GLYCOGEN SYNTHESIS IN *TETRAHYMENA PYRIFORMIS*

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CHAPTER I

INTRODUCTION

The ciliated protozoan Tetrahymena pyriformis has been maintained in bacteria-free cultures for many years. There have been many studies, both direct and indirect, concerning glycogen in T. pyriformis[1]. It is understandable why this organism has been the subject of such studies since it has been shown to contain a dry weight glycogen content as high as 50 percent (1).

The glycogen isolated from T. pyriformis is a polysaccharide with properties that closely resemble those of mammalian glycogen. In an extensive study of glycogen isolated from the GL strain, Manners and Ryley (2) found several properties of T. pyriformis glycogen to be nearly identical to those of rabbit muscle and rabbit liver glycogen. The properties that were similar were specific rotation, unit chain length, and percent conversion to maltose by β-amylase. Using a light-scattering technique, they found that the major difference between T.

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[1] The following abbreviations are used: T. pyriformis, Tetrahymena pyriformis; UDPG, uridine diphosphate glucose; ATP, adenosine triphosphate; G-6-P, glucose-6-phosphate; G-1-P, glucose-1-phosphate; UTP, uridine triphosphate; UDP, uridine diphosphate; NAD, nicotinamide-adenine dinucleotide; NADH, nicotinamide-adenine dinucleotide (reduced form); NADP, nicotinamide-adenine dinucleotide phosphate; NADPH, nicotinamide-adenine dinucleotide phosphate (reduced form); Tris, Tris-(hydroxymethyl) aminomethane; ADP, adenosine diphosphate; 6-P-gluconate, 6-phosphogluconate; PP, inorganic pyrophosphate; GSH, glutathione (reduced form); and CoA, coenzyme A.

pyriformis glycogen and mammalian glycogen is the greater molecular weight of the T. pyriformis glycogen. They determined the molecular weight of highly purified T. pyriformis glycogen to be 9.8×10^6 as compared to 6.8×10^6 for rabbit liver glycogen and 2.8×10^6 for rabbit muscle glycogen. Wagner (1), using strain E, also has studied the glycogen isolated from T. pyriformis. He reported that the glycogen occurs naturally in the organism as subcellular granules of particle weight not less than 14 million. Wagner estimated this particle weight for glycogen from sedimentation data obtained in a preparative Spinco Model L ultracentrifuge.

The remaining studies to be discussed have been performed with cells grown on a medium containing proteose peptone and salts which frequently was supplemented with glucose and/or acetate as the primary carbon source. Under certain growth conditions, the glyoxylate cycle may be important in glycogen synthesis from lipid materials. However, with carbohydrate in the medium, glycogen synthesis occurs, but the glyoxylate cycle appears to be inoperative. In view of this fact, the nature of the growth medium will be indicated for each of the experiments from the literature described below.

Using washed suspensions of T. pyriformis strain E which had been grown with aeration in a proteose peptone medium containing acetate, Wagner (1) determined that the lipid content of the cells is depleted when glycogen synthesis occurs. The increase in glycogen was found to be equivalent to the decrease in lipid. He also noted that the cells contain a large portion of their lipid as phospholipids and that all of the decrease which occurs in lipid content during glycogen synthesis is due to a decrease in phospholipids.

Wagner (1) also reported that 0.1 percent glucose (final concentration) added to a proteose peptone-acetate culture medium caused a considerable increase in glycogen accumulation in T. pyriformis. All of the increase in glycogen which occurred on the addition of glucose to the medium could be accounted for on the basis of glucose disappearance from the medium. In agreement with this conclusion, Levy (3) cites Hogg and Wagner (4) as having reported that strain E stationary phase cells could incorporate 75 percent of the label from added glucose into glycogen.

Wagner (1) has observed that the glycogen content of older cultures varies with age and nature of the growth medium. He observed that cells grown in a medium containing glucose and acetate had a glycogen content of 462, 142, and 73 μg per mg dry weight at 48, 112, and 161 hours of growth, respectively. The phase of growth of the organism was not stated for each of the time periods considered above. However, from growth curves presented by Wagner (1), it would appear that the 48 hour growth period represented logarithmically growing cells and that the 112 and 161 hour growth periods represented stationary phase cells. If this be true, then the results obtained with glucose in the medium would indicate that the glycogen content per cell decreased in going from logarithmic growth phase cells to stationary phase cells. This result is in contrast to those obtained by Wilken and Best[2] who also used strain E cells. They found a 7- to 12-fold increase in the glycogen content per cell in stationary phase cells compared to logarithmic phase cells when grown on a medium containing glucose. The glycogen content per cell in one experiment was 2.6×10^{-4} μg per cell in logarithmically growing

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[2] Dr. D. R. Wilken, personal communication of unpublished results.

cells and 18.1×10^{-4} µg per cell in stationary phase cells.

Wagner (1) also demonstrated that cells grown on the proteose peptone-acetate medium described above, but without added glucose, had a glycogen content of 49, 177, and 166, µg per mg dry weight at 48, 112, and 161 hours of growth, respectively. This apparently represents an increase in glycogen content of stationary phase cells compared to logarithmic phase cells. Similarly, in the course of other studies employing strain GL cells grown on a proteose peptone-salts medium, but without either glucose or acetate, it has been established that the glycogen content of T. pyriformis is considerably greater in stationary phase cells as compared to logarithmically growing cells (3, 5).

Levy (3) found that cells harvested in the logarithmic phase of growth had a glycogen content which ranged from 0.060 to 0.180 mg per million cells. The cells were grown in a medium containing proteose peptone, liver extract, and salts, but no glucose or acetate. In stationary phase cells the glycogen content varied from 0.07 mg to as much as 2.0 mg per million cells. When the glycogen content was expressed as percent of total dry weight, stationary phase cells contained as much as 25 times more glycogen than did logarithmic phase cells. Scherbaum and Levy (5), using the GL strain, have reported an approximate 3-fold increase in glycogen content, on a percent dry weight basis, in stationary phase cells over logarithmically growing cells. The growth medium they used was essentially the same as that used by Levy (3).

The glycogen content is not only increased in the stationary phase cells over the cells from the logarithmic phase of growth, but the rate of glycogen synthesis is also increased. Levy (3) showed that station-

ary phase cells incorporated tracer amounts of ^{14}C labeled acetate into glycogen 5 times faster than did logarithmic phase cells when compared on a per cell basis. Substrate amounts of acetate also were converted to glycogen in washed suspensions of stationary phase cells at a higher rate than in washed suspensions of logarithmic phase cells. Levy (3) also demonstrated that stationary phase cells incorporated 40 times as much labeled pyruvate into glycogen as did cells harvested in the logarithmic growth phase. It is apparent from these studies that the increase in glycogen content observed for stationary phase cells is due, at least in part, to an increase in the rate of glycogen synthesis and not merely to an accumulation of glycogen with time.

Wagner's (1) demonstration that net synthesis of glycogen could occur at the expense of phospholipids was the basis for examining T. pyriformis for the presence of the glyoxylate cycle enzymes. Evidence for the occurrence of these enzymes in T. pyriformis was first presented by Hogg (6) and later confirmed by Reeves et al. (7), Hogg and Kornberg (8), and Levy (3).

Hogg (6) made an early attempt to relate the glyoxylate cycle to glycogen synthesis from lipids. Although his results were tentative, they indicated that the amount of isocitrate lyase (E.C. 4.1.3.1) found in extracts was sufficient to account for only 10 percent of the glycogen formed. These results did not rule out the glyoxylate cycle, since some doubt existed about the stability of isocitrate lyase in the crude extracts. Levy (3) has tested the hypothesis that the increased rate of glycogen synthesis in stationary phase cells might be due to increased levels of the glyoxylate cycle enzymes. In stationary phase cells the specific activity of both isocitrate lyase and malate

synthase (E.C. 4.1.3.2) increased approximately 1.5 to 4 times compared to logarithmic phase cells. If puromycin or p-fluorophenylalanine at concentrations which completely inhibited culture growth were added to the growth medium, the increase in isocitrate lyase activity was completely or greatly suppressed. Under the same conditions; however, the ability to incorporate acetate into glycogen increased to an even greater extent than in control experiments in which these compounds were not added to the growth medium. He therefore concluded that an increase in the glyoxylate cycle enzymes is not required for increased glycogen synthesis from lipids.

