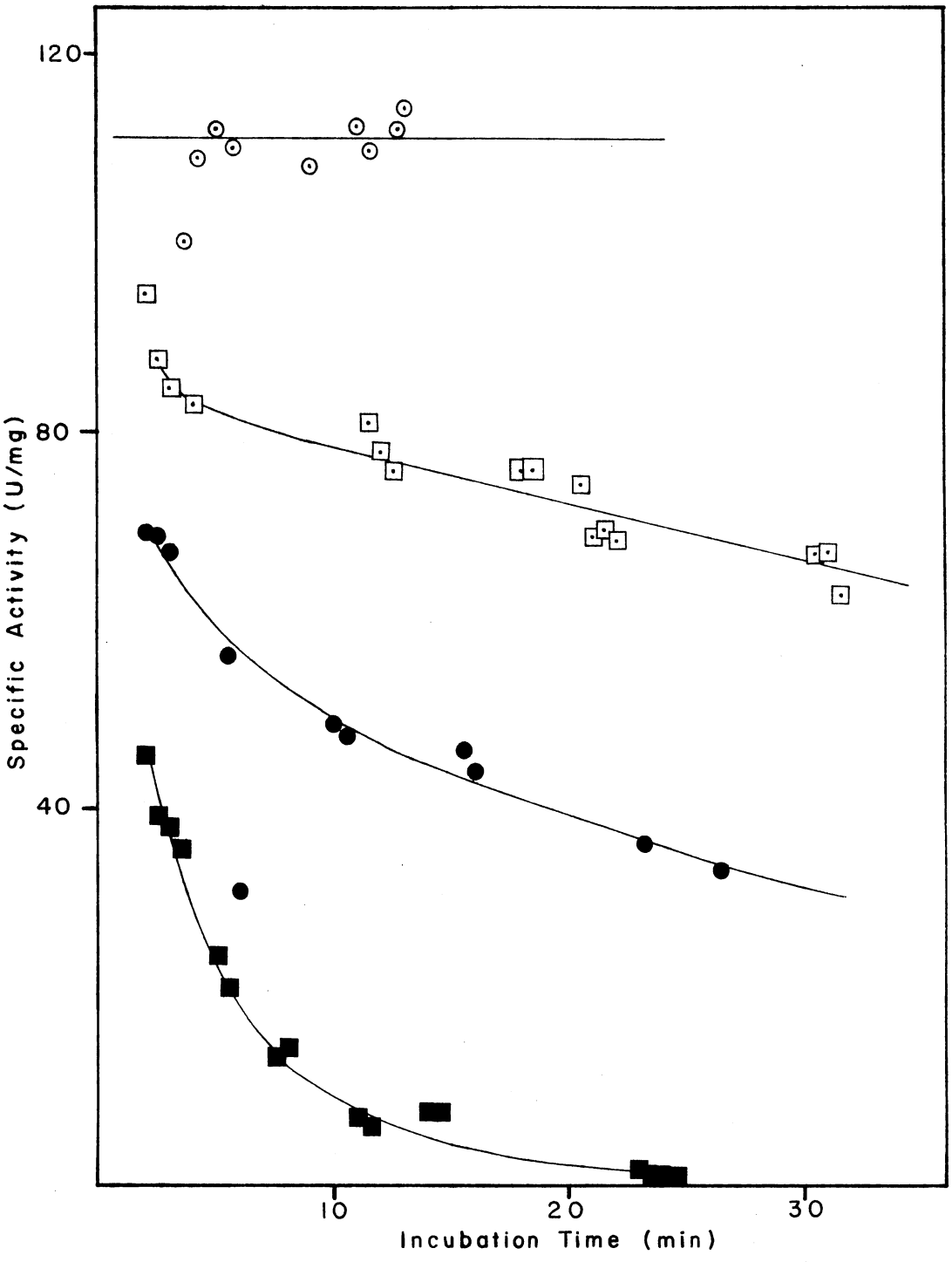
