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Regulation of Hepatic Glycogen Synthetase of *Rana catesbeiana*. I. The Effect of Insulin Treatment*

Ki-Han Kim and Lois M. Blatt†

ABSTRACT: Tadpole liver glycogen synthetase occurs only in a glucose 6-phosphate dependent form. The enzyme activity was increased two- to threefold after insulin administration. This stimulation was the result of the conversion of one enzyme form into another which still requires glucose 6-phosphate. Insulin treatment of the animal lowered the K_m for uridine diphosphate glucose from $(2.5 \pm 0.7) \times 10^{-3}$ to $(1.2 \pm 0.2) \times 10^{-4}$ M without significant changes in V_{max}. K_m's for glucose 6-phosphate for two enzyme forms were identical at 5×10^{-3} M.

Using adenosine triphosphate, which behaves kinetically as a partially competitive inhibitor of glucose 6-phosphate, additional differences in the kinetic properties of the two enzymes were determined.

Uridine diphosphate glucose:α-1,4-glucan α-4-glucosyltransferase (EC 2.4.1.11) (glycogen synthetase) has been observed to occur in two forms: one glucose 6-phosphate (G-6-P) dependent and the other independent of glucose 6-phosphate (Rosell-Perez *et al.*, 1962, 1964; Friedman and Larner, 1963; Hizukuri and Larner, 1964; Danforth, 1965; Traut and Lipmann, 1963). The ratio of the two activities occurring in different biological systems varies markedly (Goldemberg, 1966). Insulin has been reported to effect the transformation of dependent form (D form) to independent form (I form) which is accompanied by an increase in substrate affinity (Bishop and Larner, 1967). The transformation between D and I forms, which appears to involve dephosphorylation and phosphorylation of the enzyme, seems to be associated with control of glycogen synthesis (Bishop and Larner, 1967).

Mersmann and Segal (1967), however, presented evidence that the difference between these two forms of the enzyme is in their affinity for UDP-glucose (UDPG) and G-6-P and not in strict dependence upon G-6-P. Activation of the enzyme is

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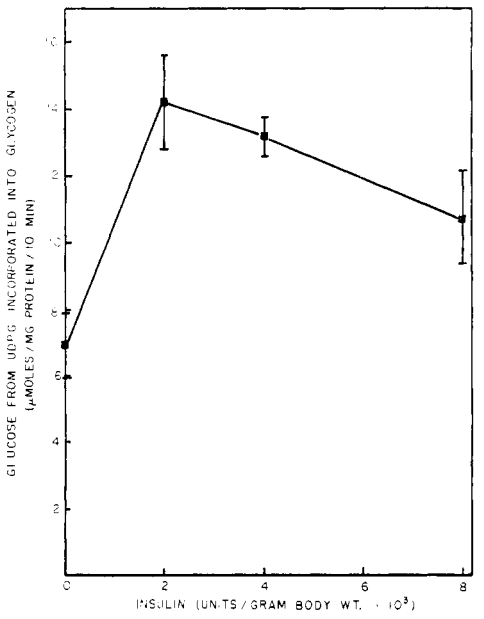

FIGURE 1: Effect of insulin on glycogen synthetase. Different concentrations of insulin were injected intraperitoneally through the tail muscle. Glycogen synthetase was assayed, as described in Methods, 6 hr after the hormone treatment. Crude enzyme preparation obtained after centrifugation at 30,000*g* for 10 min was used. The graph shows the variation of three determinations. Five animals were used for each determination.

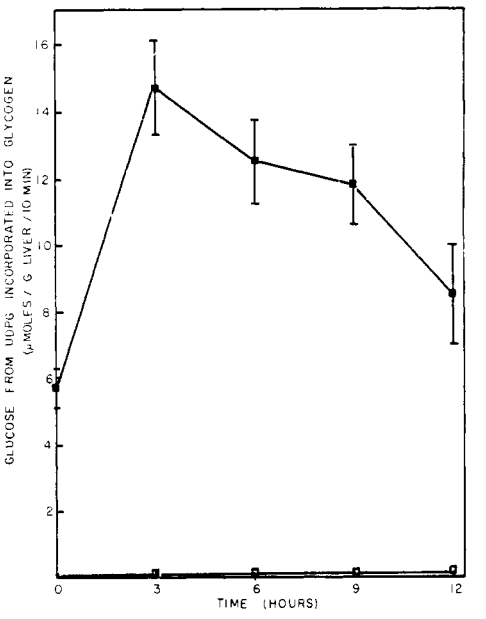

FIGURE 2: Effect of insulin on glycogen synthetase. Insulin (0.0025 IU/g of body weight) was injected intraperitoneally. Livers from five animals were taken at different periods after the hormone treatment. Glycogen synthetase was assayed either in the presence of 20 mM glucose 6-phosphate (■—■) or in the absence of glucose 6-phosphate (□- - -□). Crude enzyme preparations as described in Figure 1 were used. Graph shows the standard deviation of three determinations.

accompanied by an increased affinity for both UDPG and G-6-P. Therefore, they proposed referring to the D and I forms, respectively, as b and a forms analogous to the terminology employed for phosphorylase.