Hogg and Kornberg (8) studied the effects of added acetate and/or glucose in a proteose peptone medium on the levels of the glyoxylate cycle enzymes malate synthase and isocitrate lyase. Their findings are summarized in Table I. The addition of acetate to a proteose peptone medium resulted in a 15-fold increase in isocitrate lyase but had relatively little effect on malate synthase. The addition of glucose to the proteose peptone medium had little effect on isocitrate lyase but completely suppressed malate synthase. Glyconeogenesis from acetate was observed only in cells grown on the proteose peptone medium supplemented with acetate. A comparison of the enzyme activities from cells grown on proteose peptone-acetate medium and those grown on this medium supplemented with glucose indicated that in cells grown on the latter medium the isocitrate lyase activity was depressed 70 percent while the malate synthase activity was depressed 30 percent. There were, however, appreciable amounts of each of these enzymes. Despite this fact, the cells were not capable of glyconeogenesis from acetate. It was found, however, that if cells were grown on the acetate- and glucose-containing

TABLE 1

FORMATION OF GLYOXYLATE-CYCLE ENZYMES AS A FUNCTION OF GLUCOSE AND ACETATE IN THE GROWTH MEDIUM[a]

Composition of Medium	Specific Activity[b]	
	Isocitrate Lyase	Malate Synthase
Proteose Peptone	0.055	3.5
Proteose Peptone + Acetate	0.85	3.00
Proteose Peptone + Glucose	0.04	$<$0.01
Proteose Peptone + Acetate + Glucose	0.60	0.90

[a] Data taken from Hogg and Kornberg (8).
[b] μmoles of substrate transformed/mg of soluble protein/hour.

medium, but not harvested until all of the glucose had disappeared from the medium, the inhibitory effect of glucose on glycogen synthesis from acetate was reversed. Hence, it appears that the presence of glucose in the growth medium causes the glyoxylate cycle to be inoperative for glycogen synthesis in T. pyriformis.

Hogg and Kornberg (8) also showed that unless the glyoxylate cycle enzymes were incorporated into an organized intracellular structure, glycogen synthesis from acetate could not occur. In cells which were capable of glyconeogenesis from lipids, essentially all of the isocitrate lyase and malate synthase was contained in one of two distinguishable types of submicroscopic (0.5 to 1.0 μ) intracellular particles. In cells incapable of glyconeogenesis from lipids, however, the enzymes of the glyoxylate cycle were distributed in both of the two intracellular particles and in a soluble supernatant fraction. In the latter type of cells, 62 percent of the malate synthase activity was found in the soluble fraction. The subcellular particles from cells which could synthesize glycogen from acetate not only contained the glyoxylate cycle enzymes but also several of the tricarboxylic acid cycle enzymes. When L-[2-^{14}C] glutamine was added to a cell suspension actively converting acetate into glycogen, most of the ^{14}C was recovered as carbon dioxide while only a negligible amount was recovered in glycogen. This isotopic distribution pattern suggests that the four carbon acids produced from L-[2-^{14}C] glutamine do not equilibrate with the four carbon acids which are active intermediates in the glyoxylate cycle, and hence, that the site of oxidation and carbon dioxide production is separate from that of glyoxylate cycle activity. In view of the relatively high activities of isocitrate lyase and malate synthase in T. pyriformis capable

of glyconeogenesis, Hogg and Kornberg (8) concluded that "the glyoxylate cycle plays a necessary role in the conversion of fat into carbohydrate."

From the above discussion it appears that the relative importance of the glyoxylate cycle in glycogen synthesis in T. pyriformis is primarily related to the intracellular distribution of the enzymes involved, rather than to an increase or decrease of their concentration.

It was noted earlier in this discussion that labeled pyruvate is incorporated into T. pyriformis glycogen (3). The glyoxylate cycle also is probably the route by which T. pyriformis incorporates pyruvate into glycogen after decarboxylation to acetyl coenzyme A by the pyruvic dehydrogenase complex.

The results discussed thus far have indicated that glycogen may be synthesized from either carbohydrate or lipid carbon sources. Several of the probable intermediate enzymes involved in glycogen synthesis from either of these sources are depicted in Figure 1. Glycogen synthesis from carbohydrate is indicated as pathway 1, and glycogen synthesis from lipid precursors is indicated as pathway 2. As shown in the figure, the two pathways probably merge to form a common pathway just prior to the final synthesis of glycogen. Since the glyoxylate cycle is not operative when glucose is present in the medium (8), all of pathway 2 would not be operative. Hence, no net synthesis of glycogen from pyruvate, acetate, or lipids would occur. However, under these same conditions glucose would be utilized for net glycogen synthesis via pathway 1. When the cells are grown in the absence of glucose, hexokinase (pathway 1) is not required, but the cells may nevertheless synthesize glycogen by pathway 2. Regardless of which pathway is used, 1 or 2, both eventually must depend on the enzymes presumed to be common to both pathways.

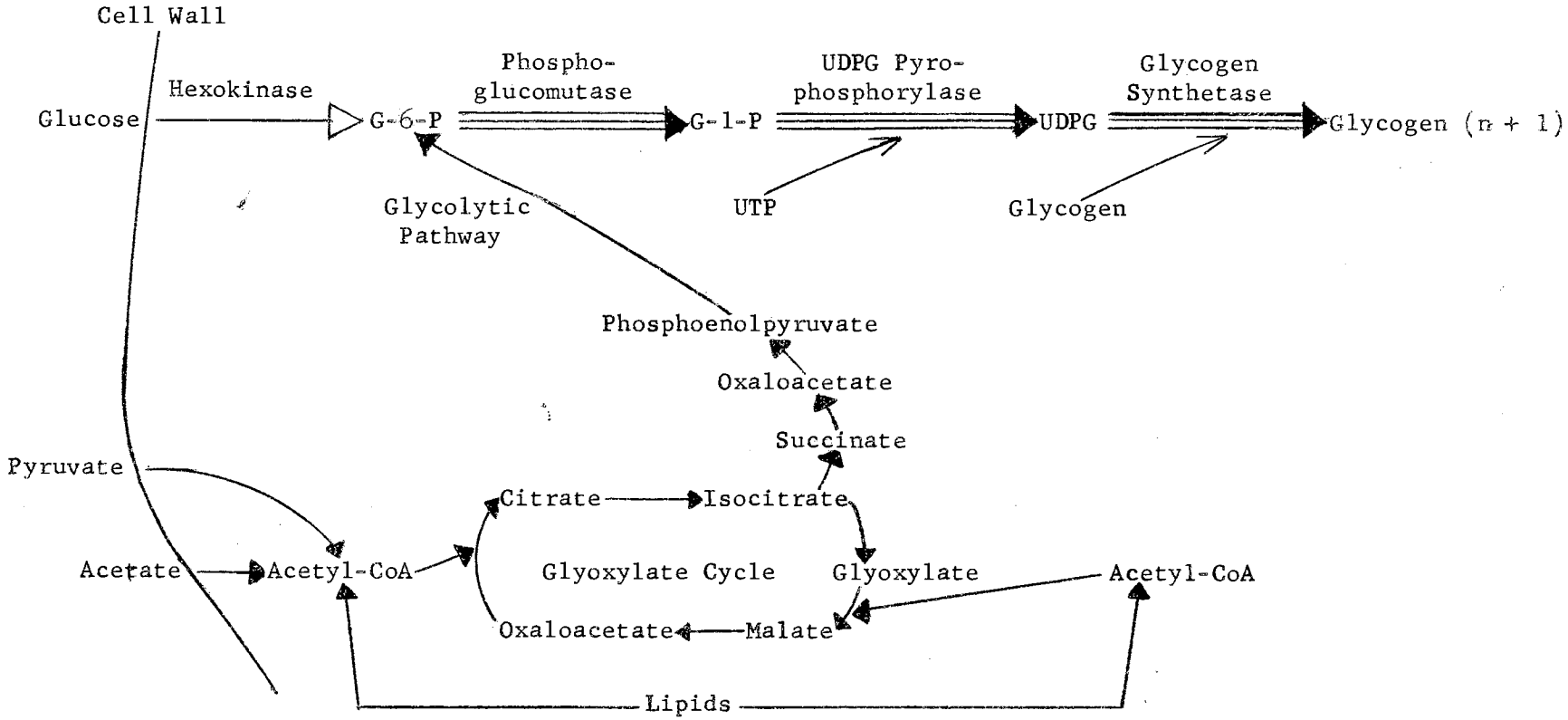

Figure 1. Glycogen Synthesis in T. pyriformis

Pathway 1: ——▷

Pathway 2: ——▶

Common Pathway: ≡▷

Control of the glyoxylate cycle via the absence or presence of glucose in the medium (as discussed earlier) is the factor controlling which of the two pathways would feed substrate into the common pathway.

Although considerable attention has been given to the possible role that the glyoxylate cycle enzymes may play in glyconeogenesis in T. pyriformis, little attention has been given to the enzymes immediately preceding the final step of glycogen synthesis or the final step itself. Similarly, little attention has been given to the enzymes involved in glycogen synthesis from glucose which would involve the enzymes hexokinase (E.C. 2.7.1.1), phosphoglucomutase (E.C. 2.7.5.1), UDPG pyrophosphorylase (E.C. 2.7.7.12), and glycogen synthetase (E.C. 2.4.1.11). Of these four enzymes, only hexokinase and phosphoglucomutase have been reported to occur in T. pyriformis. Very few reports concerning the presence of these two enzymes in T. pyriformis appear in the literature. Ryley (9) attempted to measure hexokinase, phosphoglucomutase, and phosphorylase (E.C. 2.4.1.1) as well as several other enzymes in the GL$_3$ strain. His attempt to measure hexokinase activity did not unequivocally demonstrate the presence of this enzyme, but the data obtained were consistent with its presence in T. pyriformis extracts. He was able to show the presence of phosphoglucomutase (0.016 μmoles of substrate utilized per minute per mg protein) and of phosphorylase. Warnock and van Eys (10), using strain E, reported a specific activity for hexokinase of 50 μmoles of substrate per minute per mg of protein in stationary phase cells. Neither UDPG pyrophosphorylase nor glycogen synthetase has been reported to occur in T. pyriformis.