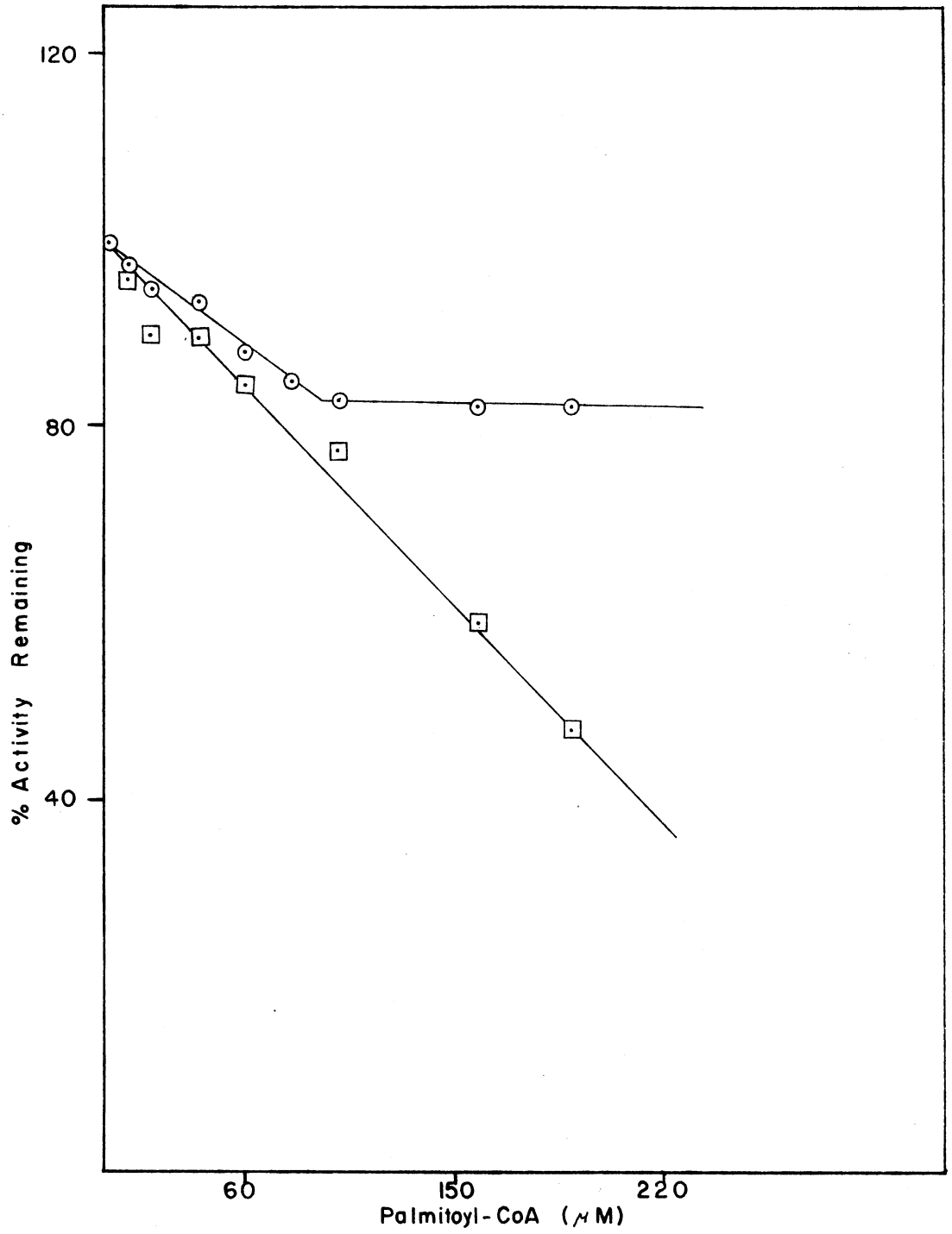