The situation in *Rana catesbeiana* tadpoles is unique in that all hepatic glycogen synthetase activity is glucose 6-phosphate dependent. In this communication, we report some properties of anuran glycogen synthetase and the effect which insulin treatment of the animal has on the enzyme. A two- to three-fold increase in glycogen synthetase activity is observed after insulin is administered to tadpoles. The enzyme isolated from insulin-treated animals has a lower K_m for UDPG than that of control animals. Other differences in the kinetic properties of the two enzymes are reported.

Materials and Methods

Chemicals. Puromycin, cycloheximide, G-6-P, UDPG, and other nucleotides were purchased from Nutritional Biochemical Corp. Shellfish glycogen, bovine pancreas insulin (24.1 IU/mg), and thyroxine were the products of Sigma Chemical Co. Radioactive [^{14}C]UDPG obtained from Schwartz Bio-Research Inc. had a specific activity of 200 μCi/μmole. Glass fiber filters (934 AH) were the product of Wilkins-Anderson Co., Clifton, N. J.

Animals. R. catesbeiana tadpoles weighing 7–10 g were purchased from Lemberger Co., Oshkosh, Wis. The stock animals were kept in a tank of dechlorinated water (15°) and fed periodically with canned spinach. Treatment of the animals with hormone was carried out, however, at 24°.

Protein Determination. Protein was determined by the method of Lowry *et al.* (1951) with crystalline bovine serum albumin as the standard.

Preparation of Homogenate and Enzyme. Pooled livers of several animals were washed once in three volumes of frog Ringer's solution. The washed livers were then homogenized in four volumes of 0.4 M sucrose solution (pH 7.4) containing 0.01 M EDTA. The tadpole enzyme was found to be extremely unstable in Tris buffer or in water; maximum stability was attained only in 0.4 M sucrose solution. The homogenate was centrifuged at 30,000*g* for 15 min and the residue was rehomogenized with two volumes of sucrose solution. After centrifugation, the supernatants were combined and used to determine total activity of glycogen synthetase in the presence of 0.02 M glucose 6-phosphate.

When purification of the enzyme was necessary, shellfish glycogen (1.5 mg ml) was added to the sucrose solution to adsorb soluble glycogen synthetase and thus facilitate subsequent purification. The supernatant described above was centrifuged at 144,000*g* for 2 hr in a Spinco Model L-2. After centrifugation, the brownish microsomal fraction which sedimented on top of the transparent glycogen pellet was carefully removed by washing with sucrose solution. About 70–90% of the total activity was recovered in the glycogen pellet fraction with about 200-fold purification. This fraction was used for kinetic studies. However, further purification without loss of activity could be obtained by the use of a DEAE column and stepwise elution by the method of Traut and Lipmann (1963). No significant differences between the qualitative kinetic properties of 500-fold-purified and less purified (200-fold) enzyme were found. The final preparation was essentially free of phosphorylase activity. The details of the purification procedure and the properties of the enzyme will be published elsewhere.

Glycogen Synthetase Assay. Glycogen synthetase activity

TABLE I: Effect of Inhibitors of Protein Synthesis.[a]

Expt	Additions	Act. (μmoles/g of liver per 10 min)
1	Control	6.2
	Insulin	18.7
	Insulin + puromycin	18.7
	Puromycin	17.5
2	Control	5.0
	Insulin	10.0
	Insulin + cycloheximide	8.8
	Cycloheximide	5.0

[a] Glycogen synthetase of the various groups of animals was assayed 6 hr after treatment as described in Methods. All reagents were injected intraperitoneally. The doses of insulin and antibiotics were 0.0025 IU and 20 μg/g of body weight, respectively. Each value was obtained with 0.1 ml of a crude homogenate prepared from the livers of at least five animals under standard assay condition.

TABLE II: Effects of Nucleotides.[a]

	Control		Insulin Treated	
Inhibitors	μmoles/mg of Protein per 10 min	% Inhibition	μmoles/mg of Protein per 10 min	% Inhibition
None	4.47	0	6.35	0
ADP	0.96	79.8	1.42	77.7
UDP	0.31	93.5	0.83	87.0
UMP	1.39	71.0	1.88	71.0
AMP	2.81	41.1	3.76	40.9
CMP	4.38	8.2	6.11	3.9
GMP	2.97	37.7	4.32	32.0
CTP	3.71	22.2	5.48	13.8
UTP	0.68	85.7	1.74	72.7
GTP	2.82	40.8	4.55	28.4

[a] Partially purified glycogen synthetase was assayed in the presence of 2×10^{-3} M nucleotide as described in Methods except 1×10^{-2} M glucose 6-phosphate was used instead of 2×10^{-2} M. Percentage inhibition was calculated on the basis of incorporation obtained in the absence of these nucleotides.