From the foregoing discussion two major deficiencies in our knowledge of the synthesis of glycogen in T. pyriformis are evident. These

deficiencies are that a direct precursor of glycogen has not been established and that all the required enzymes for glycogen synthesis by organisms grown on a medium containing glucose as the carbon source have not been detected in extracts of the organism. Therefore, the objectives of the present investigation were to determine:

A. If the enzymes hexokinase, phosphoglucomutase, UDPG pyrophosphorylase, and glycogen synthetase are present in T. pyriformis strain E.

B. If the glucosyl moiety of UDPG is incorporated into glycogen, thereby establishing UDPG as a direct precursor of glycogen in T. pyriformis.

C. If the enzymes hexokinase, phosphoglucomutase, UDPG pyrophosphorylase, and glycogen synthetase are present at sufficient levels to account for glycogen synthesis from glucose.

D. If increased glycogen synthesis in the stationary phase could be due to an increase in the level of one or more of these enzymes.

CHAPTER II

EXPERIMENTAL

Materials

UDPG-^{14}C. UDPG-^{14}C with the label in the number one carbon of glucose was synthesized enzymatically essentially as described by Anderson et al. (11). This method employs a series of enzymatic reactions to convert glucose-^{14}C into UDPG-^{14}C in a single incubation mixture. Glucose-^{14}C is phosphorylated by ATP in the presence of hexokinase, and the G-6-P formed is then converted to G-1-P by phosphoglucomutase. The G-1-P is reacted with UTP in the presence of UDPG pyrophosphorylase to form UDPG-^{14}C and inorganic pyrophosphate. Inorganic pyrophosphatase is added to hydrolyze the inorganic pyrophosphate. This removes one of the products and thereby causes the sequence of reactions to proceed in the direction of UDPG synthesis. The reaction was stopped by heating at 80° C for 2 minutes. The reaction mixture was then cooled on ice and centrifuged. The UDPG-^{14}C which was in the supernatant fluid was purified by chromatography on a 40 x 1.8 cm Dowex-1 formate resin column. The elution procedure used was similar to that of Hurlbert et al. (12). In the procedure used, the sample was applied to the column and followed by water until 100 ml of effluent had been obtained. A gradient elution was then begun by passing 1,500 ml of 4 M formic acid followed by 4 M formic acid plus 0.2 M ammonium formate through a 500 ml mixing chamber,

which was originally filled with water, until the UDPG-^{14}C was eluted. The UDPG-^{14}C in the column effluent was detected by ultraviolet absorption at 260 mμ and radioactivity measurements. The eluted UDPG-^{14}C was adsorbed on Norite and eluted with 50 percent ethanol containing one ml of concentrated ammonium hydroxide per liter. The ethanol was removed on a flash evaporator, and the remaining aqueous solution lyophilized. The UDPG-^{14}C obtained after lyophilization was dissolved in water and further purified by descending chromatography in isobutyric acid, ammonia, and water (66:1:33) all by volume (13). The UDPG-^{14}C was detected on the paper by its ultraviolet absorption properties and radioactivity measurements. After elution from the paper with water, the UDPG-^{14}C concentration was determined by its absorption at 262 mμ employing a millimolar extinction coefficient of 10 (13), and its radioactivity measured in a Packard Tri-Carb liquid scintillation counter. The UDPG-^{14}C isolated had a specific activity of 0.352 μc per μmole. It was diluted 8-fold with carrier UDPG prior to use in glycogen synthetase assays.

Additional Materials. Glycogen was isolated and purified from a T. pyriformis culture in the stationary phase by the procedure of Manners and Ryley (2). Pyruvate kinase was isolated from rabbit muscle according to Buchler and Pfleiderer (14). Other reagents and their sources were: ATP, G-1-P, phosphoenolpyruvate, UDPG, NAD, NADP, hexokinase, G-6-P dehydrogenase, and phosphoglucomutase, Sigma Chemical Co.; UTP, Pabst Laboratories; inorganic pyrophosphatase, Worthington Biochemical Corporation; glucose-1-^{14}C, California Corporation for Biochemical Research; benzoic acid-^{14}C isotopic standard, Volk Radiochemical Company; and UDPG dehydrogenase from bovine liver acetone powder (15)

and UDPG pyrophosphorylase from bovine mammary tissue, which were generous gifts from Dr. K. E. Ebner. All other chemicals used were of reagent quality.

Methods

Maintainance and Growth of T. pyriformis. Cultures of T. pyriformis, strain E, originally obtained from Dr. J. van Eys through the courtesy of Dr. L. G. Warnock, were maintained and grown in the glucose-containing medium described by Warnock and van Eys (10) except that it also contained 0.8 ml of Dow Corning antifoam B per liter. Cells were grown aseptically, generally at 27 to 29° C with vigorous aeration in a 2 liter aspirator bottle containing 1.5 liters of medium. Such bottles usually were inoculated with 7.5 to 10 ml of three day old stock culture per liter of medium to yield approximately 1 to 3 x 10^3 cells per ml. Cell counts were made in a Sedgewick-Rafter counting chamber with a Whipple ocular micrometer (16) after fixation in 0.5 percent formic acid containing 0.5 percent sodium chloride. Logarithmically grown cells were harvested when the culture reached 1 to 2 x 10^5 cells per ml (i.e., near the end of the logarithmic phase of growth which usually took approximately 24 to 30 hours), and stationary cultures were harvested after 3 to 5 days of growth when there were approximately 1.2 x 10^6 cells per ml.

Enzyme Extracts. Cells were harvested by centrifugation at 0 to 5° C for 5 minutes at 11,700 x g in a Sorvall RC-2 centrifuge. All further operations were done at 0 to 5° C. After decanting the supernatant solution, the cells were resuspended in cold 0.5 percent sodium chloride solution and centrifuged in conical centrifuge tubes in an International Clinical centrifuge at approximately 80 x g (maximum speed) for 3 to 4 minutes. The supernatant solution and small "fluffy"

layer on top of the sedimented cells were removed by aspiration. The cells were washed once again in sodium chloride solution, and finally, once in cold water, and were recovered each time by centrifugation at 80 x g. The packed wet cells were weighed, and one volume of 0.05 M Tris-HCl buffer pH 7.5 was added. The suspension was homogenized at maximum speed in a VirTis homogenizer for 10 minutes in the cold. The homogenate was centrifuged for 15 minutes at 10,000 x g, and the supernatant solution was filtered through a layer of glass wool. The filtrate usually contained 13 to 27 mg of protein per ml as determined by a biuret procedure (17).

Enzymatic Units and Specific Activity. All enzyme units are defined as that amount of enzyme which transforms one μmole of substrate per minute under the assay conditions. Specific activity is the number of units of enzyme per mg of protein.

Enzyme Assays. All assays were performed at pH 7.5 and 28° C. Spectrophotometric assays were performed using a Beckman DB spectrophotometer. The reaction rates were recorded directly on a Sargent Model SRL Linear-Log recorder. The linearity of the assay with time and protein concentration was determined for each of the assays and with each T. pyriformis extract studied. Specific activities were calculated from the linear portion of such curves. All assays were completed within 24 hours after harvesting the cells. The extract was maintained at 0 to 5° C until the assays were completed.

Hexokinase. Hexokinase activity was assayed spectrophotometrically. The reaction components and their final concentration were: Tris-HCl buffer pH 7.5, 50 mM; $MgCl_2$, 5 mM; NADP, 0.15 mM; glucose, 2 mM; G-6-P dehydrogenase, 0.15 units; T. pyriformis extract; and ATP, 0.1 mM.

ATP was added last to initiate the reaction. The final volume was one ml. The rate of the reaction was followed by measuring the reduction of NADP at 340 mμ.

Phosphoglucomutase. Phosphoglucomutase was assayed in the same manner described for hexokinase except that G-1-P, 5 mM and cysteine (adjusted to pH 7.5 just prior to use), 25 mM were added to the reaction mixture, and ATP and glucose were omitted. G-1-P was added last to initiate the reaction.

UDPG Pyrophosphorylase. UDPG pyrophosphorylase was assayed spectrophotometrically. The reaction components and their final concentrations were: Tris-HCl buffer pH 7.5, 50 mM; $MgCl_2$, 5 mM; NAD, 1 mM; G-1-P, 5 mM; UDPG dehydrogenase, 0.016 units; T. pyriformis extract; and UTP, 1 mM. The reaction was initiated by the addition of UTP. The final volume was one ml. The rate of reduction of NAD was recorded at 340 mμ.

