STUDIES ON GLYCOGEN SYNTHETASE FROM *TETRAHYMENA PYRIFORMIS*

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FROM TETRAHYMENA PYRIFORMIS

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CHAPTER I

INTRODUCTION

Glycogen serves as a nutritional resorvoir in animal and bacteria. It is a branched polysaccharide consisting of glucose residues linked primarily by α,1,4 glycoside bonds between branch points and by α,1,6 linkages at the branch points. Glycogen has variously been found to have 8 to 12 glucose residues between branch points and molecular weights ranging from 2.7×10^6 to 1×10^9.

It is synthetized by the action of the enzyme glycogen synthetase from nucleoside diphosphate glucose and primer glycogen according to equation 1:

$$1 \quad \text{NDP-glucose}^1 + (\text{glycogen})n \longrightarrow \text{NDP} + (\text{glycogen})_{n+1}$$

where n is the number of glucosyl residues. This enzyme has been shown to be present in a large number of animals, yeast, plants and bacteria. In some cases the enzyme has been purified and its properties studied. Although the protozoan

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[1]The following abbreviations are used: NDP-glucose, nucleoside diphosphate glucose; NDP, nucleoside diphosphate; G-1-P, glucose-1-phosphate; LDH, lactic dehydrogenase, PEP, phosphoenol pyruvate; DTT, dithiothreitol; A_{340}, absorbance at 340 mμ; ΔA_{340}, change in absorbance at 340 mμ. The rest of the abbreviations used are those currently accepted by the Journal of Biological Chemistry.

Tetrahymena pyriformis has been reported to contain as high as 50 per cent of its dry weight as glycogen, the glycogen synthetase of this organism has not been previously well studied. The purpose of the investigation described in this thesis was to attempt purification of glycogen synthetase and to study some of the properties of the enzyme.

CHAPTER II

LITERATURE REVIEW

Glycogen Synthesis by Glycogen Synthetase or Phosphorylase

The synthesis of polysaccharides can take place by transfer of glycosyl residues from sugars substituted at the hemiacetalic hydroxyl group (1). Thus disaccharides, glucose-1-phosphate and nucleotide sugars can serve as glycosyl donor molecules. The biosynthesis of glycogen and starch has been achieved *in vitro* with the enzyme phosphorylase as shown in reaction 2.

2 G-1-P + acceptor $\longrightarrow$ α,1,4 glycosyl acceptor + Pi.

Such a reaction leads to elongation of the α,1,4 chains of glycogen or starch. On reaching a certain length the polysaccharide may be acted upon by the branching enzyme to produce α,1,6 linkages, thus giving rise to the typical chains of glycogen or amylopectin. Since the phosphorylase reaction is freely reversible, it was once believed that glycogen synthesis or degradation *in vivo* was carried out by the same enzyme. Currently it is believed, however, that *in vivo* phosphorylase is mainly involved in breakdown of glycogen (1), rather than synthesis because of the following

reasons: In *in vitro* experiments the phosphorylase reaction reaches equilibrium when the ratio of inorganic phosphate to glucose-1-phosphate is about 3 at pH 7.0. Synthesis of glycogen takes place only when this ratio is lower than 3. In animal tissues *in vivo* this ratio has been shown to be as high as 300 (2). Adrenaline or glucagon produces an increase in the amount of active phosphorylase (3, 4) yet produces only glycogenolysis. If phosphorylase were involved in glycogen synthesis, there should have been an increase in glycogenisis but this was not the case.

An enzyme different from phosphorylase which is believed to be responsible for glycogen synthesis was first described by Leloir and Cardini (5). When UDP-glucose was incubated with a rat liver enzyme and a small amount of glycogen, approximately equal amounts of UDP and glycogen were formed according to reaction 3:

$$3\ \text{UDP-glucose} + (\text{glycogen})_n \longrightarrow \text{UDP} + (\text{glycogen})_{n+1}$$

where n is the number of glucosyl residues. This enzyme was named Uridine diphosphate glucose-glycogen transglucosylase or glycogen synthetase. The free energy change in reaction 3 is about -4000 calories, and the equilibrium constant was calculated to be 250 (6). This implies that the reaction should proceed irreversibly in the direction of glycogen synthesis.

Source and Subcellular Distribution of Glycogen Synthetase

Since the detection of glycogen synthetase in rat liver (5), the enzyme has been found in other organs and other sources such as rat (7), rabbit (8), human, turtle, lobster, lamb and frog muscle (9), sheep (10), rat and rabbit brain (11), human leuckocytes (12) and thrombocytes (13), locust (14), Neurospara (9), yeast (15), sweet corn (16), Arthrobacter (17) and Agrobacterium tumefacions (18).

Luck (19) demonstrated the distribution of the enzyme in rat liver subcellular fractions. On the basis of seventy per cent recovery of the enzyme from the subcellular fractions, the nuclear, mitochondrial, microsomal and supernatant fractions had 4 per cent, 17 per cent, 47 per cent and 2 per cent of the activity, respectively. Thus most of the enzyme activity was present in microsomes. The ratio of glycogen synthetase to glycogen content correlated closely in all fractions. The enzyme appears to be bound to glycogen (19, 20) since it was found to sediment with glycogen (20).

Substrate Specificity

Animal Glycogen Synthetase. Glycogen has generally been the acceptor substrate used in glycogen synthetase assays. Many other compounds may serve as glycosyl acceptors, however, none is as good an acceptor as is glycogen. Maltose,

maltotriose, oligosaccharides (21), phosphorylase limit dextrin from glycogen, β amylase limit dextrin from glycogen, soluble starch and potato starch (1) can serve as acceptor substrates. Of these, the compound having the best acceptor activity next to glycogen was phosphorylase limit dextrin from glycogen. It was 59 per cent as efficient as glycogen (1).

Brown et al (22) have reported that phosphorylase, β amylase, and α amylase limit dextrins prepared from glycogen were 25 per cent, 12 per cent and 24 per cent, respectively, as active as glycogen as acceptors. In order to demonstrate the acceptor ability of α amylase limit dextrin a long incubation period was required. When the ratio of UDP-glucose to α amylase limit dextrin was 2, a heptasaccharide rather than glycogen was produced as the reaction product (22). These results confirm and extend a previous report (1) that no glycogen was formed when α amylase limit dextrin from glycogen was used as the acceptor.

In other experiments, glycogen formed by rat muscle glycogen synthetase was shown to be a substrate for β amylase. Since maltose was the product, it was concluded that glycogen synthetase catalyzes only the formation of α,1,4 bonds (7).

The best glucosyl donor substrate for glycogen synthetase is UDP-glucose. An analog of UDP-glucose containing

pseudouridine in place of uridine was only 5 to 10 per cent as active as UDP-glucose when tested with rat liver enzyme (23). TDP-glucose was 5 per cent as active as UDP-glucose when tested with rat and rabbit skeletal muscle enzyme (24, 8). ADP-glucose was 50 per cent as active as UDP-glucose for the enzyme from rat skeletal muscle (21); however, CDP-glucose, IDP-glucose and ADP-maltose did not serve as glycosyl donors.

Bacterial Glycogen Synthetase. Greenberg and Preiss (17) used an enzyme purified 70 to 80 fold from Arthrobacter species to study glycogen synthesis in bacteria. They reported that ADP-glucose was about 300 times more effective a substrate than UDP-glucose. Madsen (18) had reported earlier that glycogen was synthesized by UDP-glucose in Agrobacterium tumefacions; however, ADP-glucose was not tested as a substrate for the enzyme in his experiments. In view of the results of the more recent experiments of Greenberg and Preiss (17) it appears likely that ADP-glucose may be the preferred glucosyl donor in the synthesis of bacterial glycogen.

Phytoglycogen Synthesis. Although phytoglycogen (25) is almost identical to animal glycogen in molecular structure and in physical and chemical properties, ADP-glucose was 250 times more active in the synthesis of sweet corn phytoglycogen (16) than UDP-glucose.

From the available data on bacterial and phytoglycogen synthesis it can be concluded that each of these kinds of glycogen is synthetized from ADP-glucose, rather than UDP-glucose, as the glycosyl donor.

Activators and Occurrence of Two Forms of Glycogen Synthetase

Glycogen synthetase has been shown to be activated by several compounds. The most important activator is glucose-6-P. The extent of activation obtained varies greatly according to the source of the enzyme and the type of preparation used ranging from 1.6 (9) to 250 (11) (7, 8, 9, 10, 11, 15, 20). Algranati and Cabib (15) observed that in the absence of glucose-6-P the pH activity curve of the enzyme had a maximum at pH 7.5, while in the presence of glucose-6-P, a broad pH optimum ranging from pH 7 to pH 9 was observed. Similar behavior was observed by Traut and Lipmann (9) in which lamb muscle enzyme exhibited maximal activity at pH 6.9 without glucose-6-P, but in the presence of glucose-6-P the pH optimum ranged from pH 7 to pH 9. Kornfeld and Brown (8) found that for the rabbit muscle enzyme, the pH optimum was pH 7.5 in the absence of glucose-6-P and pH 8.2 in the presence of glucose-6-P. A requirement for this cofactor has been demonstrated in animal (9) and yeast (15) glycogen synthetase, however no glucose-6-P

requirement has been demonstrated by the bacterial glycogen synthetase of Agrobacterium tumefacions (using UDP-glucose) (18) or the ADP-glucose glycogen synthetase of Arthrobacter species (17).

The mode of activation by glucose-6-P is not due to the transfer of glucose units from glucose-6-P to glycogen, since Leloir et al (7) showed that the glucose moiety of ^{14}C glucose-6-P was not incorporated into glycogen. Larner and Rossel-Perez (26) tested 30 compounds as activators of the dog muscle glycogen synthetase to establish the structural features of the most effective activator. In order to obtain data directly comparable with glucose-6-P, activators were tested at several concentrations and a coefficient of activation (CA) was calculated according to the formula:

$$CA = \frac{Va}{Vg} \times \frac{Kmg}{Kma}$$

where Va and Vg denote the maximal velocity given by the activators and glucose-6-P respectively. Kmg and Kma are the concentrations of glucose-6-P and activator that give half maximal activation. The CA for glucose-6-P was taken as 100. The values of CA for other activators were given as the percentage of the CA value for glucose-6-P (values in parenthesis below). Among the 30 compounds tested glucose-6-P was by far the best activator. Of the compounds tested,

1, 5, sorbitan-6-P (60 per cent) was the next most effective activator compared to glucose-6-P. This suggests that the hydroxyl group at carbon 1 of glucose-6-P is not crucial. The poor activation shown by mannose-6-P (5 per cent) and fructose-6-P (7.9 per cent) points out the importance of the orientation and presence of a hydroxyl group at carbon 2. The hydroxyl group at carbon 3 of glucose-6-P appears to be one of the essential features because allose-6-P (18.2 per cent and xylose-5-P (2.7 per cent) were less effective as activators compared to glucose-6-P. The hydroxyl group at carbon 4 may be a site of attachment for activation since galactose-6-P (33 per cent) was less effective than 1, 5 sorbitan-6-P (60 per cent). The attached phosphate at carbon 6 appears to be an absolute requirement since inorganic phosphate and glucose either alone or in combination did not activate. The pyranose ring configuration was important, since fructose-6-P (7.9 per cent) ribose-5-P (18 per cent), xylose-5-P (2.7 per cent) and sedoheptulose-phosphate (18 per cent) which have a furanose ring structure were relatively poor activators. After testing many other compounds Rossel-Perez and Larner (26) have concluded that important structural features of glucose-6-P as the activator of glycogen synthetase are the hydroxyl groups at carbon 2, 3 and 4 and the phosphate attached to the carbon 6 and a pyranose ring.