was assayed by the method described by Villar-Palasi *et al.* (1966) with a slight modification. The standard reaction mixture contained the following components: 0.67 μmole of [^{14}C]-UDPG (6000 cpm), 1.2 mg of shellfish glycogen, 10 μmoles of glucose 6-phosphate (G-6-P), 3.5 μmoles of sucrose, 1 μmole of EDTA, and an appropriate amount of the enzyme in a final volume of 0.5 ml. The reaction mixture was incubated at 37° for 10 min and inactivated by the addition of 1 ml of 10% trichloroacetic acid containing 2 mg of LiBr/ml. Glycogen, precipitated by the addition of two volumes of 95% ethyl alcohol, was washed twice with 5-ml portions of 67% ethyl alcohol on a glass filter. The dried filter was then counted in a Beckman CPM 100 scintillation counter using toluene scintillation fluid. When there was an excessive protein precipitate after the addition of trichloroacetic acid, the precipitate was removed by centrifugation before alcohol was added. Transfer of the glucose moiety from UDPG into primer glycogen was a linear function of protein concentration and time up to 20 min. This represented approximately 75% of the total reaction. The first 10 min of this linear portion was used for the assay of enzyme activity. Crude enzyme preparations were used for the experiments of Figures 1 and 2 and Table I. The rest of the experiments were carried out with partially purified enzyme. The enzyme concentration was varied to obtain, when possible, glucose incorporation into glycogen of the order of 500–1000 cpm (0.005–0.01 unit of the enzyme). Tadpole liver glycogen and shellfish glycogen showed no difference as the primer of the reaction.

After appropriate correction for background, the amount of glucose incorporated into glycogen was calculated. Glycogen synthetase activity was expressed as μmoles of glucose transferred to glycogen per 10 min per g of liver tissue (or per mg of protein). A unit of enzyme is defined as the amount of enzyme necessary for the incorporation of 1 μmole of glucose moiety from UDPG into glycogen in 1 min under the standard assay conditions.

Results

Glucose 6-Phosphate Dependency and the Effect of Insulin. Tadpole liver glycogen synthetase activity is completely dependent upon the presence of glucose 6-phosphate. Treatment of animals with various concentrations of insulin stimulated the enzyme activity up to threefold. Nevertheless, the enzyme from insulin-treated animals still requires glucose 6-phosphate for activity. In Figure 1, the stimulatory effect of insulin on glycogen synthetase 6 hr after hormone treatment is shown. The optimum concentration of insulin appears to be about 2×10^{-3} IU per g of body weight.

Although the normal level of insulin in the tadpole is unknown, by comparison with the rat this dosage appears to be reasonably close to the physiological level. It has been reported that rats secrete 2–3 units of insulin daily (Wagle, 1965). However, we realize that values reported for mammalian systems may not be a valid approximation for amphibians. It might be possible to effect the increase in enzyme activity in a relatively shorter period by the administration of a massive dose of insulin. However, subsequent experiments were carried out with 0.0025 IU in order to eliminate possible nonphysiological effects of a large dose of insulin. At this hormone concentration, maximum glycogen synthetase activation was found between 3 and 6 hr as shown in Figure 2. The dependence of glycogen synthetase upon glucose 6-phosphate throughout the hormone treatment is also shown in Figure 2.

To determine whether protein synthesis is involved in the stimulation of glycogen synthetase by insulin, the effects of inhibitors of protein synthesis have been examined. Puromycin and cycloheximide, at the concentrations used, inhibited tadpole hepatic protein synthesis about 95% as measured by