120
80
40
Specific Activity (U/mg)
10
20
30
Incubation Time (min)

Figure 5. The Dependence of Palmitoyl-CoA Inhibition Upon G6PD Incubation with Substrate. Assay buffer used was 10 mM potassium phosphate, 0.1 mM EDTA, pH 7.4. Incubation conditions were, [Syringe 1] and [Syringe 2] : [9 μg G6PD/ml + 0.078 mM $NADP^+$] and [0.078 mM $NADP^+$ + 0.434 mM G6P + 10 μM palmitoyl-CoA], 0—0; [9 μg G6PD/ml + 0.434 mM G6P] and [0.078 mM $NADP^+$ + 0.434 mM G6P + 10 μM palmitoyl-CoA], □—□.


120
80
40
% Activity Remaining
60
150
220
Palmitoyl-CoA (μM)

within the first 0.5-1.0 second of the reaction. If enzyme is incubated with $NADP^+$, this rapidly expressed inhibition increases to about 15% with increasing palmitoyl-CoA to about 100 μM, but higher palmitoyl-CoA concentrations are much less effective. When the enzyme is incubated with G6P, however, continuously increasing rapid inhibition with increasing palmitoyl-CoA concentrations is observed. Above 20 μM palmitoyl-CoA, enzyme inhibition becomes essentially complete at longer times whether enzyme is incubated with $NADP^+$ or not (Figure 3). The data in Figure 5, therefore, indicate that there are two or more sequential kinetic steps in the reaction of palmitoyl-CoA with enzyme. $NADP^+$ appears to significantly reduce the rate of one of these transformations.