Larner and Rossel-Perez (27) observed a decrease in Km for UDP-glucose in the presence of glucose-6-P and magnesium ion for the rat muscle glycogen synthetase. Kornfeld and Brown (8) also reported that in the presence of glucose-6-P and magnesium ion the Km for UDP-glucose was reduced five fold for the rabbit skeletal muscle enzyme. These results offer a partial explanation for the activation of the enzyme by glucose-6-P (8). Larner et al (28) prepared two different forms of glycogen synthetase from rat skeletal muscle in crude form. One form of the enzyme did not require glucose-6-P for activity and was therefore called the independent or I form. It could be prepared by incubating a crude lyophilized preparation for 60 to 70 minutes at 8°° in 0.05 M mercaptoethanol. The other form of the enzyme was called the dependent or D form since glucose-6-P was required for maximal activity. It was prepared by aging the muscle in the frozen state prior to obtaining the enzyme associated with the 100,000 xg particulate fraction. The two forms of glycogen synthetase, independent and dependent have been prepared from toadfish (29), frog (29), rabbit (30) and dog muscle (31). Both the dependent and independent forms of the enzyme have been characterized by measuring the effect of glucose-6-P and magnesium ion on the Michaelis constant for UDP-glucose and the maximum velocity of the reaction.

Such data on glycogen synthetase from several biological materials is compiled in Table I.

Several compounds other than glucose-6-P have been shown to activate glycogen synthetase. The enzyme of rabbit muscle was activated 30 to 40 per cent by magnesium ion (0.0125M) (8). The independent form of rabbit skeletal muscle enzyme has been shown to be activated by calcium ion (5×10^{-3}M to 1.5×10^{-2}M) and magnesium ion (5×10^{-3}M to 1.0×10^{-2}M) in the presence or absence of glucose-6-P (30). Phosphate and sulfate anions (2.5×10^{-3}M to 1.5×10^{-2}M) increased the activity of the independent form. On the other hand phosphate and sulfate ions decreased the activity of the dependent form of the enzyme in the presence of glucose-6-P but were stimulatory in the absence of glucose-6-P (30).

A purified preparation of the enzyme from rabbit muscle has been shown to be activated by the addition of sulfhydryl compounds such as GSH, cysteine or 2-mercaptoethanol (8). Partially purified enzymes from rat liver (7), Neurospara, rabbit, turtle, lobster, and frog muscle (9) also have been shown to require sulfhydryl compounds for maximal acitvity. These studies have suggested the necessity of sulfhydryl groups for the activity of the enzyme.

Inhibitors of Glycogen Synthetase

In addition to the stimulatory effects of compounds

TABLE I

EFFECT OF GLUCOSE-6-P AND MAGNESIUM ION ON THE Km FOR UDP-GLUCOSE AND VELOCITY OF GLYCOGEN SYNTHETASE FROM SEVERAL SOURCES

Source	Enzyme[a] form	Constants[b]	Additions to the Assay Mixture[c] None	Glucose-6-P	Mg^{++}	Glucose-6-P $+Mg^{++}$	Ref.
Rat Liver	NS	Km	-	4.8×10^{-4}	-	-	(20)
Rat Brain	NS	Km	-	2.2×10^{-3}	-	-	(11)
Rat Muscle	NS	Km	-	5.0×10^{-4}	-	-	(7)
	I	Km	1.0×10^{-3}	2.0×10^{-4}	1.7×10^{-4}	1.0×10^{-4}	(28)
	I	V	.038	.038	.036	.035	(28)
	D	Km	6.0×10^{-4}	4.0×10^{-4}	4.0×10^{-3}	2.5×10^{-4}	(28)
	D	V	.0045	.03	.0063	.03	(28)
Rabbit Muscle	NS	Km	1.85×10^{-3}	5.91×10^{-4}	1.46×10^{-3}	2.95×10^{-4}	(8)
	NS	V	.102	.14	.13	.138	(8)
	I	Km	3.3×10^{-3}	4.2×10^{-4}	2.5×10^{-3}	5.0×10^{-4}	(30)
	I	V	0.02	.02	.02	.02	(30)
	D	Km	5.0×10^{-3}	2.6×10^{-4}	7.1×10^{-3}	2.7×10^{-4}	(30)
	D	V	0.7	3.3	.07	2.8	(30)

TABLE I (Continued)

Source	Enzyme[a] form	Constants[b]	Additions to the Assay Mixture[c]: None	Glucose-6-P	Mg^{++}	Glucose-6-P $+Mg^{++}$	Ref.
Dog Muscle	I	Km	1.9×10^{-3}	6.6×10^{-4}	2.4×10^{-4}	2.3×10^{-4}	(31)
	I	V	.037	.037	.037	.037	(31)
	D	Km	1.0×10^{-2}	7.5×10^{-4}	-	2.1×10^{-4}	(31)
	D	V	.016	0.16	.016	0.19	(31)
Frog Muscle	D	Km	-	1.8×10^{-3}	-	1.1×10^{-3}	(29)
	D	V	-	.38	-	0.9	(29)
Toadfish Muscle	D	Km	-	2.5×10^{-4}	-	1.5×10^{-4}	(29)
	D	V	-	.174	-	.24	(29)
Tetrahymena pyriformis[d]	I	Km	-	-	-	1.66×10^{-3}	

a - The symbols used are: NS, not specified; I, independent form, and D, dependent form of glycogen synthetase.

b - Km, expressed as moles/liter; V, represents maximum velocity expressed as μmoles/mg protein/minute.

c - The results shown have been taken from several papers. Different assay conditions were used by different workers, therefore no attempt has been made to indicate concentration of reactants used. The column None means a complete glycogen synthetase assay mixture as described by the author except that neither glucose-6-P nor magnesium ion was added.

d - Data from this thesis.

containing sulfhydryl groups, the importance of sulfhydryl groups in glycogen synthetase has also been suggested by the inhibitory effects of sulfhydryl reagents. The frog muscle enzyme (29) was completely inhibited by p-hydroxy mercuribenzoate (4×10^{-4}M). Rabbit muscle enzyme was completely inhibited by p-hydroxy mercuribenzoate (1×10^{-5}M) in the absence of glucose-6-P but in the presence of glucose-6-P 30 per cent of the glycogen synthetase activity remained. Similarly the enzymes from toadfish, rat and dog muscle were inhibited by p-hydroxy mercuribenzoate. However this inhibitory effect was overcome by 0.05M mercaptoethanol in the reaction mixture (28-31).

Inhibition of glycogen synthetase by compounds other than sulfhydryl compounds also has been observed. Phloridizin (3.3mM) and glucose (0.05M) each inhibited the activity of rat muscle enzyme 50 per cent. Phloridizin (2×10^{-3}M) inhibited the activity of glycogen synthetase from the rabbit muscle enzyme by 90 per cent (32). Glycogen synthetase of rat muscle was inhibited by potassium borate (0.2M; 80 per cent), KCN (0.1M; 58 per cent) UDP (1mM; 31 per cent) and trehalose phosphate (0.01M; 59 per cent) (7). UMP, IMP, CMP and GMP inhibited between 20 and 50 per cent when tested at the same concentrations as the substrate (5mM). Uridine 5'-phosphate and glucose-1-P were

shown to be competitive inhibitors of glycogen synthetase with respect to UDP-glucose (32).

Control Mechanisms and the Interconversion of the Two Forms of Glycogen Synthetase

The two forms of glycogen synthetase are interconvertible (33). In mouse muscle Danforth (34) demonstrated that there is a good correlation between the amount of glycogen and the per cent of the independent form of glycogen synthetase. When the tissue glycogen content was high the enzyme existed primarily as the dependent form. Conversely, when the tissue glycogen was low, the enzyme existed primarily as the independent form. These data led Danforth (34) to suggest that glycogen controls its own synthesis by controlling the dependent form and the independent form interconversion.

One mechanism of interconversion of the dependent and independent forms of glycogen synthetase has been shown to be due to phosphorylation and dephosphorylation reactions (33). The independent form of the enzyme was phosphorylated in the presence of magnesium ion and ATP labelled with ^{32}P in the γ position. The terminal phosphate group of ATP was incorporated into the independent form of the enzyme to yield the dependent form. This reaction appeared to be enzymatic. The Km for ATP for the conversion of the independent to the

dependent form of rat muscle enzyme was shown to be 7×10^{-5}M. The conversion of the independent form to the dependent form was three times faster when UTP and magnesium ion was used instead of ATP and magnesium ion. Cyclic 3',5'-AMP enhanced the conversion of the independent to the dependent form with either ATP and magnesium ion or UTP and magnesium ion. When the ^{32}P-labelled dependent form of the enzyme prepared from the experiment described above was incubated with a crude rat muscle extract it was converted to the independent form with a concomitant liberation of ^{32}P-labelled inorganic phosphate.

Belocopitow, Appleman and Torres (35) have reported that the independent form of the enzyme was converted to the dependent form when it was incubated with calcium ion and a protein factor present in the enzyme preparation. Such a reaction would lead to a decrease in glycogen synthesis. The activation of phosphorylase b kinase which leads to glycogen breakdown also requires calcium ion and a protein factor (36). The properties of the protein factors which are required for each of these processes have been compared (35) and found to be nearly identical. This suggests that a single protein performs a regulatory function in both the synthesis and the breakdown of glycogen. Its effects would be to decrease glycogen synthesis by participating in the conversion of the

independent to the dependent form of glycogen synthetase and to enhance glycogenolysis by stimulating phosphorylase b kinase activity and ultimately phosphorylase activity.

Effect of Epinephrine on Glycogen Synthetase

Belocopitow (37) observed a decrease in the activity of glycogen synthetase when rat hemidiaphragms were incubated with epinephrine. A similar effect was also observed when rat leg muscle enzyme was incubated with cyclic 3',5'-AMP or ATP and cyclic 3',5'-AMP (37). Larner and Rossel-Perez (31) have shown that cyclic 3',5'-AMP enhanced the conversion of the independent to dependent form of glycogen synthetase in the presence of ATP and magnesium ion. This reaction leads to the formation of a less active form of glycogen synthetase. It has also been shown that epinephrine acts by increasing the formation of cyclic 3',5'-AMP (38-40) which in turn activates phosphorylase b kinase in the presence of ATP (36). Thus administration of epinephrine also leads to an increase in glycogenolysis. The effect of epinephrine is to increase the formation of cyclic 3',5'-AMP which enhances the conversion of the independent form to the dependent form of glycogen synthetase, thereby decreasing glycogen synthesis, and also activates phosphorylase b kinase which ultimately increases glycogenolysis.

Histochemical Studies

A histochemical demonstration of glycogen synthetase and amylophosphorylase in tissue slices has been reported (41, 42). Cut frozen sections of fresh tissues were incubated in a mixture containing UDP-glucose or glucose-1-P as substrate for one hour at constant temperature (25° or 37°). The tissue was then placed in a dilute Gram's iodine solution until a color due to an iodine-polysaccharide complex appeared. The polysaccharide synthesized from glucose-1-P stained reddish purple or violet purple (42a) and was digested completely by α amylase but incompletely by β amylase suggesting that it was a branched polysaccharide. To explain the formation of a branched polysaccharide from glucose-1-P, it was suggested that the polysaccharide resulted from the action of amylophosphorylase and branching enzyme. When tissue slices were incubated with glucose-1-P and 10^{-4}M $HgCl_2$ to inhibit branching enzyme the polysaccharide produced stained blue, (42a) as is typical of the linear amylose structure, and was completely digested by α and β amylases. These results indicated that polysaccharide synthesis under these conditions was due to the action of amylophosphorylase. The polysaccharide synthesized with UDP-glucose as substrate stained the same as naturally occuring glycogen (reddish brown) (42, 43). Furthermore, it was completely digested by α amylase but

incompletely digested by β amylase suggesting that the polysaccharide had a branched structure. Thus the polysaccharide probably was synthesized as a result of the action of glycogen synthetase and branching enzyme. Glycogen synthesis from UDP-glucose in the presence of $HgCl_2$ to inhibit the branching enzyme, an experiment which would test the validity of this interpretation, was not reported. It would be expected that this product would also stain blue since the enzyme is supposed to make only linear α,1,4 bonds (7).