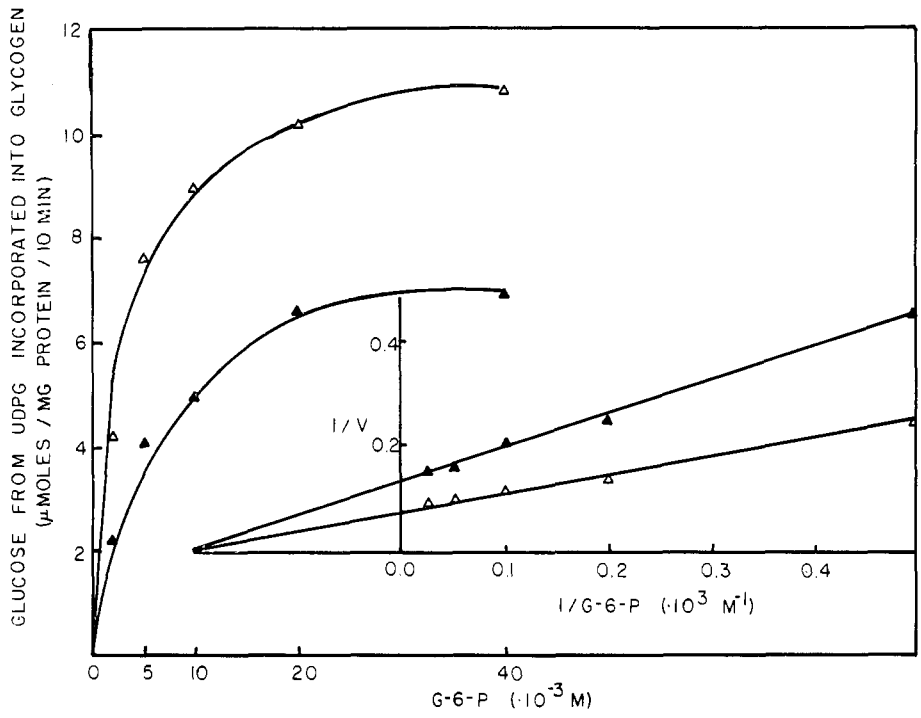

FIGURE 3: Glucose 6-phosphate concentration dependence of the enzyme and double-reciprocal plot of glucose 6-phosphate concentration and the rate of glycogen synthesis. Conditions are the same as in the standard assay (see Materials and Methods), except for the glucose 6-phosphate concentrations indicated. (▲—▲) Data obtained with the control enzyme and (△---△) with the enzyme from insulin-treated animals.

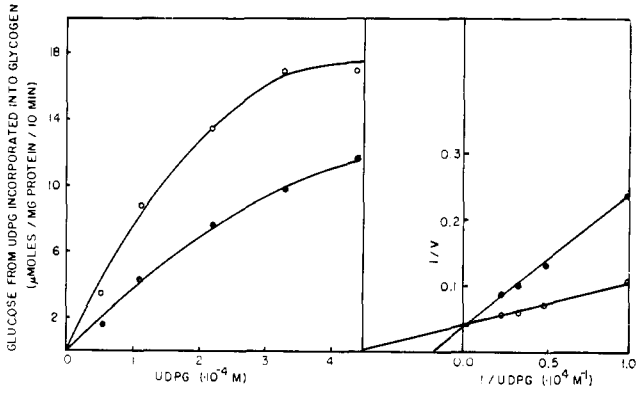

FIGURE 4: UDPG concentration dependence of the enzyme and double-reciprocal plot for glycogen synthetase. Partially purified enzyme from the control and insulin-treated animals was used. Conditions are the same as described in Methods except for the UDPG concentrations indicated. (●) Data obtained with the control enzyme and (○) with the enzyme from insulin-treated animals.

the incorporation of radioactive leucine into total liver protein. Furthermore, puromycin (10 μg/g body weight) completely inhibited the induction of hepatic enzymes by thyroxine, *i.e.*, the urea cycle enzymes[1] (Kim and Cohen, 1968). As shown in Table I, the increase in glycogen synthetase in insulin treated animals was not blocked by either puromycin or cycloheximide. This indicates that insulin stimulation of glycogen synthetase does not involve *de novo* protein synthesis.

[1] Unpublished data.

Protein synthesis was implicated in the increase in I form activity caused by insulin treatment in the rat (Steiner and King, 1964). However, subsequent reports provided no evidence for the involvement of new enzyme synthesis (Kreutner and Goldberg, 1967).

Puromycin has been reported to cause anomolous effects on carbohydrate metabolism (Appleman and Kemp, 1966). Indeed, treatment of the animal with puromycin alone stimulated glycogen synthetase to a level comparable with insulin stimulation. However, simultaneous administration of puromycin and insulin did not produce an additive effect, thus indicating that some common mechanism(s) is involved. The mechanism by which puromycin stimulates tadpole glycogen synthetase has now been clarified in our laboratory. It appears that puromycin treatment stimulates insulin secretion after a sequence of reactions, and this insulin is responsible for increased activity (Blatt *et al.*, 1969).

Subsequent studies on simple kinetic properties of the partially purified enzymes from control and insulin-treated animals revealed some differences in their properties.

Apparent K_m's for UDPG and G-6-P of the Two Enzyme Forms. The effect of glucose 6-phosphate on glycogen synthetase activity from control and insulin treated animals is shown in Figure 3. The apparent K_m's calculated for glucose 6-phosphate of the two enzymes are identical, *i.e.*, 5×10^{-3} M. A consistently higher V_{max} was observed with the enzymes from insulin-treated animals although the difference was small.

