Regulation of Hepatic Glycogen Synthetase of
Rana catesbeiana

EFFECT OF INSULIN AND HYDROCORTISONE ON GLYCOGEN SYNTHETASE IN A LIVER SYSTEM IN VITRO, AND REGULATION OF GLYCOGEN SYNTHETASE BY CELLULAR METABOLITES*

(Received for publication, May 22, 1970)

LOIS M. BLATT, J. SANDERS SEYALL,§ and KI-HAN KIM

From the Department of Biochemistry, Purdue University, Lafayette, Indiana 47907

SUMMARY

A minced liver system from Rana catesbeiana tadpoles has been developed. Treatment of this liver preparation in vitro with insulin results in the activation of glycogen synthetase. Hormonally induced activation is the result of the conversion of the less active form of the enzyme to the more active form. The insulin-mediated activation is counteracted by hydrocortisone and dibutyryl-cyclic adenosine 3',5'-monophosphate. This indicates that hydrocortisone activation of glycogen synthetase in the intact animal is a secondary effect of the gluconeogenic hormone. With this in vitro system, we have obtained experimental evidence which indicates that hepatic glycogen synthetase can be controlled by modulation of cellular metabolites without requiring activation of glycogen synthetase by insulin. Alterations of cellular metabolites were achieved by the addition of glucose and ammonium acetate to the incubation medium.

Activation of liver glycogen synthetase (uridine diphosphate-glucose:α-1,4-glucan α-4 glucosyl transferase, EC 2.4.1.11) by insulin has now been well documented (1-3). In most cases activation involves the transformation of an inactive form which is dependent on glucose 6-phosphate for enzymatic activity (D or b form) to an active form which is independent of glucose-6-P (I or a form). The situation in Rana catesbeiana tadpoles is unique since all hepatic glycogen synthetase activity is dependent on glucose-6-P. The 2- to 3-fold stimulation of activity caused by treatment of tadpoles with insulin is the result of transformation of one D-form to another which has a higher affinity for substrate, UDP-glucose (3). Although glucose-6-P plays an essential role as the activator of the D-form of glycogen synthetase and as a precursor of glycogen, its role as a controlling agent of hepatic glycogen synthesis has been questioned. This question arises since the cellular glucose-6-P concentration is relatively constant despite changes in physiological state and since different relative ratios of the D to the I (or b to a) form of glycogen synthetase occur in various biological systems.

Two mechanisms for the control of glycogen synthetase activity can be considered at the present time, the first being conversion between two enzyme forms (I --> D) which is dependent on hormonal stimulation, and secondly, the effect of metabolites on the normally occurring enzyme form. It is possible that both of these control mechanisms are available in the tissues of higher organisms, e.g. rat muscle and liver (4, 5), whereas in yeast probably only metabolite regulation exists (6, 7). However, it is generally believed that hepatic glycogen synthesis is dependent upon the conversion of the D to I-form, because the cellular concentration of UDP-glucose and glucose-6-P are too low to allow activity of the D-form (8).

Recently, Gold (5) suggested the possibility of metabolite control of liver glycogen synthetase activity on the basis of the differential effects of intermediary metabolites on the nonactivated enzyme normally present in liver and an in vitro activated form. However, the physiological significance of the activation in vitro and the relationship of this phenomena to the mechanism in vivo of endocrine control, conversion between D and I forms in rats, are still obscure (5). Glucose activation and modification of glycogen synthetase and phosphorylase in the perfused rat liver have been reported by Buschiazzo, Exton, and Park (9). However, activation of liver glycogen synthetase by insulin has not been observed with tissue preparation in vitro.

It is the purpose of this communication to report that a minced liver system in vitro from R. catesbeiana tadpoles has been developed in which both mechanisms for the control of glycogen synthetase activity can be studied. Treatment of this liver preparation with insulin results in the activation of glycogen synthetase. In addition, metabolite control of the activity of hepatic glycogen synthetase, independent of the hormonally induced transformation of the enzyme to the activated form, has been studied. With both the minced liver eviscerated and pancreactomized tadpoles, it is shown that hydrocortisone antagonizes the insulin-mediated activation of glycogen synthetase. The use of this system may help to clarify the conflicting reports of the effect of hydrocortisone on glycogen synthetase (5, 10, 11).