Experiments using tissue slices and the histochemical assay technique have in general yielded results (43) similar to those obtained in <u>in vitro</u> studies (7-15, 26-32). For example the pH optimum for polysaccharide synthesis from UDP-glucose was from pH 7.2 to pH 8.4 and synthesis rapidly decreased at pH 9.0 (43). In addition glucose-6-P increased glycogen synthesis from UDP-glucose in agreement with its known stimulatory effect on glycogen synthetase. Muscle adenylic acid and ATP enhanced polysaccharide synthesis from glucose-1-P but not from UDP-glucose. AMP and ATP are known to stimulate phosphorylase activity. Polysaccharide synthesis from glucose-1-P was optimal at pH 5.7 to 6.0. Potassium phosphate inhibited polysaccharide synthesis from glucose-1-P but not from UDP-glucose. These results are consistent with the known equilibrium for the phosphorylase catalyzed reaction.

High phosphate concentrations would shift the equilibrium toward glycogenolysis rather than glycogen synthesis.

Takeuchi and Glenner (42) were unable to explain the fact that glycogen synthetized from UDP-glucose stained reddish brown while that synthetized in the presence of UDP-glucose and glucose-6-P stained reddish purple. The color change suggests that in the latter case the polysaccharide was less highly branched. A possible explanation for these results is that the branching enzyme becomes limiting when glycogen synthetase is stimulated by glucose-6-P, thus the product is less highly branched.

Glycogen and Glycogen Synthetase from *Tetrahymena pyriformis*

Glycogen isolated from the protozoan *Tetrahymena pyriformis* is a polysaccharide with properties similar to those of mammalian glycogen (44). The organism can contain as high as 50 per cent of its dry weight as glycogen (45). The presence of glycogen synthetase in *Tetrahymena pyriformis* was demonstrated by Cook (46) by incorporation of glucose ^{14}C from UDP-glucose ^{14}C into glycogen. The best glycosyl donor for *Tetrahymena pyriformis* glycogen synthetase was UDP-glucose. ADP-glucose was 1.7 per cent effective as UDP-glucose but GDP-glucose and IDP-glucose were completely ineffective as

substrates.[2] The purpose of the present work was to extend previous studies of Tetrahymena pyriformis glycogen synthetase (46 and footnote 2) by attempting to purify the enzyme and to examine some of its properties.

[2]Unpublished results of N. H. Best and D. R. Wilken.

CHAPTER III

EXPERIMENTAL PROCEDURES

Materials

Glycogen was isolated and purified from Tetrahymena pyriformis by the method of Manners and Ryley (44). The cells were harvested in the stationary phase of growth. UDP-glucose, phosphoenol pyruvate, pyruvate kinase, lactic dehydrogenase, glucose-6-phosphate and UDP were obtained from Sigma Chemical Company, NADH from Boehringer and Soehne and GSH from Mann Research Laboratories. Proteose peptone and yeast extract from Difco Laboratories. Tween 80 from Nutritional Biochemicals and antifoam B from Dow Corning Corporation. All other chemicals used were of reagent quality.

Methods

Tetrahymena pyriformis growth medium contained 10 gm proteose peptone, 10 gm dextrose, 1 gm yeast extract, 5 ml 1M KH_2PO_4, 5 ml 1M K_2HPO_4, 0.5 ml Tween 80, 0.06 ml antifoam B in a total volume of 1000 ml (46). Cells were grown, generally at 26 to 28° with vigorous aeration either in a

carboy of 10 liter capacity containing 7 liters of medium or in a 2 liter aspirator bottle containing 1.5 liters of medium. Growth medium usually was inoculated with cell cultures to yield approximately 1 to 5 x 10^3 cells per ml. Cell counts were made in a Sedgewick-Rafter counting chamber with a Whipple occular micrometer (47) after fixation in 0.5 per cent formic acid containing 0.5 per cent sodium chloride. Early stationary phase cells were harvested when the culture reached 5.5 to 5.85 x 10^5 cells per ml and stationary phase cells were harvested when there were approximately 5.9 x 10^5 to 1.2 x 10^6 cells per ml. Protein concentrations were measured by the procedure of Lowry et al.

Preparation of Crude Extract

Cells were harvested by centrifugation at 0 to 5° for 5 minutes at 10,400 xg in a Sorvall RC-2 centrifuge. Cells were washed twice with 0.1M Tris HCl buffer pH 7.5 and the top fluffy layer was removed by aspiration. The weight of the cells was determined and an equal volume of 0.1M Tris HCl buffer pH 7.5 was added. The cell suspension was sonicated for 1 to 1.5 minutes using a Raytheon Sonic Oscillator Model DF 101. The sonicate was centrifuged at 10,000 xg for 15 minutes in a Sorvall RC-2 centrifuge at 0° and the supernatant solution was filtered through glass

wool. The filtrate from this procedure was used as the starting material in attempts to purify glycogen synthetase. This supernatant solution will be referred to as the crude extract in the text.

Assay Procedures

One Step Assay. UDP-glucose in the presence of primer glycogen and the enzyme glycogen synthetase forms glycogen and UDP (equation 3). The UDP formed was assayed in the presence of PEP, pyruvate kinase, LDH and NADH which leads to the formation of lactate and NAD as shown in equations 4 and 5.

3 $$\text{UDP-glucose} + (\text{glycogen})_n \xrightarrow[\text{synthetase}]{\text{glycogen}} \text{UDP} + (\text{glycogen})_{n+1}$$

4 $$\text{UDP} + \text{PEP} \xrightarrow[\text{kinase}]{\text{pyruvate}} \text{UTP} + \text{pyruvate}$$

5 $$\text{Pyruvate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{LDH}} \text{Lactate} + \text{NAD}^+$$

The reaction was followed by measuring the decrease in light absorption at 340 mμ due to disappearance of NADH. The assay mixture contained Tris HCl buffer pH 7.5, 50 mM; $MgCl_2$, 10 mM; purified Tetrahymena pyriformis glycogen, 1 per cent; glucose-6-P, 1 mM; glutathione, 5 mM; phosphoenol pyruvate 0.78 mM; NADH, 0.15 mM; KCl, 7.5 mM; pyruvate kinase, 2.1 units; LDH, 0.2 units; UDP-glucose, 0.4 mM and the solution being tested for glycogen synthetase activity. Water was

used to bring the final volume of the reaction mixture to 1 ml. All reagents except the extract and UDP-glucose were mixed in the cuvette and then the extract was added. A background rate of NADH oxidation was measured and the UDP-glucose was added. The rate of NADH oxidation was remeasured. The stimulation in rate of NADH oxidation in the presence of UDP-glucose compared to its absence was taken as the rate due to glycogen synthetase. The rate of NADH oxidation was measured using a Beckman DB Spectrophotometer, and a Sargeant Model SRL recorder. The reaction mixture was maintained at 25° by circulating water at 25° through the cuvette chamber housing.

Two Step Assay. The two step assay is a modification of Traut and Lipmann's procedure (9). The reaction mixture contained Tris HCl buffer pH 7.5, 50 mM; $MgCl_2$, 10 mM; purified Tetrahymena pyriformis glycogen, 1 per cent; glucose-6-P, 1 mM; glutathione, 5 mM, Tetrahymena pyriformis extract and UDP-glucose, 0.4 mM in a final volume of 0.5 ml. After temperature equilibration of the other reagents at 25°, UDP-glucose was added to initiate the reaction. The reaction was stopped after the required amount of time by heating the reaction mixture in a boiling water bath for 1 minute. Precipitated protein was removed by centrifugation for 5 minutes at full speed in a clinical centrifuge. An aliquot of the

supernatant solution was then assayed for UDP in the presence of the reagents described in the one step assay except that UDP-glucose, glycogen and glycogen synthetase were omitted.

CHAPTER IV

RESULTS

Purification Methods Attempted

The crude extract prepared as described in Methods, was centrifuged at 100,000 xg for 1 hour in a Spinco Model L Ultracentrifuge. There were three layers of precipitate in the bottom of the tube. The bottom layer was a marble-like glassy solid which probably was glycogen. The center layer was pink in color. The top layer of the precipitate was a loosely packed material. The supernatant solution was a clear yellow liquid. The supernatant solution contained 50 to 70 per cent of the total glycogen synthetase of the same specific activity as the crude extract. The enzyme in the 100,000 xg supernatant solution was more unstable than that in the crude extract. Since most of the enzyme was present in the soluble fraction, the 100,000 xg supernatant solution was used to attempt purification experiments.

The 100,000 xg supernatant solution was subjected to ammonium sulfate fractionation at 0°. During the addition of ammonium sulfate the solution was kept stirring by using a magnetic stirrer. Protein fractions were obtained between

0 to 40, 40 to 50 and 50 to 65 per cent saturation with ammonium sulfate. The 40 per cent ammonium sulfate precipitate did not have any activity. The 40 per cent supernatant solution had 180 per cent activity compared to the crude extract indicating activation of the enzyme due to ammonium sulfate (49). The protein fraction obtained between 40 and 50 per cent saturation had 15 to 20 per cent of the enzyme activity. The protein fraction obtained between 50 to 65 per cent saturation had about 40 per cent of the enzyme activity compared to the crude extract. Its specific activity was 4.2 fold higher than that of the crude extract. Very little activity was obtained in the supernatant solution obtained from the preparation of the 50 to 65 per cent ammonium sulfate fraction. This activity was not recovered in the 65 to 70 per cent ammonium sulfate fraction. Recovery in different experiments was variable. In one experiment the protein fraction obtained between 50 to 65 per cent saturation had 14 per cent yield and 3.8 fold purification compared to the crude extract. This fraction was dialyzed for 1.5 hours against 0.1 M Tris HCl buffer pH 7.5 and 1 mM DTT at 0°. No loss of enzyme occurred on dialysis. The dialyzed enzyme was chromatographed on a DEAE column (2 x 6 cm) which previously had been equilibrated with 5 mM Tris HCl buffer pH 7.5, 1 mM DTT, and 1 mM EDTA buffer in the cold room (4°).

Then the column was eluted by a linear gradient (50) between 5 mM Tris HCl and 1M Tris HCl each at pH 7.5. Initially the mixing flask contained 5 mM Tris HCl pH 7.5 plus 1MM DTT (100 ml) and the reservoir contained 1M Tris HCl pH 7.5 plus 1 mM DTT (100 ml). Two ml fractions were collected using a Beckman Model 132 fraction collector. The fractions were assayed for protein at 280 mμ. Four peaks of protein were obtained at tube numbers 2, 35, 42 and 51. Enzyme activity was detected in tubes 38 to 47. The contents of these fractions were pooled. The recovery of enzyme from the column was 25.6 per cent or 3.8 per cent compared to the crude extract and the purification was 8.4 fold. The enzyme was lyophilized. This treatment resulted in nearly complete loss in activity.

The reason for the low recovery (25.6 per cent) of the enzyme from the DEAE chromatographic step described above might have been due to inactivation of the enzyme on the column. In order to stabilize lamb muscle glycogen synthetase during DEAE chromatography, Traut and Lipmann (9) included 0.5 per cent glycogen in the eluting solutions which they used. Therefore, in another attempt to purify Tetrahymena pyriformis glycogen synthetase, 0.5 per cent glycogen was added to the eluting solutions described above. A different elution pattern compared to the experiment described

above was obtained. The protein peaks observed (tubes 11, 35, 46, 69 and 82) were not as well separated as in the previous experiment. Enzyme activity was detected in tube 4-20. These fractions were pooled. The recovery of activity from the column was 30 per cent or 12 per cent compared to the crude extract with a purification of 6.5 fold. Therefore, addition of glycogen to the eluting solutions did not appreciably stabilize the enzyme. Lyophilization of the sample resulted in an 80 per cent loss of the activity compared to the unlyophilized sample.