Figure 4 shows the dependence of the rate of glycogen synthesis upon UDPG concentration when the enzyme was assayed in the presence of a saturating concentration of glucose 6-phosphate (0.02 M). From the double-reciprocal plot of UDPG concentration and velocity, it is clear that the differ-

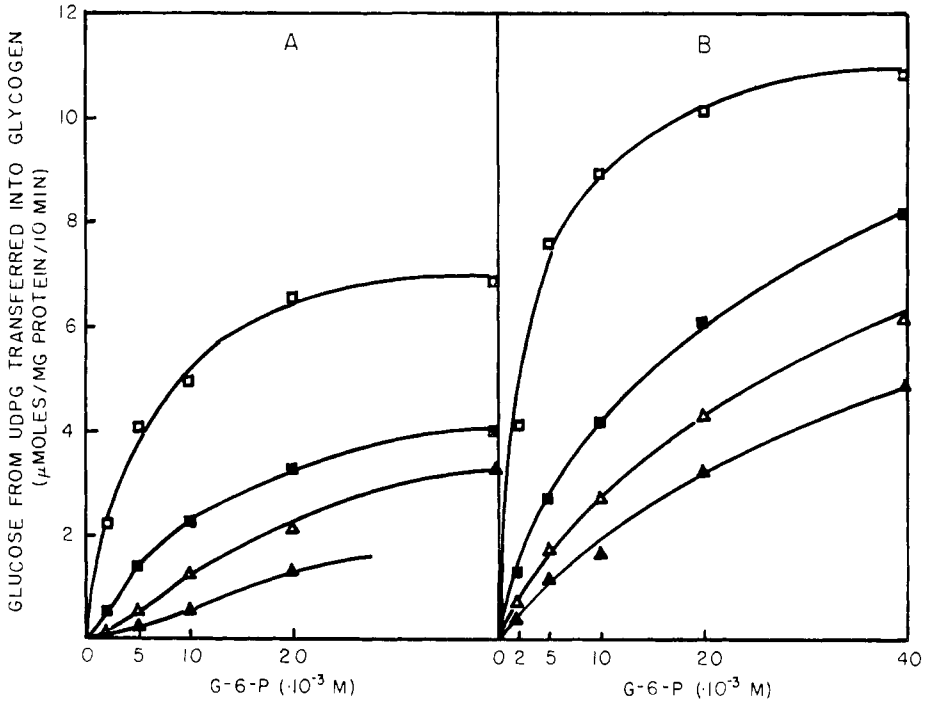

FIGURE 5: The inhibitory effect of different concentrations of ATP in the presence of glucose 6-phosphate. The rate of glycogen synthesis in the presence of different concentration of ATP and glucose 6-phosphate was determined. Part A represents the results obtained with the control enzyme in the presence of: (□—□) no ATP, (■—■) 1.5×10^{-3} M, (△—△), 3×10^{-3} M, and (▲—▲) 4.5×10^{-3} M ATP at the concentrations of glucose 6-phosphate indicated. Part B represents the effect of ATP and glucose 6-phosphate with enzyme obtained from the insulin-treated animals. Other conditions are the same as in the standard procedure described in Methods; partially purified enzyme was used.

ence between the two enzymes is the decrease in the K_m for UDPG in the case of enzyme from insulin-treated animals. The K_m's for UDPG calculated from Figure 4 are 7×10^{-4} and 1.5×10^{-4} M for the control and the enzyme from insulin-treated animals, respectively. However, the K_m for UDPG of the control enzyme varies widely, depending upon the preparation, from 0.64×10^{-3} to 3.2×10^{-3} M. This variation appears to be due to the presence of the two enzyme forms at varying ratios in the control animals. Apparent K_m's for UDPG of the control enzyme and the enzyme from insulin-treated animals were calculated from several determinations and found to be $(2.5 \pm 0.7) \times 10^{-3}$ and $(1.2 \pm 0.2) \times 10^{-4}$ M, respectively. There was only a slight change in V_{max}. The apparent K_m for UDPG for the enzyme from frog muscle has been reported to be $1.1\text{–}1.8 \times 10^{-3}$ M (Rosell-Perez and Larner, 1962). This is close to the K_m of the hepatic enzyme from control tadpoles.

Differential Inhibition by Nucleotides. Additional evidence that insulin treatment caused changes in the properties of the enzyme comes from the differential inhibitory effect of various nucleotides. Table II illustrates the degree of inhibition by nucleotides. The most potent inhibitor is UDP. It has already been reported that UDP acts as a competitive inhibitor of the substrate, UDPG, in other systems (Rothman and Cabib, 1967). This is also true for tadpole liver enzyme. More significant differential inhibition was observed mainly with nucleotide triphosphates. In general, although the differences were small, the enzyme from insulin-treated animals was consistently less inhibited than the control enzyme. Inhibition of the enzyme by ATP is partially competitive with respect to glucose 6-phosphate. ATP caused an increase in K_m for glucose 6-phosphate of both enzymes to the same extent, with minor changes in V_{max}. At a higher concentration (40 mM), glucose 6-phosphate reversed ATP inhibition of enzyme from insulin-treated animals to a greater extent than in the case of enzyme from control animals (Figure 5).