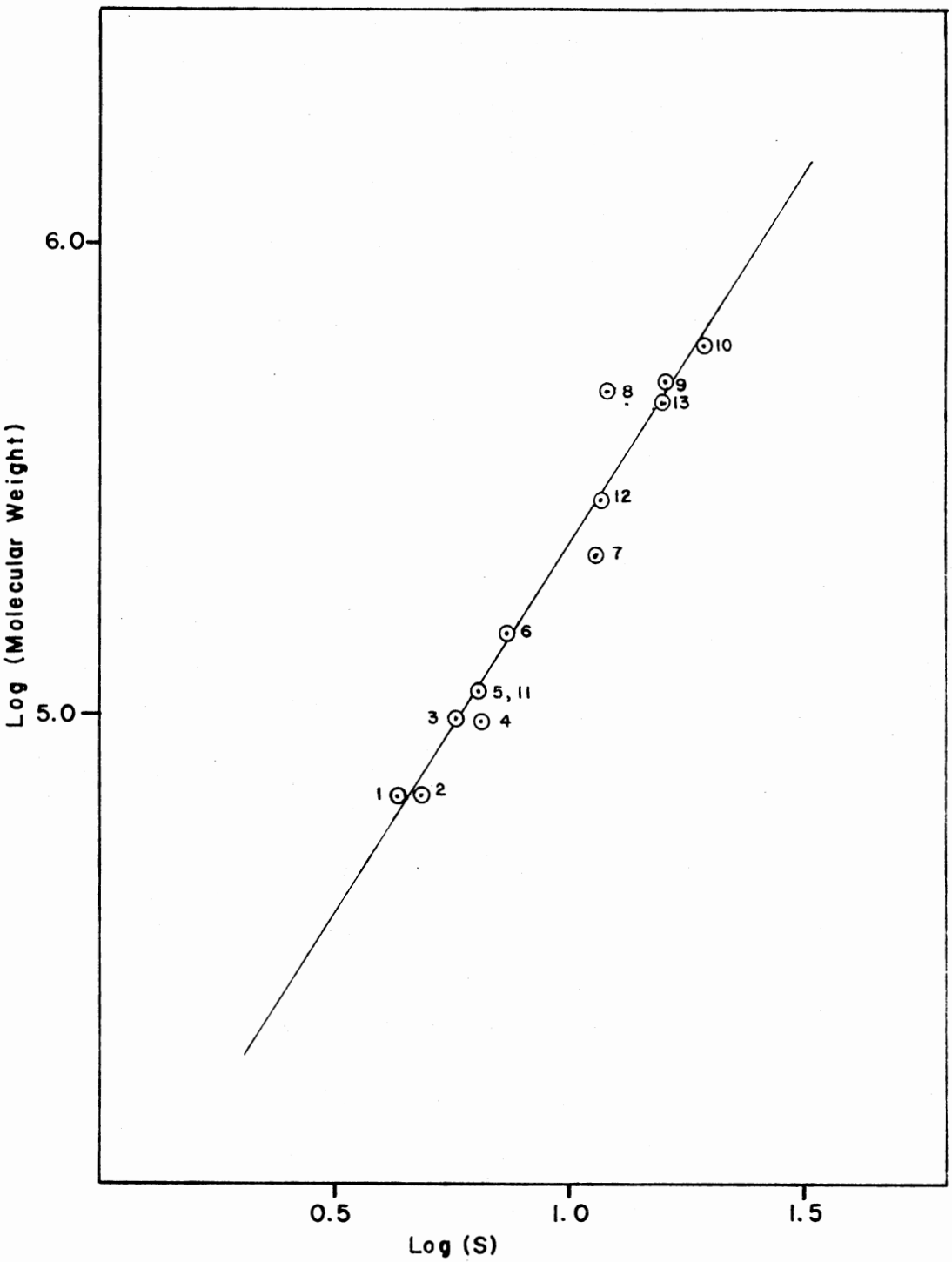
Sedimentation Velocity Measurements

Sedimentation velocity measurements performed with 1 mg G6PD/ml in 10 mM potassium phosphate, 0.12 M KCl, 10 mM NaCl, 0.1 mM EDTA, 10^{-4} M $NADP^+$, pH 7.4 gave a value of 15.7 S. A molecular weight of 466,000 can be estimated from this s value assuming G6PD is a globular protein and using calibration proteins as done in Figure 6. When 100 μM palmitoyl-CoA was added to this solution, and the measurements performed, we obtained two enzyme peaks, one corresponding to 6.4 S and the second corresponding to 11.6 S. These values correspond to molecular weight estimates of 113,000 and 286,000 respectively (Figure 4). In addition we observed a hypersharpening of the peaks, an indication of an associating system. Thus we observed an apparent dissociation of the G6PD by palmitoyl-CoA from an apparent octameric form to an apparent dimer-tetramer equilibrium.

Figure 6. Plot of Log(Molecular Weight) vs. Log(Sedimentation Coefficient) for Several Globular Proteins. Proteins are: Hemoglobin[a], 1; Albumin[a], 2; Diptheria antitoxic pseudoglobulin[a], 3; α-Amylase[b], 4; Concanavalin[b], 5; Aldolase[b], 6; Catalase[b], 7; Cytochrome C[b], 8; β-Galactosidase[b], 9; Hemocyanin[b], 10; Glucose 6-phosphate dehydrogenase (see text), 11, 12, 13.

[a]Data obtained from Cohn, E. J. and Edsall, J. T. (1943) Proteins, Amino Acids and Peptides, Reinhold Publishing Corporation, New York.

[b]Data obtained from Sober, H. A. (1968) Handbook of Biochemistry, The Chemical Rubber Co., Cleveland.


6.0
5.0
Log (Molecular Weight)
0.5
1.0
1.5
Log (S)
10
8
9
13
12
7
6
5, 11
3
4
1
2

Fluorescein Labelled G6PD

The fluorescein labelled G6PD contained 0.5 molecule of fluorescein per monomer of enzyme. The labelled enzyme behaved the same as the unlabelled enzyme. The rate of expression of the palmitoyl-CoA inhibition depended on the palmitoyl-CoA concentration, the time of incubation of the G6PD with palmitoyl-CoA, and the G6PD concentration (Figures 7 and 8).

Incubation of the fluorescein labelled G6PD at 25°C and a concentration of 82.0 μg/ml in a 10 mM potassium phosphate, 0.12 M KCl, 10 mM NaCl, 0.1 mM EDTA, pH 7.4 buffer containing 0.01 mM $NADP^+$ led to a 50 percent increase in fluorescence overnight. This also occurred when 10 μM palmitoyl-CoA was added to the mixture. Depolarization measurements were attempted with the hope that they would reveal a dissociation of the enzyme that paralleled the palmitoyl-CoA inhibition. We observed total depolarization of the enzyme even before the addition of the palmitoyl-CoA. We believe that we had tetrameric enzyme labelled with two molecules of fluorescein. Energy transfer between like molecules on an enzyme does not lead to fluorescence quenching, but instead leads to depolarization (29). We believe this is why we observed the total depolarization, which prevents us from monitoring enzyme dissociation with this fluorescence probe.