An attempt was made to increase the specific activity of the enzyme by ammonium sulfate fractionation in the presence of mercaptoethanol to protect protein sulfhydryl groups. In one experiment mercaptoethanol was added to a 100,000 xg supernatant solution from the crude extract to a final concentration of 0.05 M. Then it was fractionated with ammonium sulfate. The precipitate obtained between 40 and 50 per cent saturation had only 9 per cent of the enzyme compared to the crude extract and showed a 10 fold decrease in specific activity. The 50 per cent ammonium sulfate supernatant solution contained 95 per cent of the enzyme activity.[3]

---

[3]Addition of the activity observed in the 40 to 50 per cent ammonium sulfate precipitate and supernatant fractions adds up to 104 per cent. This may be due to slight activation by ammonium sulfate (49).

Attempts to recover the enzyme from the supernatant solution at higher concentrations of ammonium sulfate were unsuccessful in that only 10 per cent of the activity could be recovered.

In another experiment an attempt was made to stabilize glycogen synthetase by chelating metal ions with EDTA and by protecting protein sulfhydryl groups with mercaptoethanol. EDTA (mM) and mercaptoethanol (0.05M) were added to the 100,000 xg supernatant solution prior to fractionation with ammonium sulfate. The precipitate obtained between 40 and 50 per cent saturation had 32 per cent of the enzyme activity but only a 1.1 fold purification compared to the crude extract. This approach to stabilize the enzyme to ammonium sulfate fractionation was not pursued since there was only loss of enzyme activity.

Kornfeld and Brown (8) purified an ammonium sulfate fraction (dialyzed) of rabbit muscle glycogen synthetase by using calcium phosphate gel. Two experiments were conducted to purify the Tetrahymena pyriformis glycogen synthetase by using calcium phosphate gel. Four samples of dialyzed ammonium sulfate fraction (50-65 per cent) were treated with 0.5 mg, 1.0 mg, 1.5 mg or 2.0 mg of calcium phosphate gel per mg of protein. The samples in which 0.5 mg gel per mg protein and 1.0 mg gel per mg protein were used absorbed 75 per cent and 82 per cent of the enzyme, respectively. The other two

gel concentrations absorbed all of the enzyme. Several attempts to elute the enzyme from the calcium phosphate gel using 0.2M phosphate mM EDTA buffer pH 7.5 (8) were not successful. Therefore, calcium phosphate gel fractionation attempts were not continued.

Lipmann and Traut (9) have purified lamb muscle glycogen synthetase using ethanol fractionation as one of their purification methods. A similar type of experiment was conducted on a 100,000 xg supernatant solution of crude extract. Two experiments were performed. In one experiment the 100,000 xg supernatant solution was adjusted to 10 per cent ethanol by adding absolute ethanol at -5 to -8°. The temperature was maintained at -5° while the mixture was stirred for 15 minutes. Then it was centrifuged at 20,000 xg for 15 minutes. No activity was detected in the supernatant solution (supernatant 1). The precipitate was suspended in 0.4 volume (compared to the amount of enzyme solution before alcohol treatment) of 0.1 M Tris HCl buffer pH 7.5 and again centrifuged at 20,000 xg for 15 minutes. The clear supernatant solution (supernatant 2) had 75 per cent of the enzyme activity compared to the 100,000 xg supernatant solution and 37.5 per cent of the activity compared to the crude extract. In another experiment the 100,000 xg supernatant solution was treated with absolute ethanol to a final concentration of 20

per cent and supernatant 2 was prepared as described above. The supernatant 2 from this experiment had 100 per cent of the enzyme from the 100,000 xg supernatant solution or 50 per cent recovery compared to the crude extract and a 2 fold purification. Supernatant 2 lost all of its activity within 2 hours, however two additional attempts to fractionate the enzyme with alcohol as described were unsuccessful.

Some experiments were tried to fractionate the crude extract using ethanol. In one experiment ethanol was added to the crude extract to a final concentration of 20 per cent, as described above, and supernatant 2 was prepared. Supernatant 2 had 32.8 per cent recovery of the enzyme with a 1.7 fold purification compared to the crude extract. Then supernatant 2 was fractionated with ammonium sulfate. The precipitate obtained at 45 per cent saturation of ammonium sulfate yielded 67.5 per cent of the activity compared to alcohol fractionation or 22.2 per cent compared to the crude extract with 3.5 fold purification. The supernatant solution from the addition of ammonium sulfate contained only 10 per cent of the activity of the crude extract with a 7.6 fold purification. An attempt to recover the enzyme from the 45 per cent ammonium sulfate supernatant solution by precipitation at a higher ammonium sulfate concentration was unsuccessful. The precipitate obtained at 45 per cent saturation of

ammonium sulfate lost 75 per cent of its activity on storing in the deep-freeze overnight. Another attempt to fractionate the crude extract with alcohol at a final concentration of 40 per cent yielded 51 per cent recovery of the enzyme but no purification. On further fractionation of the supernatant 2 of this experiment with ammonium sulfate (as described in the previous experiment) no purification was obtained and there was a 90 per cent loss of activity.

Stabilization of the Enzyme. The major problem encountered during attempts to purify glycogen synthetase was its instability. The crude extract and the 100,000 xg supernatant solution lost activity within a day when stored at 4° or in the deep-freeze. In some experiments the crude extract, when lyophilized in 2 ml portions and stored in the deep-freeze, was stable for at least 3 days. When the lyophilized powder of the crude extract was dissolved in 0.1 M Tris HCl buffer pH 7.5 the enzyme remained stable when it was frozen and thawed twice within 3 days. In other experiments lyophilization of the crude extract in 5 ml portions resulted in 40 per cent loss of activity.

When the 100,000 xg supernatant solution was lyophilized, 50 per cent of the enzyme activity was lost. By addition of glycogen to the 100,000 xg supernatant solution to a final concentration of 0.01 per cent before lyophilization, the loss

of activity was decreased to 25 per cent. In the presence of 0.11 per cent glycogen the enzyme in the 100,000 xg supernatant solution was stable to lyophilization and the lyophilized powder could be stored for at least 3 days without loss in activity. When the lyophilized powder was dissolved in 0.1 M Tris HCl buffer pH 7.5, 50 per cent of the enzyme activity was lost within 2 days on storing frozen in the deep-freeze.

Properties of Glycogen Synthetase Using the Crude Extract

Attempts to purify glycogen synthetase were not generally successful. Therefore, the crude extract which contained the enzyme was used to study some of its properties.

Validity of Glycogen Synthetase Assays. The reaction catalyzed by glycogen synthetase leads to the production of UDP as one of its products. Both of the assays for glycogen synthetase used are based on the measurement of the production of UDP. Therefore, it is necessary to be able to quantitatively and accurately assay for UDP. UDP was measured spectrophotometrically by following the decrease in light absorption at 340 mμ due to the disappearance of NADH in the presence of PEP, pyruvate kinase and lactic dehydrogenase as was shown in equations 4 and 5.

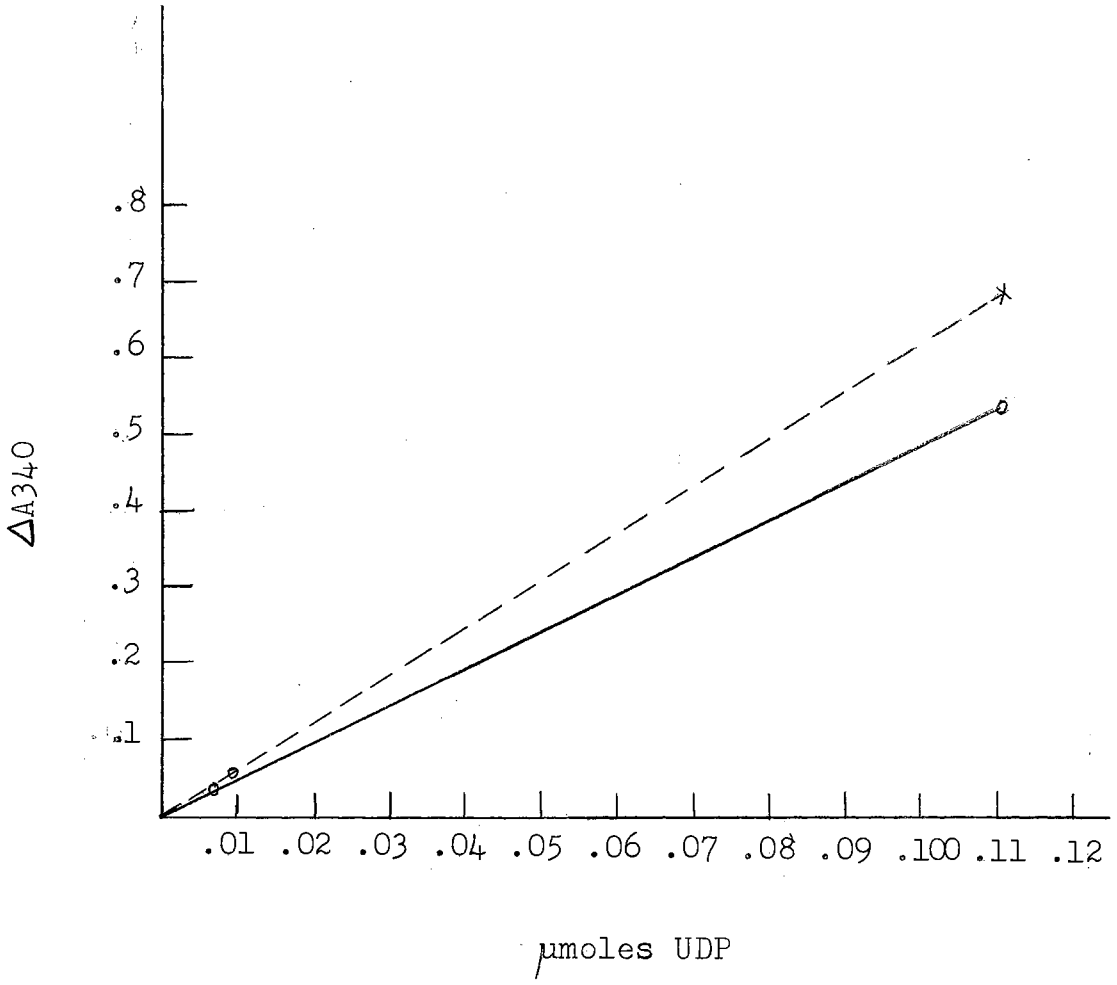
In Figure 1 the total change in A340 versus the μ moles of UDP added to the reaction mixture is shown. A straight line was obtained indicating that the change in A340 in the assay was directly proportional to the amount of UDP added. Although only three points are shown for this curve similar results were obtained in other experiments. The figure shows that the values obtained were 18 per cent less than the theoretically expected values. The underestimation of UDP may be more apparent than real since the UDP used in these assays contained a small amount of UMP as was demonstrated by paper chromatography. These results demonstrate that the assay used for UDP was valid but that it may slightly underestimate UDP concentrations.

In the one step spectrophotometric assay it is necessary to use non-rate limiting amounts of pyruvate kinase and LDH. That LDH and pyruvate kinase were in excess was suggested by the fact that in a glycogen synthetase assay mixture lacking only UDP-glucose and crude extract, the initial reaction rate was equal to a change in A340 of 1.2 per minute. This rate is much higher than the rate normally measured in assays of glycogen synthetase (usually about 0.03 absorbance unit per minute). However, a change in absorbance per minute of 0.03 corresponds to the production of only 0.005 μmoles of UDP per minute. Therefore, it was necessary to show that the

Figure 1

Enzymatic Estimation of UDP

The decrease in A340 is shown as a function of the number of μ moles of UDP added to an assay mixture. The standard one step assay reaction mixture was used except that UDP-glucose, glycogen, and the crude extract were omitted. The dashed line shows the expected curve and the solid line shows the experimentally obtained curve. The ΔA340 was linear with increasing concentrations of UDP between .006 and .115 μmoles of added UDP, however, the estimation of UDP was about 18 per cent less than expected. See text for a discussion of this point.