Glycogen Synthetase. The glycogen synthetase assay of Traut (18) was modified to contain: Tris-HCl buffer pH 7.5, 50 mM; $MgCl_2$, 10 mM; purified T. pyriformis glycogen, 1 percent; T. pyriformis extract; G-6-P, 1 mM; glutathione, 5 mM; and UDPG-^{14}C, 0.4 mM. UDPG-^{14}C was added last to initiate the reaction. In certain experiments, either G-6-P, glutathione, or both were omitted from the assay system. These assays are indicated in the text. Water was used to bring the final volume of the reaction mixture, which was contained in a 12 ml conical centrifuge tube, to 0.5 ml. At the end of the desired reaction time, the reaction was stopped by adding 0.5 ml of 60 percent KOH to the reaction mixture, followed by heating for 20 minutes in a boiling water bath. Then, 0.1 ml of saturated Na_2SO_4 and 1.5 ml of 95 percent ethanol were added and thoroughly mixed. The glycogen suspension which formed was cooled on

ice with occasional mixing for 20 minutes to allow complete precipitation of the glycogen. The precipitate was collected by centrifugation for 5 minutes at maximum speed in an International Clinical centrifuge. After the supernatant solution was decanted, the precipitate was redissolved in one ml of water, and the glycogen was reprecipitated by addition of 1.5 ml of 95 percent ethanol. The mixture was cooled on ice with occasional mixing for 20 minutes. The precipitated glycogen was collected by centrifugation as described above, the supernatant solution decanted, and the purified glycogen dissolved in one ml of water. The glycogen solution and washings were transferred to a planchet and dried in preparation for the determination of the radioactivity in the sample. Each series of glycogen synthetase assays also included tubes which contained the same components and which were carried through the same procedures as the normal assays, except that the UDPG-^{14}C was added just prior to transferring the glycogen solution to planchets. Counts observed in the experimental samples were compared to these samples to directly obtain the amount of glucose from UDPG-^{14}C incorporated into glycogen.

Radioactivity Measurements

Monitoring Radioactivity During UDPG-^{14}C Isolation and Purification. Radioactivity was determined in liquid solutions by plating small aliquots on aluminum planchets. The samples were dried and counted as described below for glycogen synthetase assays. Radioactivity was located on paper during chromatography with a gas-flow strip counter.

Determination of UDPG-^{14}C Specific Activity. UDPG-^{14}C specific activity was determined by dissolving a 0.01 ml aliquot of UDPG-^{14}C with 0.19 ml of water in 10 ml of scintillation fluid. The scintillation fluid contained the following components: 4 g of 2,5-diphenyloxazole,

200 mg of 1,4-bis-2-(phenyloxazole)-benzene, 400 ml of absolute ethanol, and 600 ml of toluene. The counts observed were corrected for the efficiency of the system used. The efficiency was determined by counting a benzoic acid standard containing 88.2×10^3 dpm in the same system. The counting was done in a Packard Tri-Carb liquid scintillation counter.

Radioactivity Measurements for Glycogen Synthetase Assay. The glycogen-^{14}C isolated and purified from glycogen synthetase incubation mixtures, as described earlier (also see 18), was plated on aluminum planchets. The dried planchets were counted with a gas-flow Gieger Müller tube having a thin end-window. The counting times employed were such that the counting error was never more than 10 percent (usually much less) in the glycogen synthetase assays.

CHAPTER III

RESULTS AND DISCUSSION

T. pyriformis Growth Curve

A typical growth curve for T. pyriformis is shown in Figure 2. The results of two separate experiments are shown. Logarithmic growth phase cells were harvested near the end of the logarithmic phase of growth at a concentration of approximately 1 x 10^5 cells per ml, and stationary phase cells were harvested at a concentration of 1.2 x 10^6 cells per ml or at some time after this concentration was reached. The concentrations of cells per ml at which the cells were harvested are indicated in Figure 2. After the cell population reaches a concentration of 1.2 x 10^6 cells per ml, the cell concentration remains essentially constant; however, there are still a few dividing cells at this phase of growth as revealed by microscopic examination of culture samples.

Enzyme Assays

Hexokinase. Hexokinase activity was determined by coupling the hexokinase catalyzed reaction (equation 1) with an excess of G-6-P dehydrogenase (E.C. 1.1.1.49) which catalyzes the reaction shown in equation 2.

(1) ATP + glucose $\longrightarrow$ G-6-P + ADP

(2) G-6-P + $NADP^+$ $\rightleftharpoons$ NADPH + H^+ + 6-P-gluconate

(sum) ATP + glucose + $NADP^+$ NADPH + H^+ + 6-P-gluconate + ADP

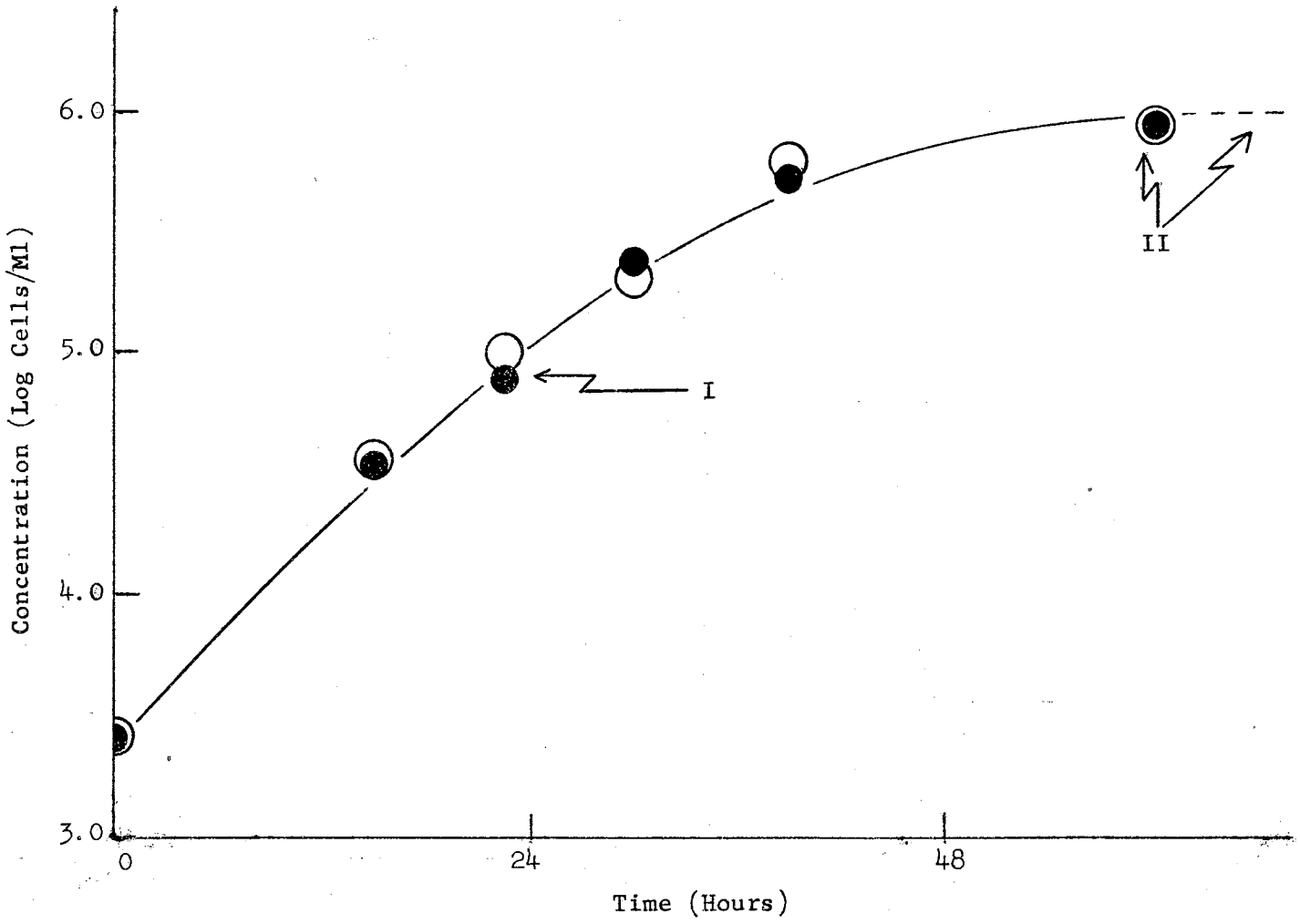

Figure 2. T. pyriformis Growth Curve
I. Cell concentration when logarithmic phase cells were harvested.
II. Cell concentration when stationary phase cells were harvested.