Measurements of the reaction rate as a function of glucose 6-phosphate concentration gave sigmoidal curves when ATP was present in the case of yeast enzyme (Rothman and Cabib, 1967). In tadpoles, however, sigmoidal behavior was found only with the control enzyme (Figure 5). The cooperative inhibitory effect of ATP becomes strikingly clear with enzyme from the control animals, when the reciprocal of velocity is plotted against the concentrations of ATP (Figure 6). However, sigmoidal behavior becomes apparent with the enzyme from insulin-treated animals when the data of Figure 5 were replotted as in Figure 6 or in Hill plots (Atkinson, 1966). Calculation of the slope (n number) for ATP from Hill plots at 2 mM G-6-P gave 3.9 and 1.1 for the control and insulin-treated enzyme, respectively (Figure 7A,B).

Effect of P_i. At a suboptimum concentration of G-6-P (10 mM), P_i stimulated the enzymes from both control and insulin-treated animals (Figure 8). The optimum concentration of P_i under these conditions appears to be 4–10 mM; higher concentrations of P_i result in a decrease in activity. P_i cannot completely replace G-6-P. The apparent K_m for G-6-P (5×10^{-3} M) obtained in the absence of P_i was reduced to 2.5×10^{-3} and 1.1×10^{-3} M for the control and insulin-treated enzymes, respectively (Figure 9), when determined in the presence of 10 mM P_i. (The intracellular concentration of P_i in the

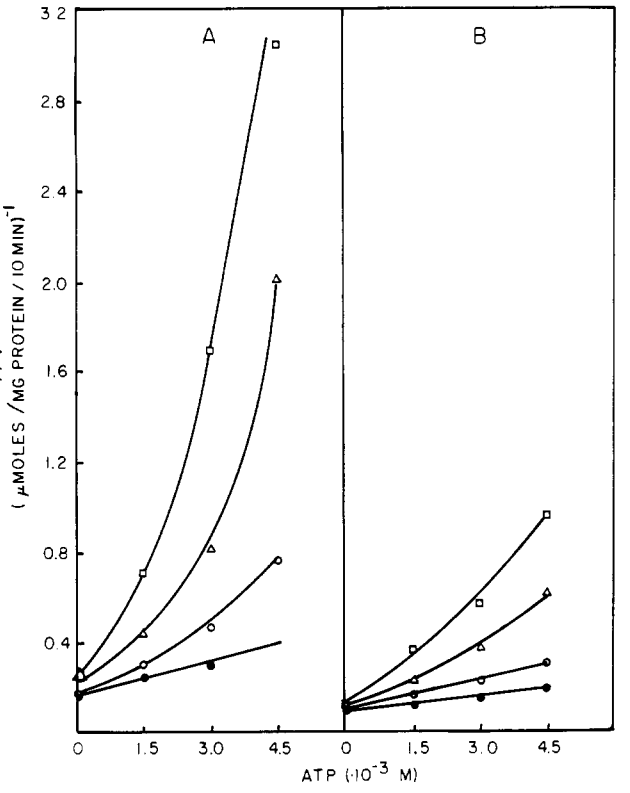

FIGURE 6: ATP inhibition of glycogen synthetase. The data obtained from Figure 5 were replotted as a Dixon plot to show the effect of ATP on the reaction rates of the two enzymes (A from control and B from insulin-treated animals) in the presence of several concentrations of glucose 6-phosphate. Curves represent the data obtained in the presence of 0.005 M (□—□), 0.01 M (△—△),0.02 M (○—○), and 0.04 M (●—●) glucose 6-phosphate. Other components were the same as in the standard reaction mixture.

tadpole liver is about 20 mM.)[2] Since the microdistribution of P_i in the cell is not known, the physiological significance of this observation must await further experimentation.

Other Properties. Because of the inherent difficulty in obtaining glycogen-free enzyme, it is impossible at this time to determine whether a difference in glycogen affinity or change in the degree of glycogen branching is involved in the hormonal control of the enzyme. Admittedly, the experimental results obtained on total soluble glycogen are too crude to estimate critically the degree of glycogen branching peculiar to those glycogen molecules attached to the enzyme; however, the percentage branching obtained from the glycogen preparations of both control and insulin-treated animals was identical, *i.e.*, 8.75%.

Since addition of insulin directly to the isolated enzyme produced no stimulatory effect, it appears that some other factor(s) is involved in the stimulation of the enzyme.

A second injection of insulin at the time when the originally stimulated activity was maximal produced no further increase in activity. The two enzymes have the same pH optimum.

Discussion

The occurrence of two forms of glycogen synthetase (D and I or b and a forms) in most biological systems, and control of transformation between the two forms by the pancreatic hormone provide one of the mechanisms controlling glycogen synthesis. It is generally believed that an early and biochemically important effect of insulin is activation of glycogen synthetase by means of the conversion of D into I form (b into a form).

In this communication, we have presented evidence that

[2] J. S. Sevall and K. Kim, unpublished data.