* This investigation was supported in part by United States Public Health Service Research Grant AM 12865 from the National Institute of Arthritis and Metabolic Diseases and by Grant GH 8020 from the National Science Foundation. This is Journal Paper 4062 of the Purdue Agricultural Experiment Station.

† Postdoctoral fellow of the National Institutes of Health (2-F02-GM-39,634-02).

§ David Ross Fellow.
Effect of insulin concentration on glycogen synthetase in vitro

Minced tadpole livers were incubated in 5 ml of Amphibian medium with various concentrations of insulin at 25°C for 3 hours. Glycogen synthetase activity with standard deviation was obtained from three independent experiments.

<table>
<thead>
<tr>
<th>Insulin concentration (i.u./ml)</th>
<th>Glycogen synthetase (µmoles/g liver/10 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>5.5 ± 0.5</td>
</tr>
<tr>
<td>0.04</td>
<td>7.7 ± 0.5</td>
</tr>
<tr>
<td>0.24</td>
<td>9.5 ± 0.7</td>
</tr>
<tr>
<td>0.48</td>
<td>8.0 ± 0.5</td>
</tr>
<tr>
<td>0.96</td>
<td>7.5 ± 0.7</td>
</tr>
</tbody>
</table>

MATERIALS AND METHODS

Chemicals—Puromycin, cycloheximide, glucose-6-P, UDP-glucose, and other nucleotides were purchased from Nutritional Biochemicals. Dibutyryl-cyclic AMP was the product of Boehringer Mannheim. 14C-UDP-glucose, obtained from Schwarz BioResearch, had a specific activity of 200 µCi per µmole. 14C-Glucose 1-phosphate, purchased from New England Nuclear, had a specific activity of 200 µCi per µmole. Glass fiber filters (904 HA) were purchased from Wilkins-Anderson Company, Clifton, New Jersey. Amphibian culture medium (12), which is a complex synthetic medium containing fetal calf serum (10%), amino acids, vitamins, glucose (4.4 mg), and balanced salts suitable for the maintenance of amphibian tissues, was the product of Grand Island Biological Company. Hydrocortisone, purchased from Calbiochem, was dissolved in dimethyl sulfoxide and then, just prior to use, 0.9% saline solution was added so that the final concentration of dimethyl sulfoxide was 5%. Shellfish glycogen, bovine insulin (24.1 i.u. per mg), thyroxine, UDP-glucose dehydrogenase, and luciferin-luciferase for ATP assay were obtained from Sigma Chemical Company.

Animals—R. catesbeiana tadpoles, weighing 5 to 8 g, were purchased from Lemberger Company, Oshkosh, Wisconsin. The stock animals were kept in a tank of dechlorinated water at 18°C. During experiments tadpoles were kept at 25°C.

Glycogen Synthetase and Phosphorylase Assays—Glycogen synthetase activity was assayed by a modification (3) of the method of Villar-Palasi et al. (13). Phosphorylase activity was assayed by the method of Sutherland (14) except that 14C-glucose 1-phosphate was used. Incorporation of radioactivity into glycogen was measured as described for the glycogen synthetase assay.

Preparation of Minced Liver—Livers were washed by shaking in Amphibian culture medium (12) for 30 min at 25°C. The washed livers were drained and then minced in a Petri dish with two scalpels until all the tissue was reduced to pieces less than 2 mm in any dimension. Portions of the minced liver were placed in Amphibian medium and incubated, with shaking (150 rpm) at 25°C in a New Brunswick water bath shaker (New Brunswick Scientific, New Brunswick, New Jersey).