Enzyme-Concentration-Dependent Specific Activity

We hope that in the near future we will be able to perform light scattering measurements to correlate the enzyme inhibition with a dissociation of the enzyme. A laser light scattering instrument,

Figure 7. The Dependence of Fluorescein-Labelled-G6PD Activity on Time and on Palmitoyl-CoA Concentration with 0.672 µg Enzyme/ml. Conditions are described in Figure 3. Palmitoyl-CoA concentrations are: 0 µM, 0—0; 10 µM □—□; 20 µM, Δ—Δ; 30 µM, ●—●.

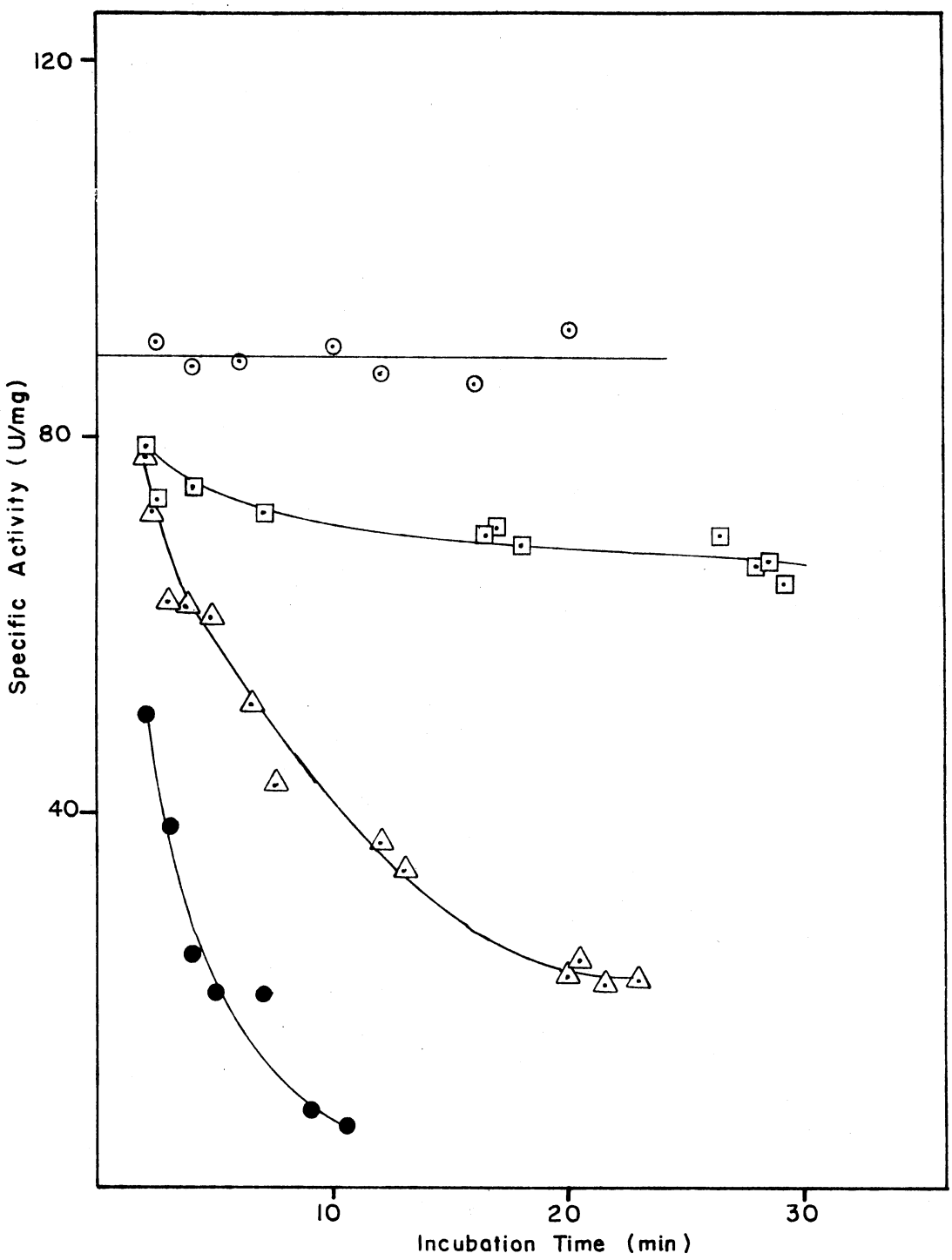
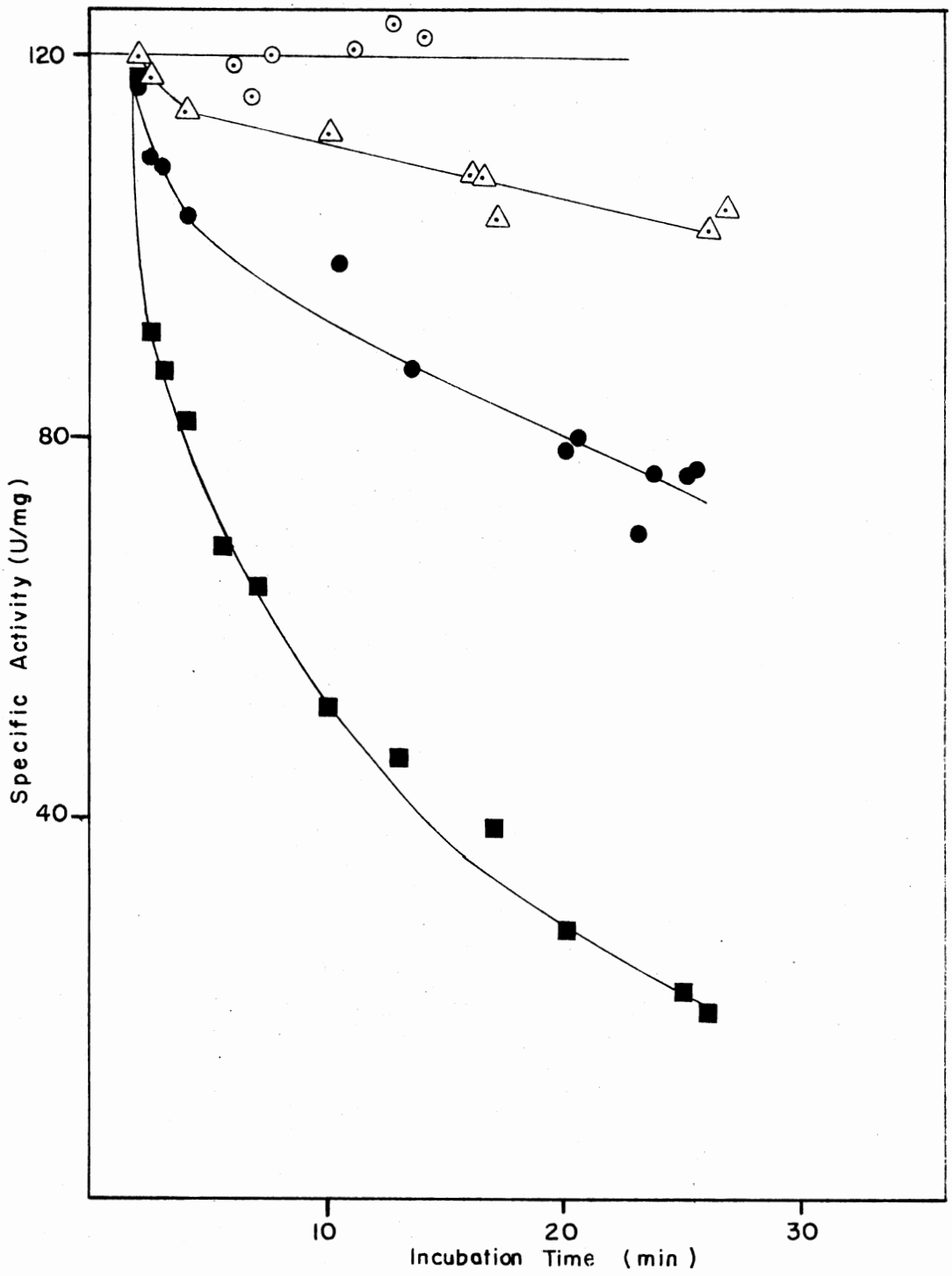