ΔA340
.8
.7
.6
.5
.4
.3
.2
.1
.01 .02 .03 .04 .05 .06 .07 .08 .09 .100 .11 .12
μmoles UDP

coupling enzymes were in excess even when only a very small amount of UDP was available to them. For this purpose 0.006 μmoles of standard UDP was assayed in the usual assay system omitting only UDP-glucose and crude extract. The reaction was complete within 20 seconds and the total change in A340 observed was 0.03. Thus the reaction rate under these conditions must have been equal to or greater than 0.09 per minute. This value is about 3 fold greater than normally observed in glycogen synthetase assays. Thus the enzymes pyruvate kinase and LDH were in excess and the one step assay employed for measuring glycogen synthetase was valid.

Effect of Protein Concentration and Incubation Time on the Reaction Rate Using the One Step Assay. Initial reaction rates were measured at several protein concentrations to test the linearity of the reaction rate with protein concentration. The results shown in Figure 2 indicate that the reaction rate was linear at low protein concentrations. In this figure the initial rates are given as the ΔA340 per minute, however, the ΔA340 was usually linear for 5 to 8 minutes.

Effect of Protein Concentration on the Reaction Rate Using the Two Step Assay. The number of μmoles of UDP produced in the first incubation of the two step assay system described in Methods was measured as a function of crude extract protein concentration. The results shown in Figure 3

Figure 2

Effect of Protein Concentration on the Rate of Glycogen Synthesis by Glycogen Synthetase using the One Step Assay

The reaction rate of glycogen synthetase is represented by ΔA340 per minute.

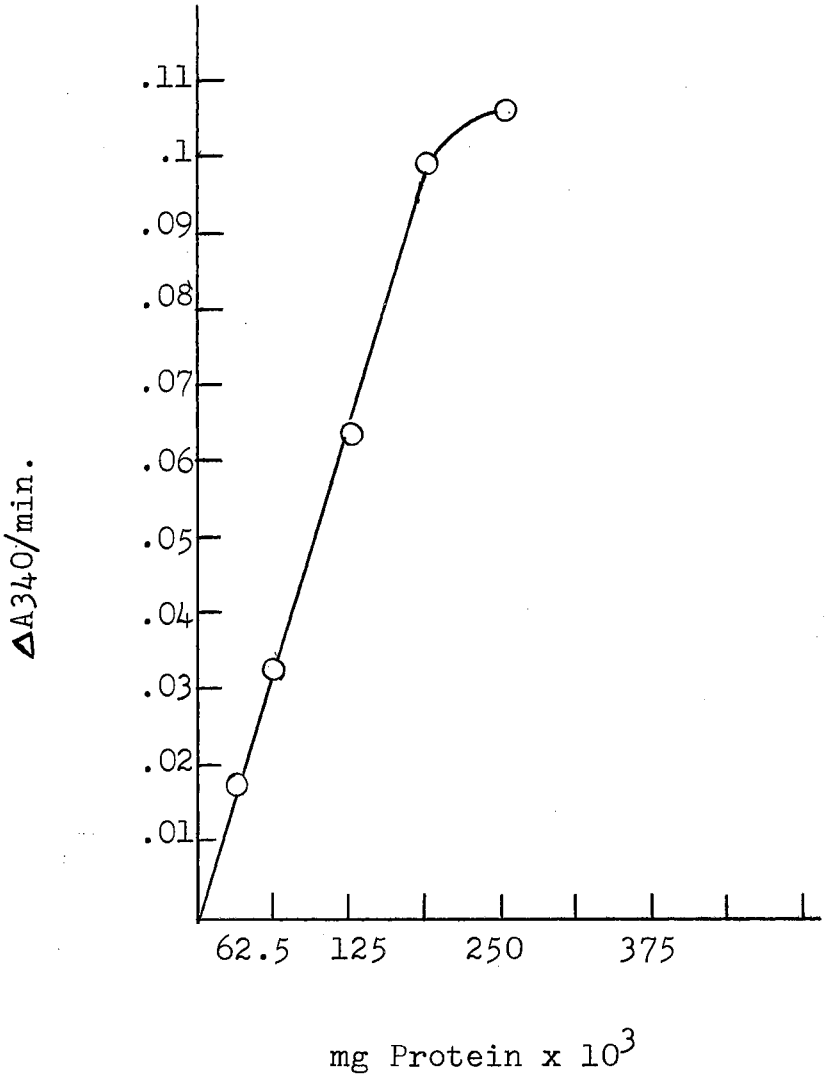
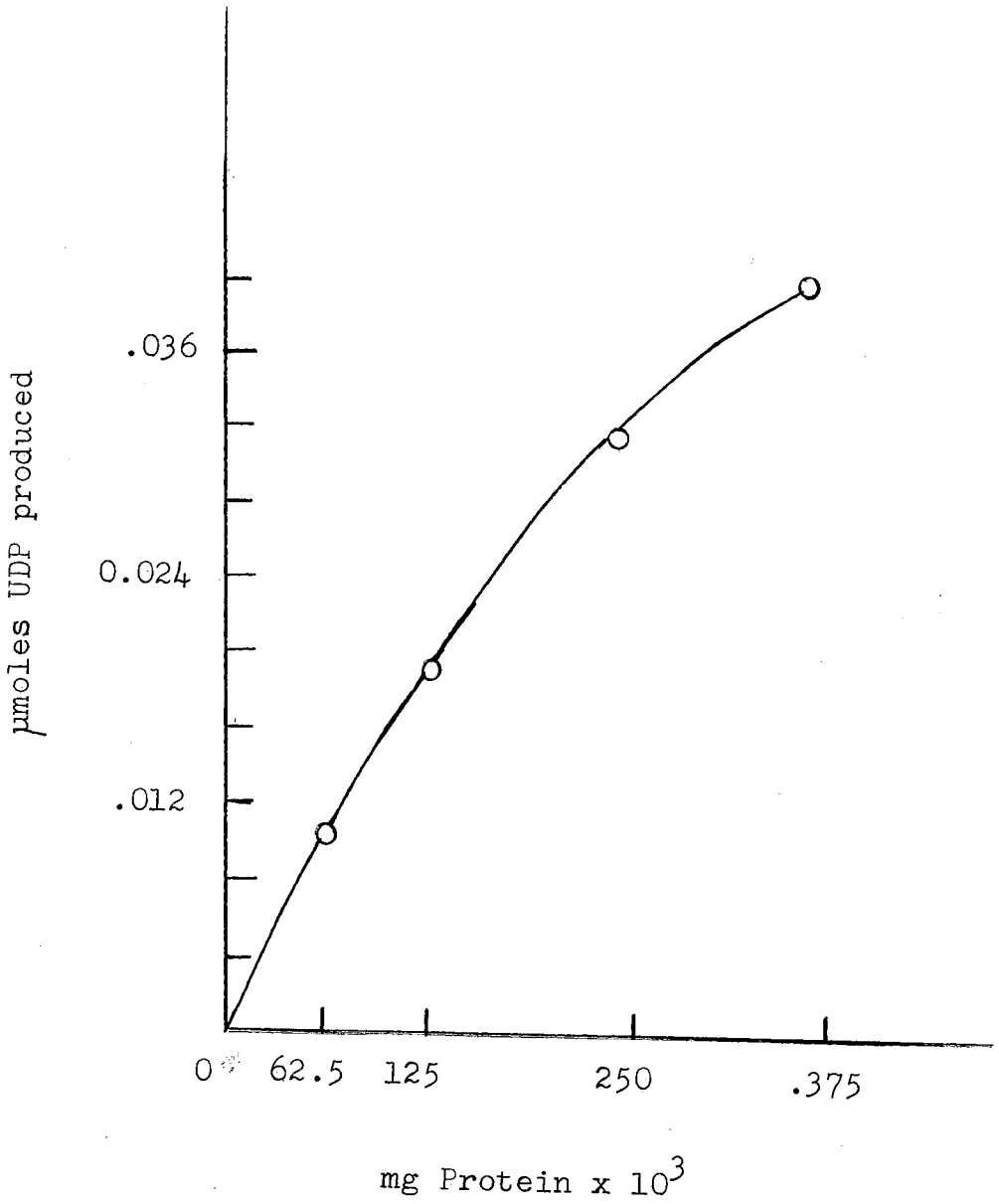

.11
.1
.09
.08
.07
.06
.05
.04
.03
.02
.01
ΔA340/min.
62.5
125
250
375
mg Protein x 10³

Figure 3

Effect of Protein Concentration on Glycogen Synthesis by Glycogen Synthetase Using the Two Step Assay

The plot shows the number of umoles of UDP produced *versus* the crude extract protein concentration. The initial incubation of the reaction mixture was 15 minutes.


μmoles UDP produced
.036
0.024
.012
0
62.5
125
250
.375
mg Protein x 10^3

indicate that the formation of UDP during the initial incubation was nearly linear in the range of 0.0625 to 0.25 mg protein. The initial incubation period was 15 minutes in the experiment shown.

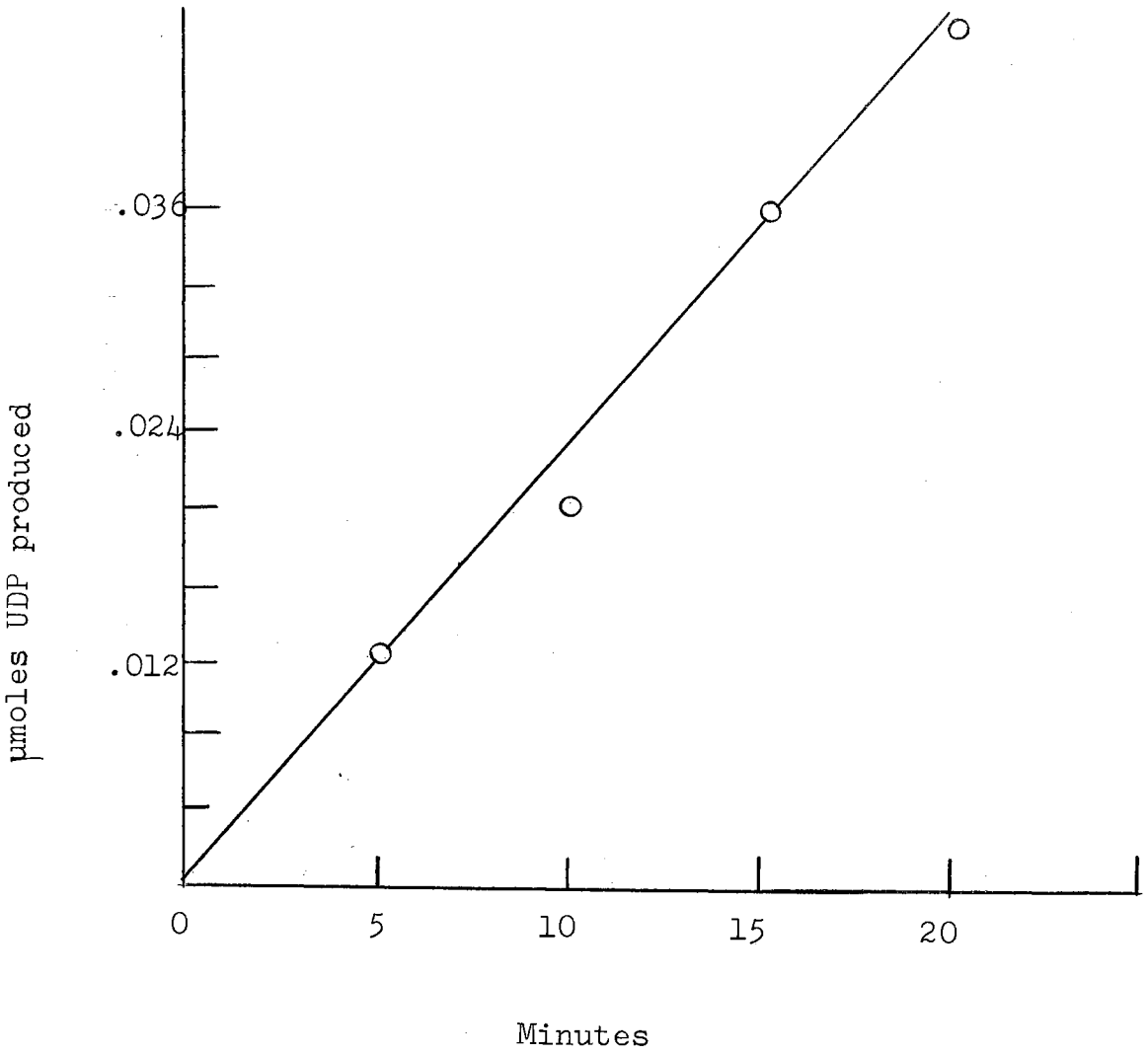
Effect of Time on the Reaction Rate. The number of μmoles of UDP produced in the first incubation of the two step assay was measured as a function of incubation time. The results shown in Figure 4 indicate that the production of UDP was linear for at least 20 minutes at the protein concentration tested (0.125 mg).