Assay of Metabolites—Acid extracts of liver were prepared by the method of Rothman and Cabib (7). Glucose-6-P and UDP-glucose in the extract were measured enzymatically with glucose 6-phosphate dehydrogenase and UDP-glucose dehydrogenase

Effect of Insulin on Glycogen Synthetase in Vitro—Incubation of minced tadpole liver preparations with insulin at 25°C resulted in the activation of glycogen synthetase. The data in Table I show that at the optimum concentration of insulin, 0.24 i.u. per ml, glycogen synthetase activity is stimulated about 2-fold. Further increase in the hormone concentration slightly diminished the degree of activation. These data can be compared with those obtained in vivo; about the same degree of stimulation is obtained by treating tadpoles with 0.0024 i.u. of insulin per mg of body weight (3). It is likely that in these preparations, which contain many broken cells, insulin may be degraded and absorbed by nonfunctional cells and cellular debris. In experiments with rabbit liver slices, Snyder and Cahill demonstrated that insulin promoted incorporation of 14C-glucose into glycogen (18). In their system, the effective concentration of insulin was 1 i.u. per ml of incubation medium.

The time course for activation of glycogen synthetase is similar in vitro and in vivo (3). Maximum activity is obtained in 3 hours. Under the conditions used, enzyme activity in untreated minced livers remained constant for at least 4 hours (Fig. 1A).


Effect of Protein Synthesis Inhibitors on Glycogen Synthetase Activity

Minced livers were incubated in 5 ml of Amphibian medium with the reagents indicated for 3 hours at 25°. The concentrations of the reagents were as follows, insulin, 0.48 i.u. per ml, puromycin, 10 μg per ml, cycloheximide, 20 μg per ml. Glycogen synthetase activity with standard deviation was obtained from three independent experiments.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Activity (μmoles/2 liver/10 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6.0 ± 0.5</td>
</tr>
<tr>
<td>Puromycin</td>
<td>6.7 ± 0.6</td>
</tr>
<tr>
<td>Cycloheximide</td>
<td>6.0 ± 0.5</td>
</tr>
<tr>
<td>Insulin</td>
<td>12.0 ± 0.0</td>
</tr>
<tr>
<td>Insulin + puromycin</td>
<td>11.7 ± 0.9</td>
</tr>
<tr>
<td>Insulin + cycloheximide</td>
<td>11.6 ± 0.8</td>
</tr>
</tbody>
</table>

results were obtained when glycogen synthetase levels were measured in minced livers incubated in Ringer's solution (Fig. 1B). With or without insulin, glycogen synthetase activity dropped about 50% in the first hour of incubation and fell to about 25% of the original activity after 4 hours. Supplementation of Ringer's solution with 10% fetal calf serum, the amount present in Amphibian medium, partially stabilized glycogen synthetase activity. In this case, glycogen synthetase activity fell to about 66% of the original value after a 4-hour incubation. Although there was only a slight stimulation of enzyme activity, the insulin-treated sample was 1.6-fold more active than the control sample after 3 hours of incubation (Fig. 1C). These results can be interpreted in at least two ways. The addition of large quantities of serum proteins to the culture medium may provide a sufficient condition for maintenance of the liver tissue. Alternatively, some component in the serum may be necessary for survival of the tissue and may act together with insulin in the activation of glycogen synthetase.

Amphibian medium and an incubation temperature of 25° were used in all experiments discussed in this report. At 37°, with or without insulin, enzyme activity decreased by at least 50% during 4 hours of incubation of liver tissue in Amphibian medium.

Effect of Inhibitors of Protein Synthesis—The effect of inhibitors of protein synthesis on activation of glycogen synthetase by insulin in minced tadpole livers was investigated. The data in Table II show that puromycin and cycloheximide have no effect on glycogen synthetase activity in minced liver preparations nor do they inhibit activation of the enzyme by insulin. The inhibitors, at the concentrations used, reduce total tadpole hepatic protein synthesis about 95% as measured by the incorporation of radioactive leucine into total liver protein. Therefore, the mechanism by which insulin stimulates hepatic glycogen synthetase activity in vitro cannot involve de novo enzyme synthesis. A similar conclusion has previously been reported for the effect of insulin in whole animal experiments (3).