120
80
40
Specific Activity (U/mg)
10
20
30
Incubation Time (min)

Figure 8. The Dependence of Fluorescein-Labelled-G6PD Activity on Time and on Palmitoyl-CoA Concentration with 6.72 μg Enzyme/ml. Conditions are described in Figure 3. Palmitoyl-CoA concentrations are: 0 μM, 0—0; 20 μM, Δ—Δ; 30 μM, ●—●; 50 μM, ■—■.

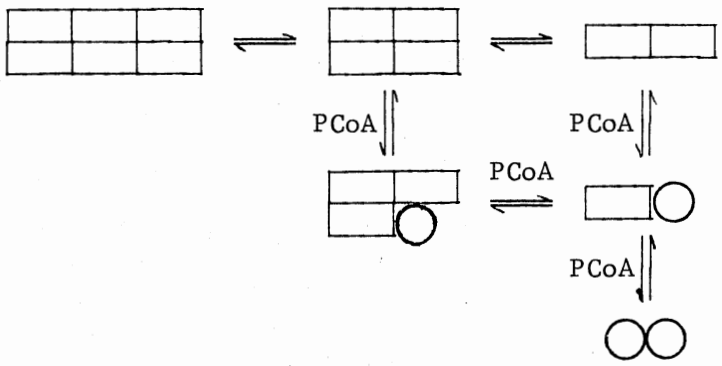

120
80
40
Specific Activity (U/mg)
10
20
30
Incubation Time (min)

such as that manufactured by Chromatix, Mountain View, California, will be needed, however, to give the desired sensitivity with sufficiently small volumes to be practical. Our current Phoenix-Brice instrument requires large volumes (about 30 ml in comparison to a few tenths for the laser instruments) and thus excessively large quantities of enzyme.

We observed an apparent dependence of the G6PD upon its 10-fold increase in concentration (Table XVII). This apparent concentration dependence was observed with both unlabelled and fluorescein labelled enzyme. Thus protein-protein interactions play a role in determining the enzyme activity.

Proposed Model

The ultracentrifugation data indicate that we have three molecular weight species. This along with the palmitoyl-CoA inhibition data leads us to propose a model along the following lines:



Hexameric and higher forms can occur and equilibrate with tetrameric and dimeric forms of the enzyme amounts of each form depending on the enzyme concentration. The binding constant for palmitoyl-CoA must be higher with the dimeric form of the enzyme than the tetrameric form.

TABLE XVII

THE DEPENDENCE OF SPECIFIC ACTIVITY ON ENZYME CONCENTRATION[a]

Fluorescein-G6PD µg/ml	G6PD µg/ml	Specific Activity
-	0.7	90.8
-	7	109.8
0.672	-	88.4
6.5	-	120.3

[a]The assays were performed in a 0.01 M potassium phosphate, 0.12 M KCl, 0.01 M NaCl, 0.1 mM EDTA, pH 7.4 buffer at 25°C. The assay contained 0.434 mM G6P and 0.01 mM $NADP^+$ and was initiated by mixing [G6PD + $NADP^+$] + [G6P + $NADP^+$].

The conformational change induced by the palmitoyl-CoA is rapid and is followed by a slower dissociation of the enzyme and binding of palmitoyl-CoA to totally inactivate it. Activation of the enzyme by BSA alone is possibly by protein-protein interactions stabilizing higher, and more active, molecular weight forms of the enzyme. Palmitoyl-CoA at a concentration where we notice an apparent activation of the G6PD does not exist in a micellular form as it does at higher concentrations and it is probably the micellular form that is inhibitory (30).