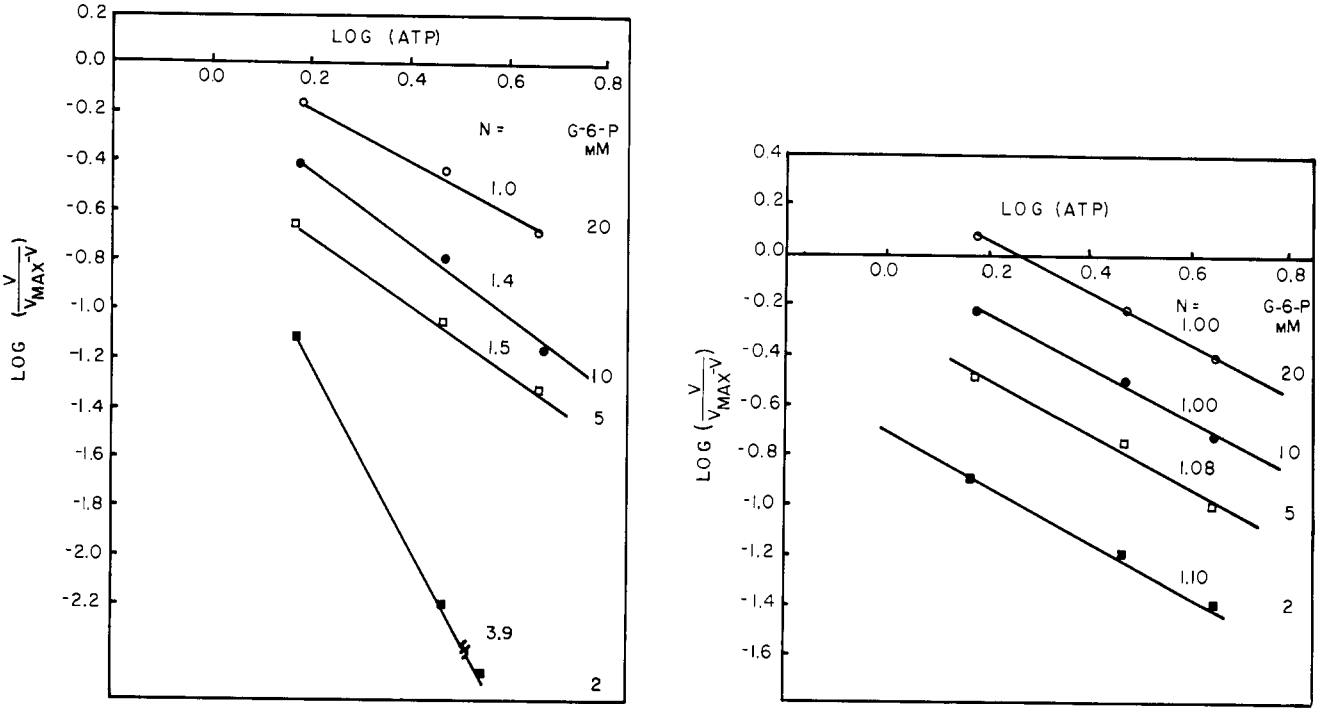

FIGURE 7: Hill plots for the curves of Figure 5 A (control enzyme) and -B (the enzyme from insulin-treated animals). G-6-P concentrations are indicated in the graph.

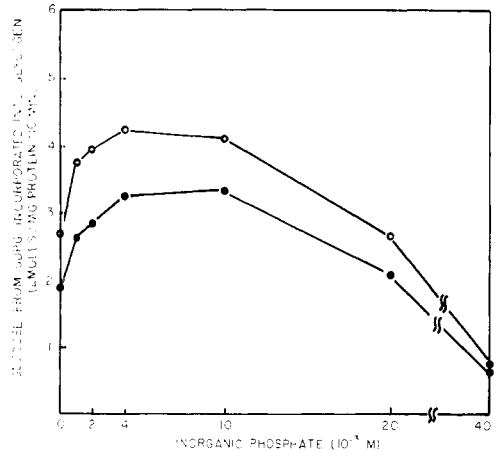

FIGURE 8: Effect of inorganic phosphate on glycogen synthetase. Partially purified glycogen synthetases from control (●—●) and from insulin-treated animals (○—○) were assayed as described in Methods except for the presence of 10 mM glucose 6-phosphate and P_i (as indicated).