The reaction was followed by observing the increase in absorbance at 340 mμ due to the formation of NADPH. Figure 3 shows the effect of T. pyriformis extract protein concentration on the reaction rate of the hexokinase catalyzed reaction. In this figure the change in absorbance per minute is shown as a function of T. pyriformis protein concentration. The absorbance change per minute in the linear portion of the plot is very small. In order to obtain a large enough total change in absorbance to be read reasonably accurately from the recorder paper, it was often necessary to allow the rate of NADP reduction to be recorded for as long as 15 minutes. During such assays the rate of NADP reduction remained linear with respect to time. The specific activities of hexokinase in T. pyriformis extracts reported later were calculated from the linear portion of the reaction rates versus protein concentration curves.

Phosphoglucomutase. Phosphoglucomutase activity was determined by coupling the phosphoglucomutase catalyzed reaction (equation 3) with an excess of G-6-P dehydrogenase which catalyzes reaction 4.

(3) G-1-P $\rightleftharpoons$ G-6-P

(4) G-6-P + $NADP^+ \rightleftharpoons$ NADPH + H^+ + 6-P-gluconate

(sum) G-1-P + $NADP^+ \rightleftharpoons$ NADPH + H^+ + 6-P-gluconate

The reaction was followed by observing the increase in absorbance at 340 mμ due to the formation of NADPH. Figure 4 shows the effect of T. pyriformis extract protein concentration on the reaction rate of the phosphoglucomutase catalyzed reaction. Each reaction rate point was taken from an individual assay in which the rate of NADPH formation was linear with time. The phosphoglucomutase activity, shown as absorbance change per minute, was linear with respect to protein concentration at a higher protein concentration than exhibited by hexokinase. In addition,

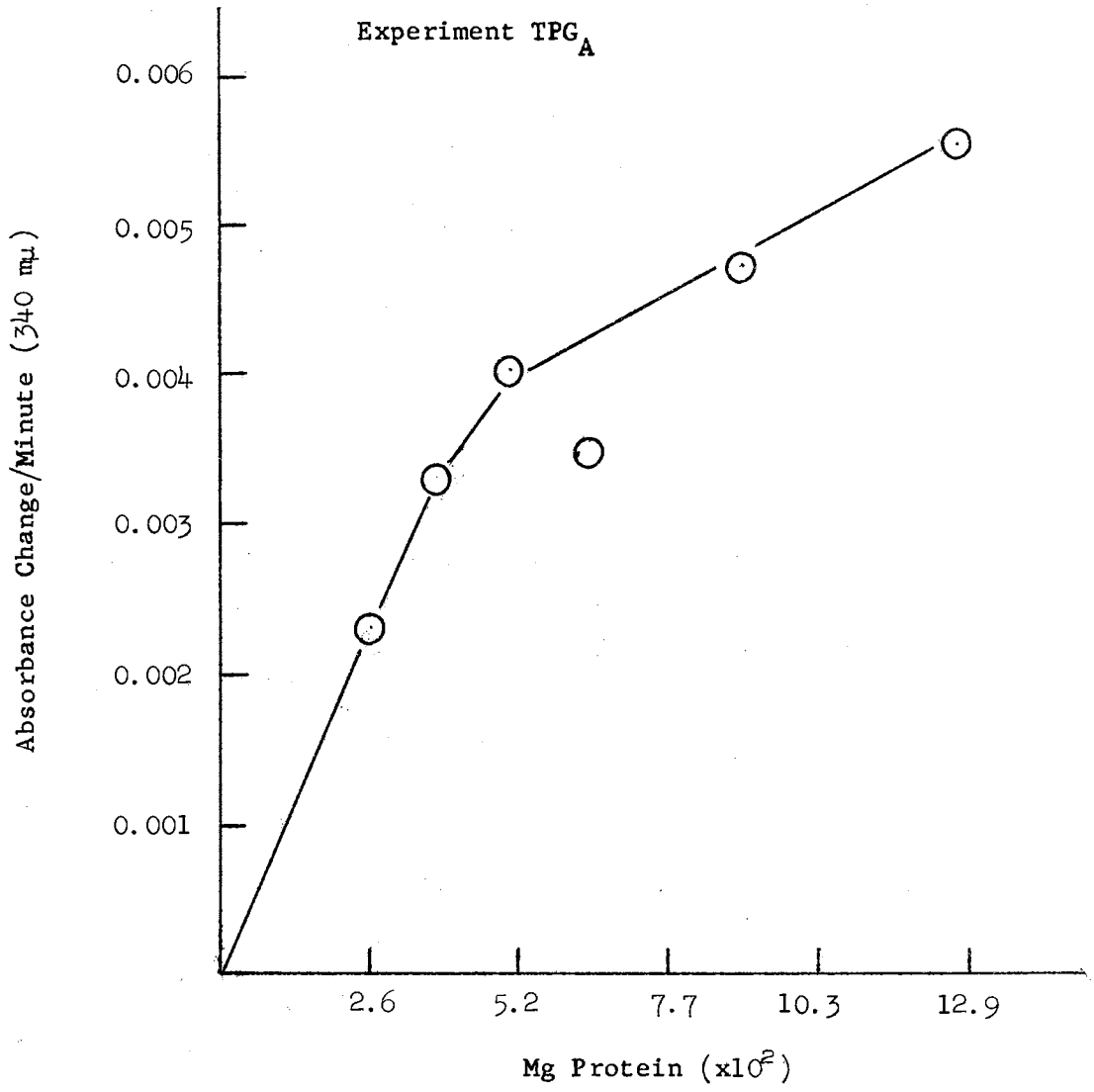

Figure 3. Hexokinase Activity as a Function of Protein Concentration in T. pyriformis Extract.

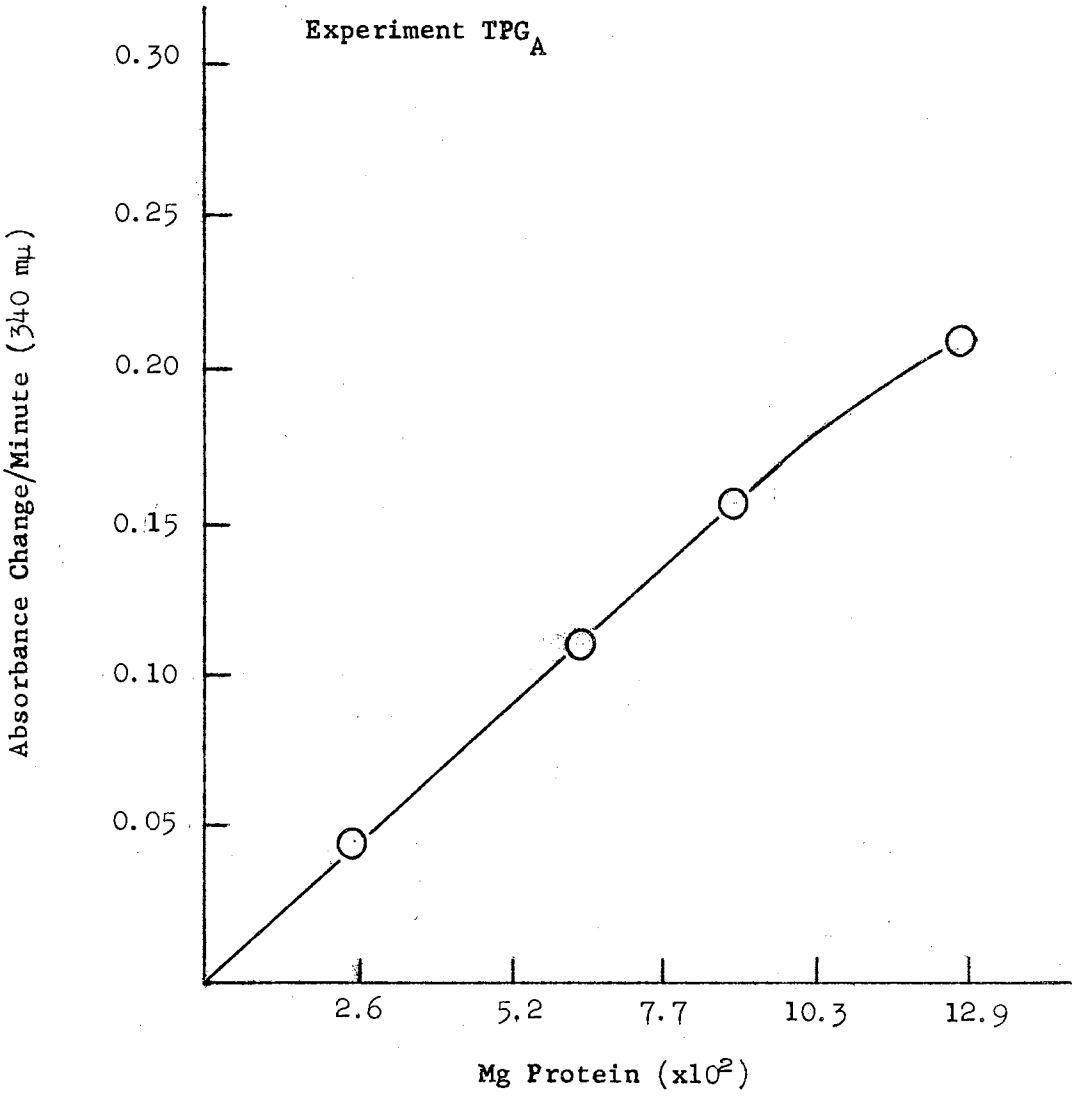

Figure 4. Phosphoglucomutase Activity as a Function of Protein Concentration in T. pyriformis Extract.

the change in absorbance per minute in the linear portion of the curve was considerably higher than for an equal protein concentration in hexokinase assays. These two factors resulted in easier and more accurate phosphoglucomutase assays than the hexokinase assays. Because of the greater activity, the phosphoglucomutase assays were carried out for only 3 minutes or less in order to determine the absorbance change per minute from the linear portion of the plot. Phosphoglucomutase specific activity was determined from the linear portion of plots such as shown in Figure 4.