Summary and Conclusion

The question of whether palmitoyl-CoA can be considered as an inhibitor of any physiological importance in light of its reversal at 25°C by cellular concentrations of $MgCl_2$ can be answered affirmatively as incubation at 37°C allows the expression of the palmitoyl-CoA inhibition. The palmitoyl-CoA inhibition of rat liver G6PD depends upon the enzyme concentration, the palmitoyl-CoA concentration, the incubation time before initiating the reaction, and if the enzyme is incubated in the absence of palmitoyl-CoA, the substrate with which it is incubated. We also notice an increased specific activity of the enzyme with increased enzyme concentration. This leads us to propose a model involving a conformational change induced by the palmitoyl-CoA, followed by a slow dissociation.

REFERENCES CITED

(1) Eggleston, L. V., and Krebs, H. A. (1974) Biochem. J., 138, 425-435.

(2) Matsuda, T., and Yugari, Y. (1967) J. Biochem., 61, 535-540.

(3) Gibson, D. M., Lyons, R. T., Scott, D. F., and Yasutoshi, M. (1972) in Advan. Enzyme Regul. (Weber, G., ed.) Vol. 10, pp. 187-204.

(4) Taketa, K., Kaneshige, Y., and Kosaka, K. (1972) Enzyme, 14, 105-115.

(5) Mack, D. O., Watson, J. J., Chandler, A. M., and Johnson, B. C. (1974) J. Nutr., 104, 12-17.

(6) Sassoon, H. F., Dror, Y., Watson, J. J., and Johnson, B. C. (1973) J. Nutr., 103, 321-335.

(7) Szepesi, B. (1973) Can. J. Biochem., 51, 1604-1615.

(8) Gozukara, E. M., Frolich, M., and Holten, D. (1972) Biochim. Biophys. Acta, 286, 155-163.

(9) Nace, C. S., and Szepesi, B. (1976) J. Nutr., 106, 285-291.

(10) Yugari, Y., and Matsuda, T. (1967) J. Biochem., 61, 541-549.

(11) Yagil, G., Shimron, F., and Hizi, A. (1974) Eur. J. Biochem., 45, 189-200.

(12) Hizi, A., and Yagil, G. (1974) Eur. J. Biochem., 45, 211-221.

(13) Kelly, D. S., Watson, J. J., Mack, D. O., and Johnson, B. C. (1975) Nutr. Rep. Int., 12, 121-135.

(14) Thompson, R. E., Spivey, H. O., and Katz, A. J. (1976) Biochemistry, 15, 862-867.

(15) Kawaguchi, A., and Bloch, K. (1974) J. Biol. Chem., 249, 5793-5800.

(16) Kuby, S. A., Wu, J. T., and Roy, R. N. (1974) Arch. Biochem. Biophys., 165, 153-178.

(17) Bonsignore, A., and DeFlora, A. (1972) in Current Topics in Regulation (Horecker, B. L., and Stadtman, E. R., eds.) Vol. 6, pp. 21-62, Academic Press, New York.

(18) Nevaldine, B. H., and Levy, H. R. (1965) Biochem. Biophys. Res. Commun., 21, 28-33.

(19) Holten, D. (1972) Biochim. Biophys. Acta, 268, 4-12.

(20) Yoshida, A. (1966) J. Biol. Chem., 241, 4966-4976.

(21) Taketa, K., and Pogell, B. M. (1966) J. Biol. Chem., 241, 720-726.

(22) Bonsignore, A., DeFlora, A., Mangiavolti, M. A., Lorenzoni, I., and Alema, S. (1968) Biochem. J., 106, 147-154.

(23) Holten, D., personal communication.

(24) Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem., 193, 265-275.

(25) Morrison, J. F., and Uhr, M. L. (1966) Biochim. Biophys. Acta, 122, 57-74.

(26) Shore, J. D., and Chakrabarti, S. J. (1976) Biochemistry, 15, 875-879.

(27) Greenbaum, A. L., Gumaa, K. A., and McLean, P. (1971) Arch. Biochem. Biophys., 143, 617-663.

(28) DeWeer, P., personal communication.

(29) Gennis, L. S., personal communication.

(30) Manley, E. R., personal communication.

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