Requirements for Glycogen Synthetase. The reagent requirements for the assay of glycogen synthetase have been determined and compared for both one and two step assays (Table II). Despite the fact that each assay was linear with time and protein concentration the one step assay gave nearly double the reaction rate observed in the two step assay. Therefore, the effect of omitting reaction mixture components in each type of assay is given as the percentage activity observed in the complete assay. The reason for this discrepancy in reaction rates was not determined. However, phosphoenol pyruvate, KCl, pyruvate kinase, lactic dehydrogenase; LDH and NADH, which were present in the one step assay were not present in the initial incubation mixture of the two step assay. One of these could have a stimulatory

Figure 4

Effect of Time on the Glycogen Synthesis by Glycogen Synthetase Using the Two Step Assay

The number of μmoles of UDP formed versus the time of initial incubation of the reaction mixture is shown. The reaction was linear for at least 20 minutes at the protein concentration tested (0.125 mg).


μmoles UDP produced
.036
.024
.012
0
5
10
15
20
Minutes

effect on glycogen synthetase. KCl, but not NaCl, has been shown to stimulate bacterial glycogen synthetase approximately 30 per cent (17).

Results shown in Table II demonstrate an absolute requirement for UDP-glucose. In the absence of glycogen the activity was decreased to 10 to 20 per cent of the activity observed in a complete assay system. The residual activity in the absence of glycogen may be at least partially due to the glycogen added in the enzyme extract itself. In the absence of glucose-6-P (in the experiment shown in Table II) the activity was decreased to 91 per cent of the activity observed in the complete system. The effect of glucose-6-P was variable with different preparations of the Tetrahymena pyriformis crude extract. Table III shows that the glycogen synthetase was usually found at least 90 per cent in the independent form but occasionally it was found in lesser amounts. The lowest amount observed was 50 per cent. Similar results were obtained in the one and the two step assays (Table II).

In the absence of GSH, the enzyme exhibited 35 per cent of the activity observed in the presence of GSH (Table II). This value varied between 35 to 50 per cent with different preparation. Similar results were observed both in the one and the two step assays. (Table II).

The dependence of magnesium ion was measured by the two

TABLE II

REQUIREMENTS FOR GLYCOGEN SYNTHETASE

Reaction Mixture

Conditions	One Step Assay	Two Step Assay
	Rate of UDP Formation as a Per Cent of Complete Assay Mixture	
Complete[a]	100[b]	100[b]
-UDP-glucose	0	0
-Glycogen	17	10
-GSH	40	37
$-Mg^{++}$	-	45
-Glucose-6-P[c]	91	91

a Complete means the standard assay conditions as described in Methods.
b The rate of formation of UDP using 0.125 mg of crude extract protein was 5.15 mμmoles per minute in the one step assay and 2.42 mμmoles per minute in the two step assay.
c The values given in this table are from one experiment only.

TABLE III

PERCENTAGE OF THE INDEPENDENT FORM OF GLYCOGEN SYNTHETASE IN DIFFERENT PREPARATIONS OF TETRAHYMENA PYRIFORMIS CRUDE EXTRACT

Number of Experiments	Per Cent I Form*
12	91-100
3	81-88
1	65
2	50

* The one step assay procedure was used.

step assay. In the absence of magnesium ion, activity decreased to 45 per cent of the activity observed in the presence of magnesium chloride.

Effect of Glycogen Concentration on the Reaction Rate. Since glycogen is one of the substrates for the enzyme, the effect of varying the concentration of glycogen was studied. The results shown in Figure 5 indicate that the reaction rate increased up to 12.5 mg of glycogen per ml of the reaction mixture and that above this concentration of glycogen the rate decreased. Similar results were obtained using a 4.2 fold purified preparation of the enzyme obtained by ammonium sulfate fraction (the 50-65 per cent saturated ammonium sulfate fraction of the 100,000 xg supernatant solution obtained from the crude extract). In that experiment maximum activity was observed at 10 mg of glycogen per ml. From these results 10 mg of glycogen per ml was chosen for the standard spectrophotometric assay.

A Lineweaver-Burk plot of the data from Figure 5 is shown in Figure 6. From this plot the Km for glycogen was determined to be 2.06×10^{-2}M, calculated as non-reducing end groups. The data of Manners and Ryley (44) was used to calculate the number of non-reducing end groups per mg of Tetrahymena pyriformis glycogen. The maximum activity corresponds to the utilization of 0.116 μmole of UDP-glucose per minute per mg protein.

Figure 5

Effect of Glycogen Concentration on the Rate of Glycogen Synthesis by Glycogen Synthetase

The effect of glycogen concentration on the reaction rate was studied using the one step assay procedure except that the glycogen concentration was varied as shown.

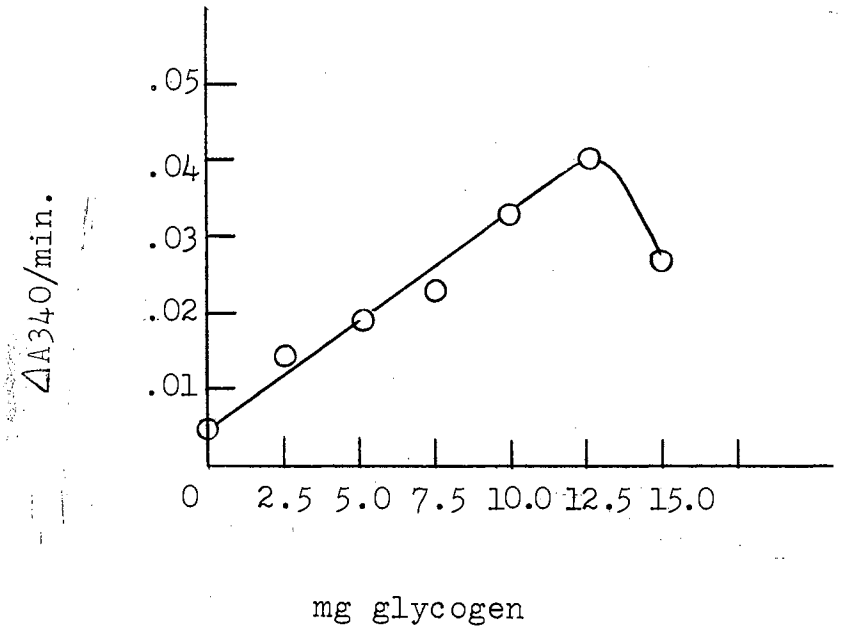
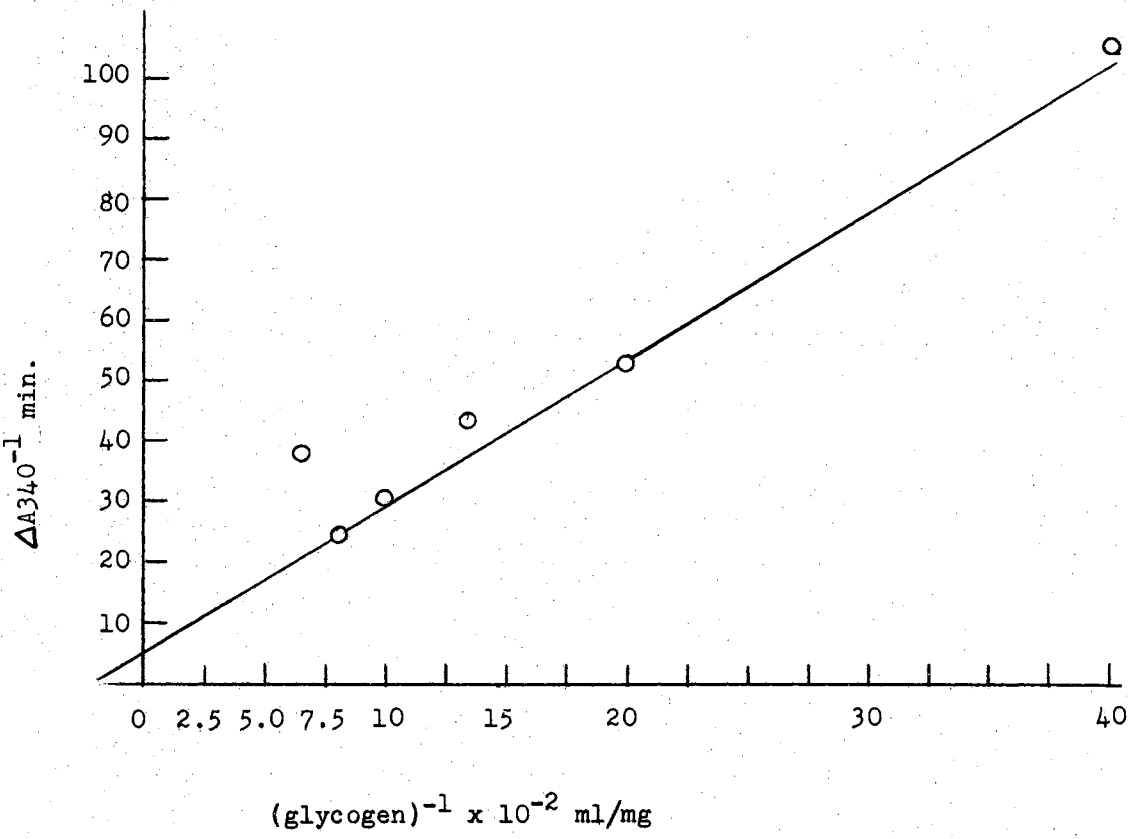

.05
.04
.03
.02
.01
ΔA340/min.
0
2.5
5.0
7.5
10.0
12.5
15.0
mg glycogen

Figure 6

Lineweaver-Burk Plot for Determining the Km of Glycogen

A double reciprocal plot of the reaction rate versus the glycogen concentration is shown. The Km of glycogen was 2.06×10^{-2}M (calculated as non-reducing end groups). The maximum velocity corresponds to the utilization of 0.116 μmoles of UDP-glucose per min. per mg of protein when the UDP-glucose concentration was 0.4 mM.