UDPG Pyrophosphorylase. UDPG pyrophosphorylase activity was determined by coupling the UDPG pyrophosphorylase catalyzed reaction (equation 5) with an excess of UDPG dehydrogenase (E.C. 1.1.1.22) which catalyzes the reaction shown in equation 6.

(5) $UTP + G\text{-}1\text{-}P \rightleftharpoons UDPG + PP$

(6) $UDPG + 2NAD^+ \longrightarrow UDP\ glucuronate + 2\ NADH + 2H^+$

(sum) $UTP + G\text{-}1\text{-}P + 2\ NAD^+ \longrightarrow UDP\ glucuronate + PP + 2NADH + 2H^+$

The reaction was followed by observing the increase in absorbance at 340 mμ due to the formation of NADH. Because of an initial lag in the rate of NAD reduction, it was necessary to employ a 6 to 10 minute time period for each assay. A constant rate of reduction of NAD was reached within this time period. The rates obtained as a function of T. pyriformis extract protein concentration are shown in Figure 5. UDPG pyrophosphorylase specific activity was determined from the linear portion of plots such as the one shown in Figure 5.

Glycogen Synthetase. The details of the glycogen synthetase assay are given in the experimental section. Unlike the assays for hexokinase, phosphoglucomutase, and UDPG pyrophosphorylase, the glycogen synthetase

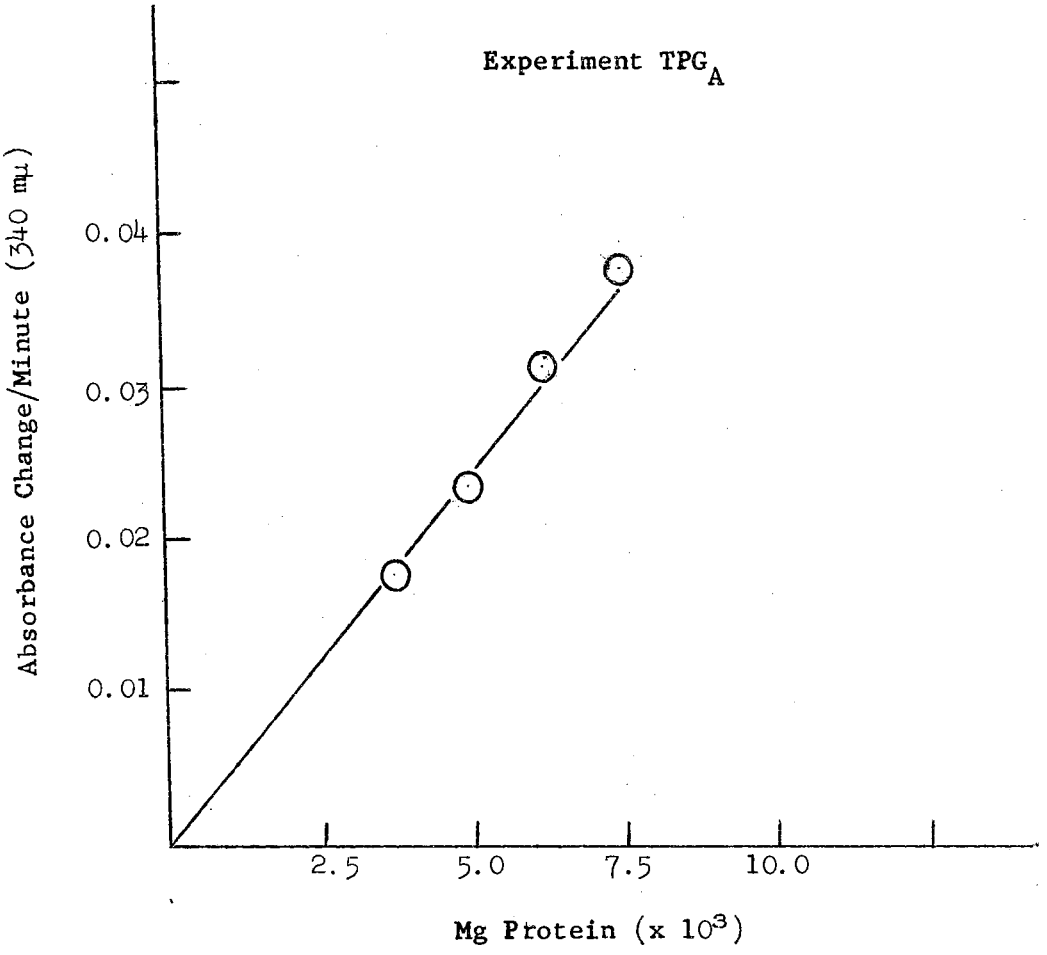

Figure 5. UDPG Pyrophosphorylase Activity as a Function of Protein Concentration in T. pyriformis Extract

assay was not a continuous spectrophotometric assay. The glycogen synthetase assay was based on the incorporation of the label from UDPG-^{14}C into primer glycogen. Figure 6 shows the incorporation as a function of time. The results shown in this figure were obtained when one mg of protein was incubated in each of six identical but separate assay mixtures for the indicated lengths of time. The results recorded are based on the incorporation above a zero-time control. In this manner, the actual incorporation of label into primer glycogen was obtained. The incorporation of ^{14}C into glycogen was linear with respect to time. Figure 7 shows the incorporation as a function of protein concentration. The results shown in this figure were obtained when the indicated amounts of protein were incubated in identical but separate assay mixtures for the same period of time (30 minutes). The results shown are based on the incorporation above a zero-time control sample. The figure shows that the incorporation of ^{14}C into glycogen was linear with respect to protein concentration. The specific activity of glycogen synthetase was calculated using data from the linear portion of plots such as those shown in Figures 6 and 7 where the incorporation was linear with respect to both time and protein concentration.

The results from the typical enzyme assays shown in Figures 3 - 7 show that all of the enzymes necessary for the conversion of glucose to glycogen are present in T. pyriformis strain E, including UDPG pyrophosphorylase and glycogen synthetase, neither of which has previously been reported to occur in T. pyriformis. These assays establish that a pathway is available in T. pyriformis for the synthesis of glycogen from glucose in the growth medium.

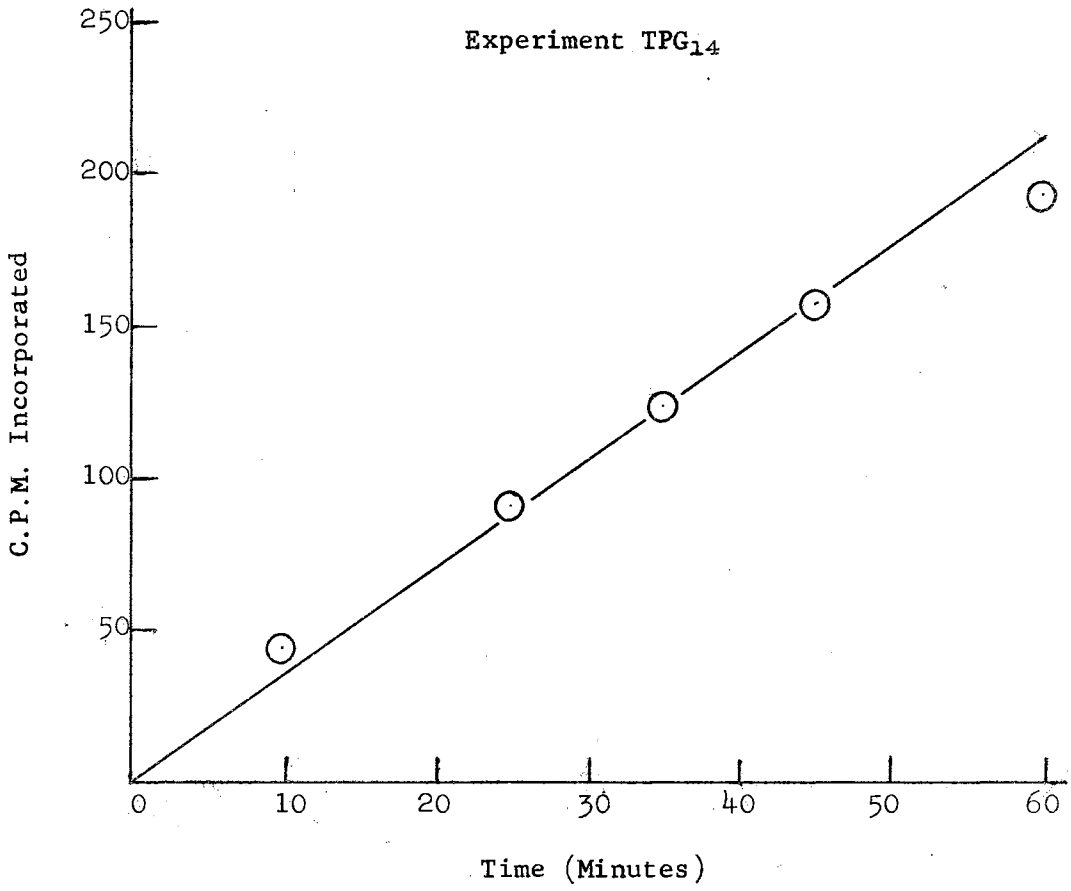

Figure 6. ^{14}C Incorporation as a Function of Time During Glycogen Synthetase Assays of T. pyriformis Extract.