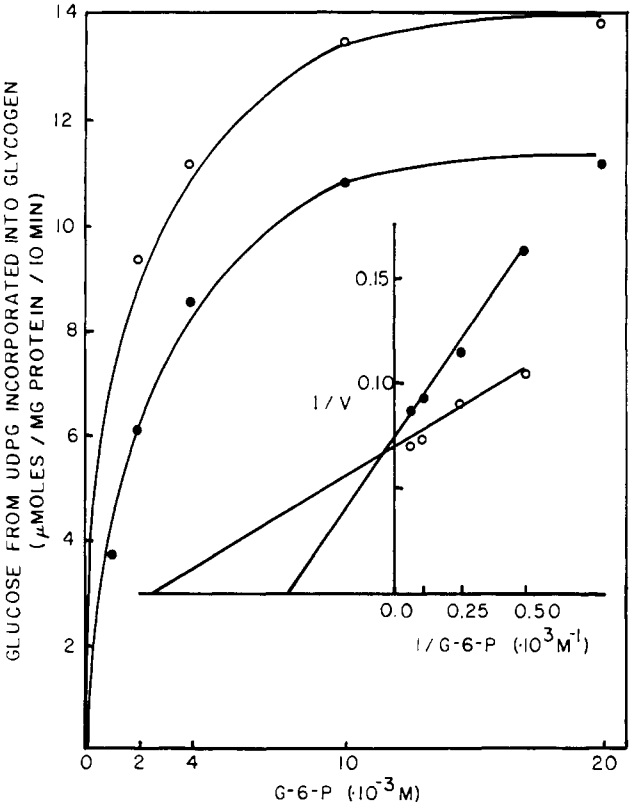

FIGURE 9: Effect of different concentrations of glucose 6-phosphate on the rate in the presence of P_i. Glucose 6-phosphate dependence of partially purified enzymes from control (●—●) and from insulin-treated animals (○—○) was determined as described in the Methods except for the presence of P_i (10 mM) and glucose 6-phosphate (as indicated).

tadpole liver glycogen synthetase is completely dependent upon glucose 6-phosphate and that treatment of the animal with insulin stimulates the enzyme without protein synthesis or the transformation of D form to I form. Stimulation of the enzyme appears to be due to transformation of a D-form enzyme to one with a higher affinity for UDPG.

It has been suggested that an analogy exists between glycogen synthetase and phosphorylase since both enzyme systems exist in two forms, one of which requires a cofactor. Transformation between the two forms involves phosphorylation and dephosphorylation. Glycogen synthetase activation requires dephosphorylation whereas activation of phosphorylase requires phosphorylation by phosphorylase kinase (Fischer and Krebs, 1955). Whether dephosphorylation is involved in the activation of tadpole glycogen synthetase, as is the case in transformation of D to I form in other systems, has yet to be determined. However, the requirement for G-6-P by both forms of the enzyme suggests some deviation from the general analogy between the two systems.

According to Mersmann and Segal (1967), activation of rat liver glycogen synthetase is accompanied by an increase in the affinity for both G-6-P and UDPG. However, insulin treatment does not change the K_m of tadpole liver glycogen synthetase for G-6-P. The presence of two enzyme forms in control tadpoles may be responsible for the wide range of the K_m determined for UDPG. The K_m for UDPG of the control enzyme varied within a range from 0.64×10^{-3} to 3.2×10^{-3} M, depending upon the particular preparation, in contrast to the K_m for UDPG, of the enzyme from insulin-treated animals. The intracellular concentration of UDPG in tadpole liver does not change significantly with insulin treatment; *i.e.*, $(2.24 \pm 0.05) \times 10^{-4}$ M for control and $(2.48 \pm 0.05) \times 10^{-4}$ M for insulin treated.[2] Insulin treatment, therefore, increases the ratio of S/K_m from 0.1 to 2, where the enzyme can be expected to function at maximum velocity. The intracellular G-6-P concentration, about 4×10^{-4} M, was not significantly affected by insulin treatment.[2] Since the K_m for UDPG is also affected by G-6-P, a higher K_m for UDPG is expected in the presence of a physiological concentration of G-6-P than found with saturating G-6-P.

In the absence of inorganic phosphate, the K_m for G-6-P of both the control and insulin-treated enzyme is 5×10^{-2} M, which is 10 times greater than its physiological concentration. In the presence of 10 mM P_i, however, this K_m is reduced to 2.5×10^{-3} and 1.1×10^{-3} M for control and hormone-treated enzymes, respectively. Thus the decreases in the K_m's for UDPG and G-6-P produce a cumulative effect on glycogen synthetase activity. Detailed studies on other factors involved in the control of glycogen synthetase are now in progress.

Although most studies of glycogen synthetase have been performed on mammalian enzymes, some properties of the frog muscle enzyme have been reported (Rosell-Perez and Larner, 1962). It has been postulated that the activation of rat enzyme involves the conversion of activities (D → I) whereas the activation of an inactive enzyme or inactive precursors is involved in the case of frog enzyme (Rosell-Perez and Larner, 1962). While the possibility remains that the activation of frog muscle enzyme by Mg^{2+} and mercaptoethanol involves such a mechanism, insulin treatment of tadpoles clearly produces hepatic glycogen synthetase with an increased substrate affinity. Treatment of the hepatic enzyme with Mg^{2+} and mercaptoethanol resulted in complete inactivation of the enzyme.

Since other kinetic properties of the insulin-stimulated en-

zyme are different from those of the control enzyme the possibility of transformation of inactive enzyme to functional enzyme, which would increase the total amount of enzyme, is not likely in the case of tadpole liver enzyme. Further kinetic studies on the nature of activation of tadpole synthetase are being carried out.

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