100
90
80
70
60
50
40
30
20
10
$\Delta A340^{-1}$ min.
0
2.5
5.0
7.5
10
15
20
30
40
(glycogen)$^{-1}$ x 10^{-2} ml/mg

Km for UDP-Glucose. The dependence of the glycogen synthetase reaction on the concentration of UDP-glucose was studied using the independent form of the enzyme. The results are shown in Figure 7. A Lineweaver-Burk double reciprocal plot of the same data is shown in Figure 8. From the Lineweaver-Burk plot the Km for UDP-glucose was determined to be 1.66×10^{-3} M. Substrate inhibition was observed above 0.6×10^{-3} M UDP-glucose.

Effect of Temperature on the Reaction Rate and Energy of Activation. The reaction rate of the enzyme was determined at temperatures 5°, 15°, 20°, 25°, 35° and 40°. The cuvette chamber of the Beckman DB Spectrophotometer was adjusted to the desired temperature by circulating water at the desired temperature through the cuvette chamber housing for 15 minutes prior to conducting the assays. The reagents used were also equilibrated to the proper temperature prior to performing the assays. The reaction rate versus temperature is shown in Figure 9. The temperature optimum was 25°. An Arrhenius plot of the same data is shown in Figure 10. The energy of activation was calculated to be 4740 calories.

Effect of pH on the Reaction Rate. The effect of pH on the reaction rate was studied by varying the pH from 6.0 to 9.0. Tris-maleate buffer was used for pH 6.0 and pH 6.5 and Tris HCl buffer was used to study the rate of the glycogen

Figure 7

Effect of UDP-glucose Concentration on the Reaction Rate

The effect of UDP-glucose concentration on the reaction rate was studied using the one step assay procedure except that the UDP-glucose concentration was varied as shown.

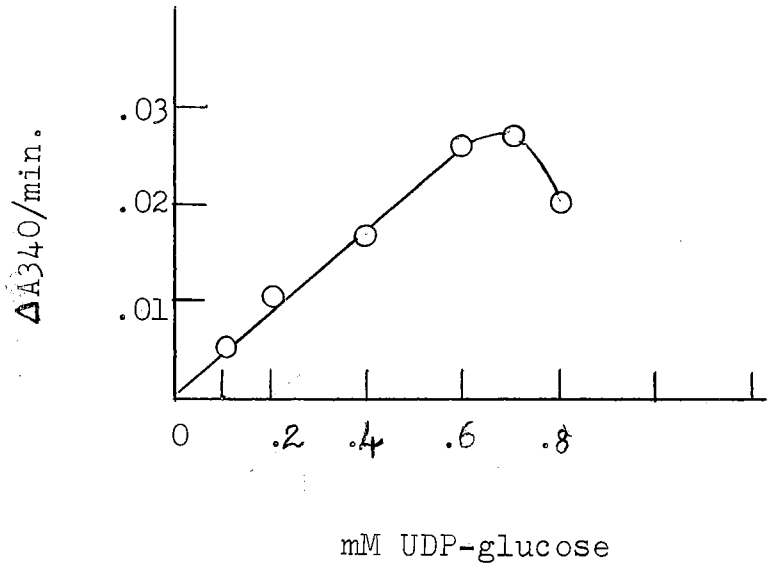

ΔA340/min.
.03
.02
.01
0
.2
.4
.6
.8
mM UDP-glucose

Figure 8

Lineweaver-Burk Plot for Determining the Km of UDP-Glucose

The reciprocal of the reaction rate versus the reciprocal of the UDP-glucose concentration is shown. The Km of UDP-glucose was 1.66×10^{-3}M. The maximum velocity corresponds to the utilization of .05 µmole of UDP-glucose per minute per mg of crude extract protein when the glycogen concentration in the assay mixture was 10 mg per ml.

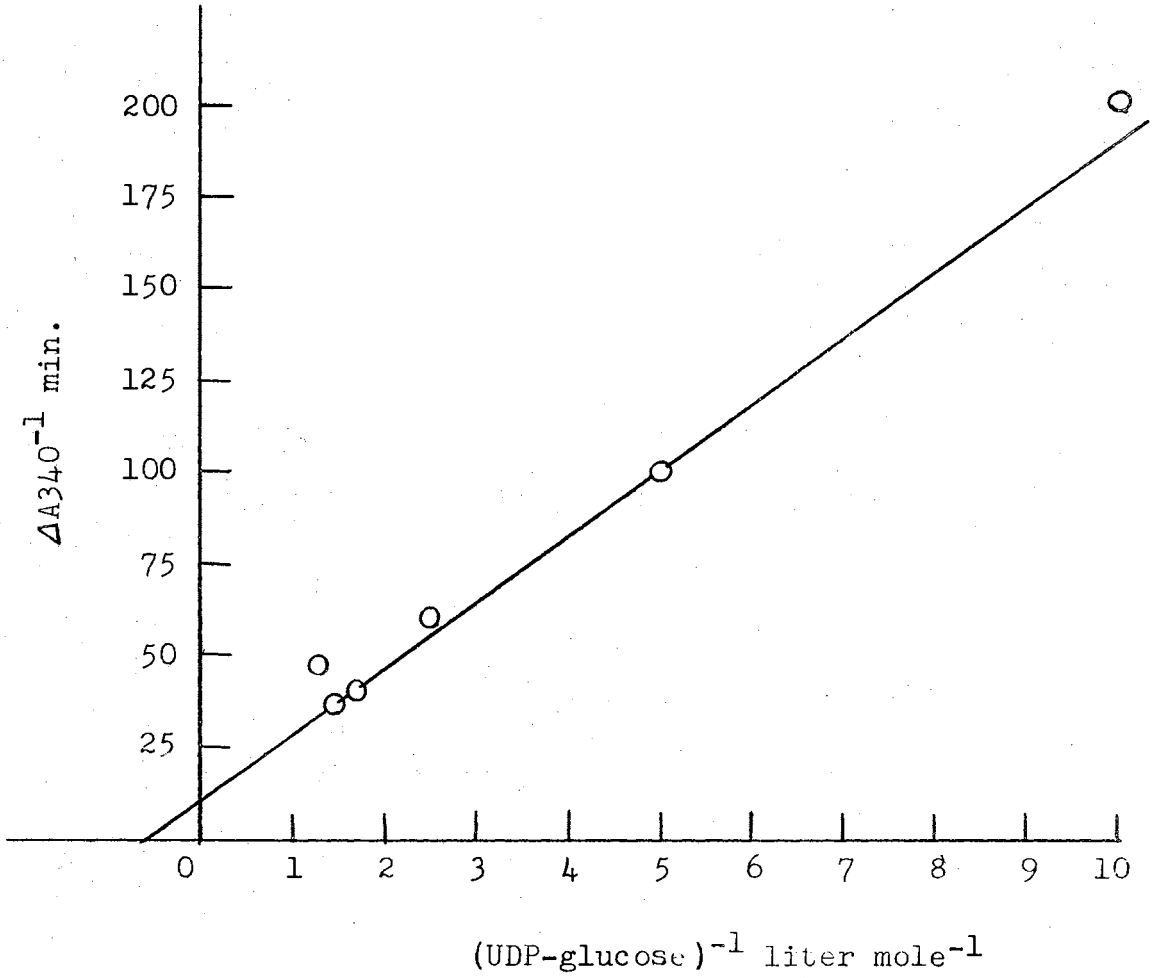

200
175
150
125
100
75
50
25
$\Delta A340^{-1}$ min.
0
1
2
3
4
5
6
7
8
9
10
(UDP-glucose)$^{-1}$ liter mole^{-1}

Figure 9

Effect of Temperature on the Reaction Rate

The effect of temperature on the reaction rate catalyzed by glycogen synthetase was studied using the one step assay. The enzyme showed a maximum activity at 25° and was inactivated above 34°.

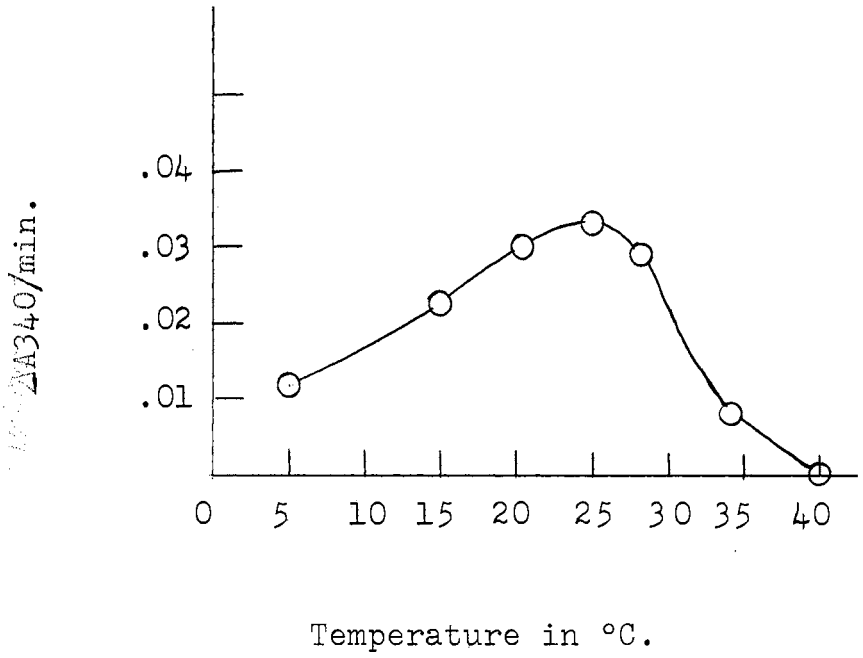
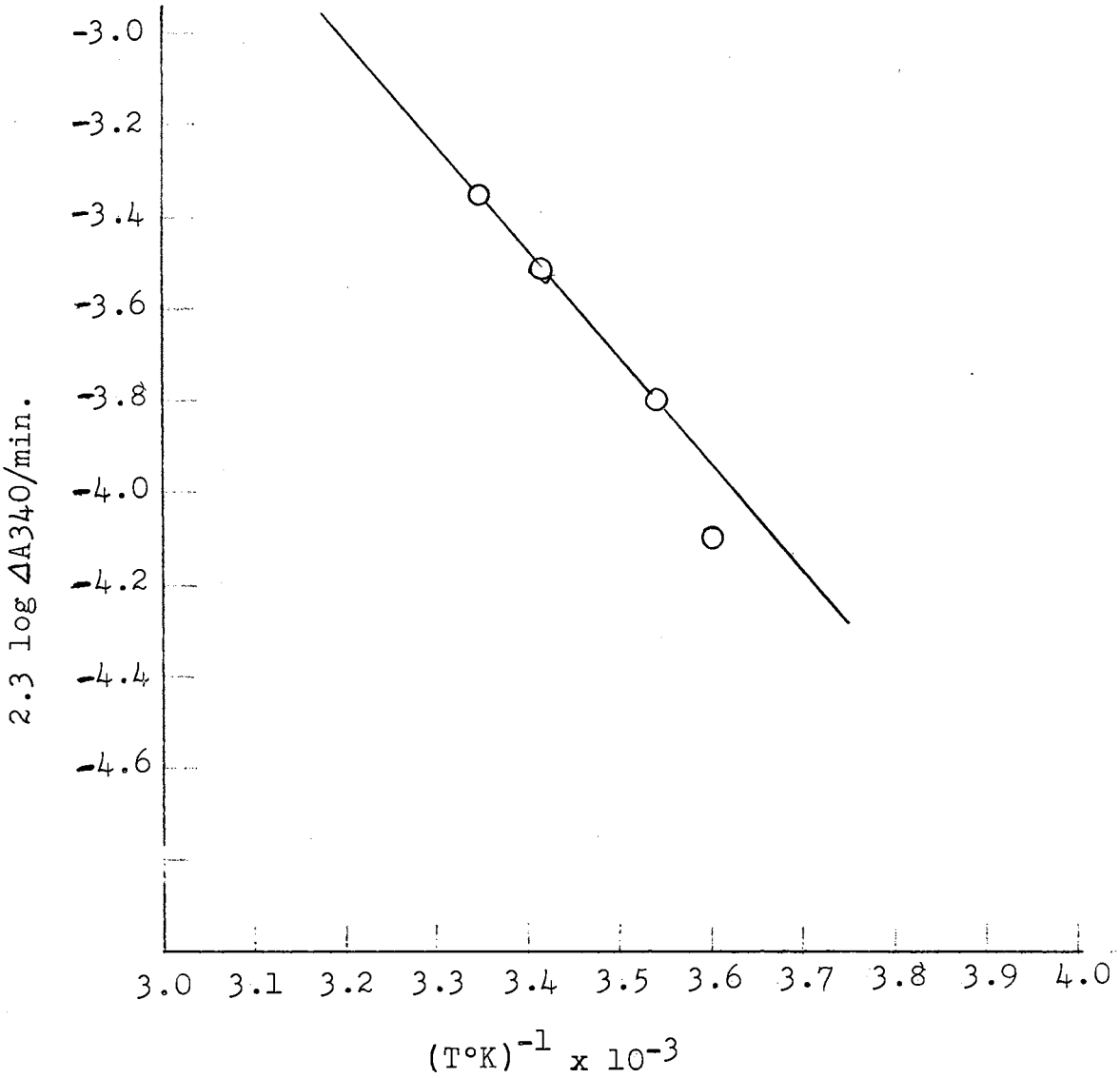

ΔA340/min.
.04
.03
.02
.01
0
5
10
15
20
25
30
35
40
Temperature in °C.