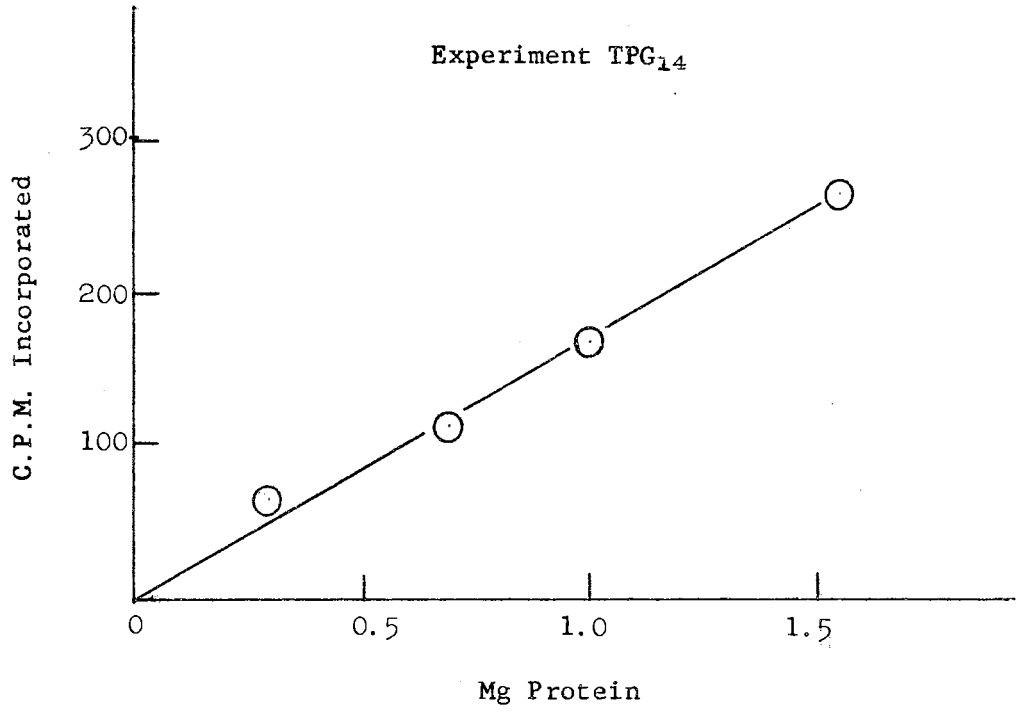

Figure 7. ^{14}C Incorporation as a Function of Protein Concentration During Glycogen Synthetase Assays of T. pyriformis Extract.

UDPG - A Direct Precursor of Glycogen in T. pyriformis

The results obtained in the glycogen synthetase assay suggested that UDPG is a direct precursor of glycogen in T. pyriformis. Other possibilities which might explain the results were considered, however. The possibility that the counts observed in the experiments described were due to UDPG-^{14}C which was carried through the procedure was eliminated because the small incorporation observed in zero-time controls was subtracted from the incorporation observed in experimental tubes. Another possibility which might explain the observed incorporation is that either free glucose-^{14}C or G-1-P-^{14}C might have been formed from UDPG-^{14}C during the incubation. If either of these compounds was carried through the procedure used to isolate glycogen from the incubation mixtures, the same results as those which were obtained would have been observed. This possibility seems unlikely, however, since neither of these compounds should have been insoluble under the conditions used to precipitate the glycogen. The most plausible explanation for the observed incorporation, other than by the direct incorporation of glucosyl residues from UDPG-^{14}C into glycogen, would be that G-1-P-^{14}C was formed from UDPG-^{14}C during the incubation period and then incorporated into glycogen by the action of phosphorylase. This possiblity was tested by comparing ^{14}C incorporation into glycogen in glycogen synthetase assays conducted in the presence or absence of 5mM unlabeled G-1-P. The results of two such experiments are shown in Table II. In experiment T-7, extract from logarithmic growth phase cells was employed as the source of glycogen synthetase, and in experiment T-5, extract from stationary phase cells was used. The addition of G-1-P to standard glycogen synthetase assays had very little effect on the amount of ^{14}C incorporated

TABLE II

EFFECT OF G-1-P ON THE INCORPORATION OF UDPG-^{14}C INTO GLYCOGEN

Experiment	Cells per ml at Harvest	μg Protein per Assay	Assay Time	Incorporation of ^{14}C into Glycogen	
				-G-1-P[a]	+G-1-P[b]
			minutes	mμmoles	
T-7	1.05×10^5	105	20	39.5	35.2
T-5	1.3×10^6	67	30	18.3	17.9

[a] Standard glycogen synthetase assay conditions were employed (see Methods).

[b] Standard glycogen synthetase assay conditions were employed except that 5 μmoles of unlabeled G-1-P also were added to the incubation mixtures.

into glycogen in either experiment. At the beginning of each experiment, the ratio of unlabeled G-1-P to UDPG-^{14}C in the incubation mixtures was 25. If the route of incorporation of ^{14}C had been via G-1-P and phosphorylase, the incorporation in tubes to which unlabeled G-1-P was added should have been markedly depressed. It may be concluded, therefore, that the incorporation of ^{14}C into glycogen in the glycogen synthetase assays is due to a direct transfer of glucose residues from UDPG to primer glycogen.

Enzyme Levels as a Function of the Growth Phase

In order to establish if the level of one or more of the enzymes, hexokinase, phosphoglucomutase, UDPG pyrophosphorylase, or glycogen synthetase, is increased in the stationary phase over the logarithmic phase of growth, the levels of these enzymes in the logarithmic and stationary phases of growth were compared. The results of such experiments are shown in Table III. Cells used to prepare enzyme extracts for each of the experiments were grown in separate containers. If the level of a limiting enzyme increases in the stationary phase of growth over that in the logarithmic phase of growth, this could be a factor controlling the increased rate of glycogen synthesis in the stationary phase of growth compared to the logarithmic phase of growth. Table III shows that there is considerable variation in enzyme activity with respect to a single enzyme among the several experiments. This variation in enzyme activity among the experiments precludes any firm conclusions concerning relative levels of enzymes in logarithmically growing cells as compared to cells in the stationary phase of growth. In spite of the variability of enzyme activity among experiments, the data in Table III show that phosphoglucomutase and UDPG pyrophosphorylase always exhibited 60 or more

TABLE III

COMPARATIVE ACTIVITIES OF ENZYMES ON THE PATHWAY OF GLYCOGEN SYNTHESIS

		Specific Activity[a]			
Growth Phase	Experiment	Hexokinase	Phospho-glucomutase	UDPG Pyro-phosphorylase	Glycogen Synthetase
Logarithmic	TPG_A	16	280	390	1.9
	TPG_C	10	680	540	9.2
	TPG_D	6	660	690	5.0
	TPG_{14}	--	---	---	0.4
Stationary	TPG_E	5	94	89	3.5
	TPG_F	15	146	127	1.5
	TPG_4	--	---	---	0.6
	TPG_{13}	--	---	---	0.2

[a] mμmoles/minute/mg protein

times as high a specific activity as glycogen synthetase. Although in some experiments the specific activity of hexokinase and glycogen synthetase were very nearly the same, in other experiments hexokinase exhibited a specific activity several times that of glycogen synthetase. Therefore, if any of these enzymes are limiting in logarithmic cells, it would appear that glycogen synthetase would probably be the limiting enzyme.

The hexokinase activity reported here is 3- to 100-fold less than that reported by Warnock and van Eys (10) who used the same strain of the organism. The reason for the low values of hexokinase activity reported in Table III as compared to their value is not understood. This difference is particularly puzzling since the spectrophotometric assay used in these experiments is inherently more sensitive than the spectrophotometric assay (19) which they used. The latter assay measures the change in color of the indicator cresol red due to increased acid production during the hexokinase catalyzed reaction. The difference between the hexokinase activity reported here and that reported by Warnock and van Eys (10); however, has no direct bearing on glycogen synthesis from glucose since it has already been suggested that glycogen synthetase activity in T. pyriformis is as low or lower than hexokinase activity.

Potential Importance of the Glycogen Synthetase Pathway for Glycogen Synthetase in T. pyriformis

If glycogen synthetase is the limiting enzyme, the potential importance of the pathway of glycogen biosynthesis involving this enzyme may be estimated. For example, 4.124 g of dry 64.3 percent pure glycogen were isolated from 10 liters of growth medium containing 1.3×10^6 cells per ml at harvest. Because these cells had been grown for 5.5

days and were very fragile, it was estimated that approximately one-half of the cells were lost during harvest. Hence, twice the amount of glycogen actually obtained should have been obtained had no cell breakage occurred. The total glycogen content, therefore, was probably nearly 5.3 g of pure glycogen per 10 liters of cells for 5.5 days of growth. In another experiment, one liter of medium was harvested when the cell concentration was 1.7×10^6 cells per ml. From this liter of cells, 286 mg of protein were extracted which had a glycogen synthetase specific activity of 0.6 mμ moles per minute per mg protein (Experiment TPG_4 of Table III). If the cells from which the glycogen was isolated contained the same amount of protein per cell and had the same specific activity as the cells from experiment TPG_4, then 10 liters of cells would have had the capacity to synthesize 1.68 g of glycogen in 5.5 days. To obtain this number, it had to be assumed that glycogen synthesis per day was linear and that the specific activity of glycogen synthetase was constant throughout a 5.5 day period. These assumptions are not entirely valid. If the assumptions are reasonably close to reality, then the calculations indicate that the glycogen synthetase activity was high enough to account for about 32 percent of the glycogen synthesized.

The glycogen synthetase specific activity observed in four different experiments employing extracts from stationary phase cells ranged from 3-fold less to 6-fold more than the activity used in the above calculation. The average glycogen synthetase specific activity of these four experiments was more than 2-fold greater than the specific activity used in the above calculations. The calculations, even with their assumptions and approximations, serve to indicate that the levels of the enzymes necessary to convert glucose into glycogen, as depicted in Figure 1, are

sufficient to account for a large portion, if not all, of the glycogen produced by the cells.

Effect of GSH and G-6-P on Glycogen Synthetase Activity

Traut and Lipmann (20) have detected glycogen synthetase in a variety of organisms. They observed that stimulation of glycogen synthetase by G-6-P is a general property of the enzyme regardless of its source. In crude extracts of frog, turtle, lobster, and a preparation from Neurospora, Traut and Lipmann (20) established that activation of glycogen synthetase required the presence of a sulfhydryl compound. In addition, activation by G-6-P was dependent on the presence of a sulfhydryl compound, such as GSH, in the reaction mixture. Glycogen synthetase from other sources such as lamb muscle were not stimulated by sulfhydryl compounds nor was a sulfhydryl compound required to observe stimulation of activity by G-6-P. In agreement with other studies (21, 22, 23, 24, 25), they found that the degree of stimulation of glycogen synthetase from different sources by G-6-P was variable. They found that variation was also frequently encountered in different preparations of the enzymes from the same source.

The effects of G-6-P and a sulfhydryl compound, GSH, on glycogen synthetase activity of T. pyriformis are shown in Table IV. In all cases tested, stimulation by GSH was observed. Attempts to establish if G-6-P caused stimulation of glycogen synthetase alone and in the presence of GSH yielded variable results. In some experiments, however, stimulation was observed. These experiments indicate that glycogen synthetase in crude extracts of T. pyriformis has characteristics similar to those of the same enzyme isolated from some multicellular organisms.

TABLE IV

EFFECT OF GSH AND G-6-P ON T. PYRIFORMIS GLYCOGEN SYNTHETASE ACTIVITY

Addition	^{14}C Incorporation		
	Experiment		
	TPG_A	TPG_4	TPG_F
	cpm		
Enzyme Alone[a]	202	106	82
Enzyme + GSH	299	222	---
Enzyme + G-6-P	214	139	163
Enzyme + GSH + G-6-P	360	274	280

[a] Glycogen synthetase assays were conducted as described in Methods except that GSH and G-6-P were omitted. These two components were added to assays as indicated. The incubations shown all contained one mg of protein and were assayed for 30 minutes. In experiment TPG_A extract from logarithmically growing cells was used, and in experiments TPG_4 and TPG_F, extracts from stationary phase cultures were used.

CHAPTER IV

SUMMARY

The occurrence of the enzymes hexokinase, phosphoglucomutase, uridine diphosphate glucose pyrophosphorylase, and glycogen synthetase was demonstrated in Tetrahymena pyriformis strain E and at levels sufficient to account for a significant amount, if not all, of the glycogen synthesized by the organism. The presence of the latter two enzymes was demonstrated to occur in Tetrahymena pyriformis for the first time. The direct incorporation of glucose-^{14}C residues from uridine diphosphate glucose-^{14}C into glycogen by cell-free extracts was demonstrated, thus establishing uridine diphosphate glucose as a direct precursor of glycogen in Tetrahymena pyriformis. In order to explore the possibility that the rapid rate of glycogen synthesis in stationary phase cells compared to logarithmic phase cells might be due to variation in the amount of one or more of the four enzymes required for glycogen synthesis from glucose, the relative amount of each of these enzymes as a function of growth phase was determined. Although variation in enzyme activity from one preparation to another precluded the conclusion that an elevation in the level of any of these enzymes is responsible for the increased glycogen synthesis in stationary phase cells, the activities of phosphoglucomutase and uridine diphosphate glucose pyrophosphorylase were always high compared to hexokinase and glycogen synthetase activities. Gluta-

thione was observed to stimulate glycogen synthetase activity. Although the results were variable, in some experiments glucose-6-phosphate stimulated glycogen synthetase from Tetrahymena pyriformis as has been demonstrated for the glycogen synthetase of some multicellular organisms.

BIBLIOGRAPHY

1. Wagner, C., Thesis, University of Michigan, University Microfilms, Inc., Ann Arbor, Michigan, 1956.

2. Manners, D. J. and Ryley, J. F., Biochem. J., 52, 480 (1952).

3. Levy, R. M., Thesis, University of Californis, Los Angeles, University Microfilms, Inc., Ann Arbor, Michigan, 1964.

4. Hogg, J. F. and Wagner, C., Federation Proc., 15, 275 (1956).

5. Scherbaum, O. H. and Levy, M., Pathologie-Biologie, 9, 514 (1961).

6. Hogg, J. F., Federation Proc., 18, 247 (1959).

7. Reeves, H., Papa, M., Seman, G., and Ajl, S., J. Bact., 81, 154 (1961).

8. Hogg, J. F. and Kornberg, H. L., Biochem. J., 86, 462 (1963).

9. Ryley, J. F., Biochem. J., 52, 483 (1952).

10. Warnock, L. G. and van Eys, J., J. Cell. and Comp. Physiol., 60, 53 (1962).

11. Anderson, E. P., Maxwell, E. S., and Burton, R. M., J. Am. Chem. Soc., 81, 6514 (1959).

12. Hurlbert, R. R., Schmitz, H., Brumm, A. F., and Potter, U. R., J. Biol. Chem., 209, 23 (1954).

13. Pabst Laboratories Circular OR-10, Milwaukee, Wisconsin (1956).

14. Bucher, T. and Pfleinderer, G., in S. P. Colowick and N. O. Kaplan (Editors), Methods in Enzymology, Vol. I, Academic Press, Inc., New York, 1955, p. 435.

15. Strominger, J. L., Maxwell, E. S., Axelrod, J., and Kalchar, H. M., J. Biol. Chem., 224, 79 (1957).

16. Hall, R. P., Johnson, D. F., and Loefer, J. B., Trans. Am. Microscop. Soc., 54, 298 (1935).

17. Gornall, A. G., Bardawill, C. S., and David, M. M., J. Biol. Chem., 177, 751 (1949).

18. Traut, R. R., Thesis, Rockefeller Institute, University Microfilms, Inc., Ann Arbor, Michigan, 1962.

19. Darrow, R. A. and Colowick, S. P., in S. P. Colowick and N. O. Kaplan (Editors), Methods in Enzymology, Vol. V, Academic Press, Inc., New York, 1962, p. 226.

20. Traut, R. R. and Lipmann, F., J. Biol. Chem., 238, 1213 (1963).

21. Leloir, L. F., Olavarria, J. M., Goldemberg, S. H., and Carminatti, H., Arch. Biochem. Biophys., 81, 508 (1959).

22. Leloir, L. F., and Goldemberg, S. H., J. Biol. Chem., 235, 919 (1960).

23. Algranati, I. D. and Cabib, E., Biochim. Biophys. Acta, 43, 141 (1960).

24. Madsen, N. B., Biochim. Biophys. Acta, 50, 194 (1961).

25. Larner, J., Villar-Palasi, C., and Richman, D. J., Ann. N. Y. Acad. Sci., 82, 345 (1959).

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