Figure 10

Arrhenius Plot for Determining the Energy of Activation

The logarithm of the reaction rate (times 2.303) *versus* the reciprocal of the absolute temperature is shown. The energy of activation was 4740 calories.

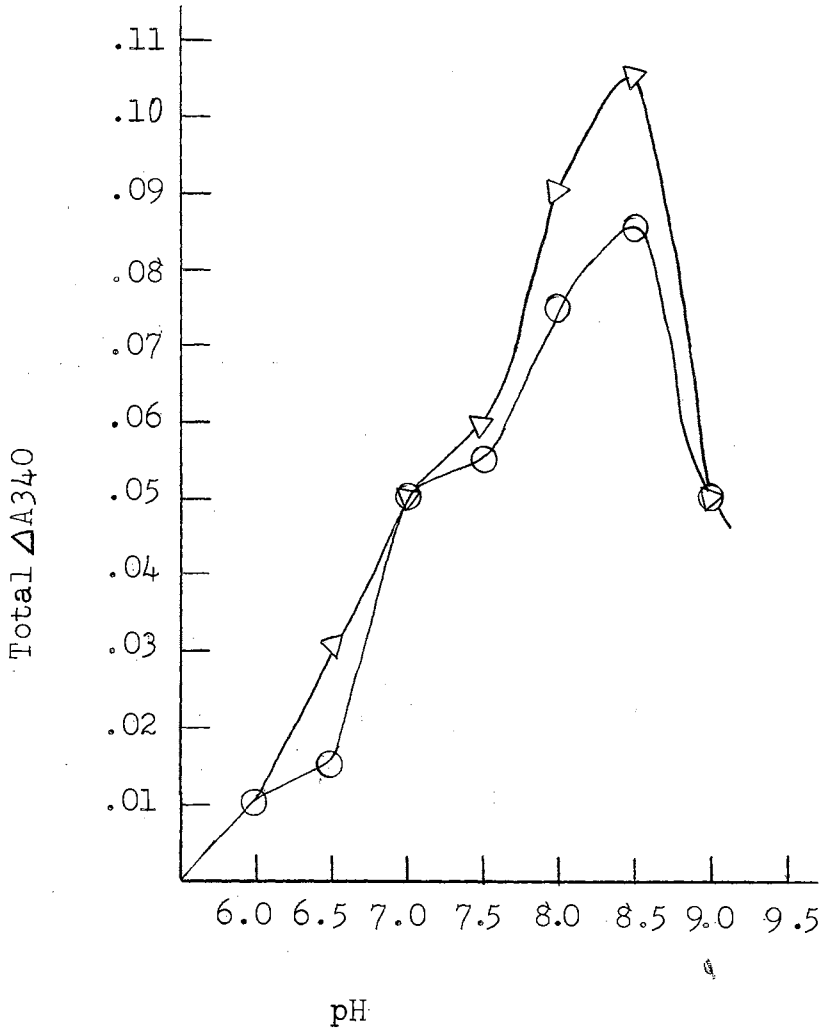

-3.0
-3.2
-3.4
-3.6
-3.8
-4.0
-4.2
-4.4
-4.6
2.3 log ΔA340/min.
3.0
3.1
3.2
3.3
3.4
3.5
3.6
3.7
3.8
3.9
4.0
(T°K)$^{-1}$ x 10^{-3}

synthetase reaction at all other pH values. The effect of pH on the reaction rate of the enzyme was performed using the standard two step assay except that a buffer of the required pH was used instead of Tris HCl buffer pH 7.5. The total change in A340 versus pH is shown in Figure 11. The contour of the pH curve as well as the pH optimum for the enzyme were the same whether the assays were conducted in the presence or absence of glucose-6-P. In this experiment 25 per cent stimulation by glucose-6-P was observed at pH 8.5 and 9 per cent at pH 7.5. Thus the enzyme was primarily in the independent form. The pH optimum was 8.5.

Figure 11

Effect of pH on the Reaction Rate

The effect of pH on the reaction rate of the glycogen synthetase catalyzed reaction was studied using the two step assay except that buffers of varying pH were used. Tris-maleate buffer was used for pH 6 and 6.5 and Tris HCl buffer was used for all other pH values. The production of UDP (shown as ΔA340) in an initial incubation mixture in the two step assay is shown versus pH. In one set of assays the standard two step assay conditions were used (Δ). In another set of assays glucose-6-P was omitted (O) from the standard two step assay.


.11
.10
.09
.08
.07
.06
.05
.04
.03
.02
.01
Total ΔA340
6.0 6.5 7.0 7.5 8.0 8.5 9.0 9.5
pH

CHAPTER V

DISCUSSION

The glycogen synthetase of Tetrahymena pyriformis appears to be present mostly as the independent form in the early stationary and stationary phase of growth of the organism. The reported Km values of UDP-glucose for glycogen synthetase from different sources have been compiled in Table I. The effect of glucose-6-P on the dependent form of glycogen synthetase when assayed in the presence of magnesium ion is to increase Vmax greatly and to decrease the Km of UDP-glucose. On the other hand glucose-6-P has no effect on Vmax for the independent form of the enzyme and decreases the Km of UDP-glucose usually only about 2 fold. The present determination of the Km of UDP-glucose for Tetrahymena pyriformis glycogen synthetase (primarily the independent form) was carried out in the presence of glucose-6-P and magnesium ion. The Km observed (1.66×10^{-3}M) appears to be about 3 to 10 fold higher than the value reported for glycogen synthetase from other animal sources except for the dependent form of the enzyme from frog muscle (1.1×10^{-3}M).

The Km for glycogen for Tetrahymena pyriformis glycogen

synthetase was 2.06×10^{-2}M (expressed in non-reducing end groups). This is about 200 fold higher than the reported Km for glycogen for the rat muscle glycogen synthetase (21).

Stimulation of glycogen synthetase by GSH and other sulfhydryl compounds has been observed with the enzyme from rat liver (20), rat (7), rabbit (8), turtle, lobster muscle, sheep brain (10) and Neurospara (9). No stimulation by GSH or sulfhydryl compounds has been observed with the lamb muscle enzyme (9). The present investigation of the Tetrahymena pyriformis showed that the enzyme is similar to the enzyme from most other sources and also that it shows about 50 to 65 per cent stimulation by GSH is in agreement with an earlier report by Cook (46).

Cook (46) has reported that the glycogen synthetase of Tetrahymena pyriformis has some characteristics similar to those of the enzyme isolated from multicellular organisms, for example, the effects of glucose-6-P and GSH. His studies were done using cells which had been harvested either during the logarithmic or the stationary phase of growth. The present studies on the Tetrahymena pyriformis glycogen synthetase from cells harvested at the early stationary phase and stationary phase of growth indicate that the enzyme is primarily in the glucose-6-P independent form. In this respect it is similar to the enzyme from other unicellular organism such as

yeast (15), Agrobacterium tumefacions (18) and Arthrobacter species (17).

Kornfeld and Brown (8) reported that magnesium ion stimulated the activity of the rabbit muscle glycogen synthetase by 35 to 40 per cent. Others (10, 11, 26-31) have also reported activation by magnesium ion. Tetrahymena pyriformis glycogen synthetase is similar to glycogen synthetase from most sources in that it was stimulated about 45 per cent by magnesium ion.

The pH optimum for Tetrahymena pyriformis glycogen synthetase is pH 8.5 both in the presence and absence of glucose-6-P. The stimulation with glucose-6-P varies with pH as has been observed by Kornfeld and Brown (8), Traut and Lipmann (9) and Algranati and Cabib (15). The shape of the pH curve was the same in the presence or absence of glucose-6-P for the Tetrahymena pyriformis glycogen synthetase in contrast to the enzyme from other sources (8, 9, 15 primarily independent form) in which the pH optimum was generally broader in the presence of glucose-6-P and extended to higher pH values.

It appears that there is substrate inhibition of Tetrahymena pyriformis glycogen synthetase at high concentrations of UDP-glucose (0.8 mM) or glycogen (15 per cent). Substrate inhibition by either UDP-glucose or glycogen of glycogen synthetase has not been reported for the enzyme obtained from

other sources (7-15, 20, 26-32).

In the absence of added glycogen, the glycogen synthetase had 10 to 20 per cent of the activity as that in the complete system. This residual activity was suspected to be due to the presence of endogenous glycogen in the enzyme extract (0.87 mg glycogen per mg of extract protein). However, the amount of glycogen added to the assay mixture as part of the crude extract (generally 0.1 to 0.2 mg) appears to be too low to fully account for the activity observed in the absence of added glycogen. It was calculated that the amount of glycogen added as part of the extract should have yielded only approximately 2 per cent of the activity observed in the complete assay system. The reason for the higher activity (10 to 20 per cent) in the absence of added glycogen is not understood.

The glycogen synthetase of Tetrahymena pyriformis appears to have maximum activity at 25° and it becomes completely inactive at 40°. There is approximately a two fold increase in activity between the temperatures 15° and 25° which is equivalent to a Q_{10} of 2. This value is similar to the value usually observed in enzyme catalyzed reactions.

The in vitro temperature effects on Tetrahymema pyriformis glycogen synthetase are in contrast to the in vivo experiments reported by Scherbaum and Levy (51). They showed

that glycogen synthesis is increased four fold when the cells were maintained at 34° compared to 28°. The present *in vitro* studies reveal that glycogen synthetase loses about 60 to 70 per cent of its activity at 34° when compared to its activity at 25°. The apparent discrepancy of results suggests that the glycogen synthetase of *Tetrahymena pyriformis* may be more stable to an increase in temperature in the living cell than in cell extracts.

SUMMARY

Glycogen synthetase was obtained from Tetrahymena pyriformis cells growing at stationary and early stationary phases. Some attempts were made to purify the enzyme. These experiments yielded little success primarily due to the instability of the enzyme to the fractionation procedures employed. The properties of the enzyme were studied using a crude extract. The Km for UDP-glucose was shown to be 1.66×10^{-3}M and temperature optimum was 25°. The energy of activation was calculated to be 4,740 calories. Maximal activity was observed at pH 8.5 measured with or without glucose-6-P. The enzyme was stimulated about 65 per cent by GSH and 45 per cent by magnesium ion. The dependence of activity on glucose-6-P was variable but usually was less than 20 per cent. Thus it appears to be mostly the independent form of glycogen synthetase. At high concentration, substrate inhibition was observed both by UDP-glucose and by glycogen.

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