In intact tadpoles, puromycin treatment caused activation of glycogen synthetase. It has recently been suggested that puromycin exerts this effect by causing the release of insulin which then activates glycogen synthetase (19). The results obtained with the minced liver system support such an indirect role for activation of glycogen synthetase by puromycin.

Effect of Hydrocortisone, Dibutyryl-cyclic Adenosine 3',5'-Mono-

phosphate and Other Factors on Glycogen Synthetase Activity in Minced Tadpole Liver Preparations—The effect of several factors which stimulate glycogen synthetase when administered to intact tadpoles has been studied in the minced liver system. These include thyroxine, dibutyryl-cyclic AMP, theophylline (20), puromycin, glucose (19), glucagon, and hydrocortisone. We found that none of these compounds activates glycogen synthetase when added to the incubation medium of minced liver preparations. When administered to alloxan-treated or pancreatectomized tadpoles (19, 20), these agents also fail to activate hepatic glycogen synthetase. This suggests that the activation in vivo of glycogen synthetase by these factors requires the presence of the intact pancreas, presumably for the secretion of insulin.

With the minced liver system, we found that hydrocortisone and dibutyryl-cyclic AMP differed from the other factors studied since the insulin-induced activation of glycogen synthetase is actually inhibited by the administration of hydrocortisone or dibutyryl-cyclic AMP with insulin (Tables III and IV). Because of this interesting property, the effects of dibutyryl-cyclic AMP and hydrocortisone were studied in more detail than those of the other factors. As shown in Table III, although hydrocortisone stimulates glycogen synthetase activity 2-fold when administered to intact tadpoles, it has no stimulatory effect on the enzyme activity of pancreatectomized tadpoles and minced liver preparations. However, hydrocortisone blocks the insulin-mediated activation of the enzyme in both the in vitro system and in pancreatectomized tadpoles. When added to minced liver preparations with insulin at the beginning of the 3-hour incubation period, hydrocortisone (1 to 100 μg per ml) blocked activation of glycogen synthetase. Inhibition also occurred if hydrocortisone was administered to samples which had been incubated with insulin alone for the first 1 or 2 hours of the incubation period. The addition of hydrocortisone to an insulin-treated sample 15 min prior to the end of the 3-hour incubation period did not block the insulin activation of glycogen synthetase. This indicates that the inhibition brought about by hydrocortisone may involve a reversal of the synthetase activation process.

These results suggest that, at the tissue level, insulin and hydrocortisone play antagonistic roles in the control of glycogen synthetase, in accordance with their opposing physiological actions on carbohydrate metabolism. Previous studies in more complex systems have failed to detect antagonistic actions of these two hormones on glycogen synthetase (5, 10, 11). Although the stimulation of hepatic glycogen deposition in the rat was attributed to a steroid-induced transformation of the D to the I-form of glycogen synthetase (21), subsequent work with alloxan diabetic rats indicated that insulin is required for activation (10). A recent report claims that hydrocortisone is necessary for the maintenance of the enzyme system which activates glycogen synthetase (11). However, at the level of isolated tissue hydrocortisone clearly opposes insulin-mediated activation of tadpole liver glycogen synthetase.

The activation of glycogen synthetase by insulin in vitro, is also inhibited by the cyclic AMP derivative, dibutyryl-cyclic AMP. As shown in Table IV, addition of dibutyryl-cyclic AMP to minced liver preparations at concentrations from 5 to 50 μg per ml did not stimulate glycogen synthetase activity. Addi
The glycogen synthetase activity reported is the average value obtained from 10 animals. See the legend of Fig. 1 for the conditions employed for the *in vitro* system used in Experiments 3 and 4.

### Table III

**Effect of hydrocortisone on glycogen synthetase activity**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Concentration</th>
<th>Activity (µmoles/g liver/10 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Insulin</td>
<td>0.0024 i.u./g</td>
<td>6.2 ± 0.5</td>
</tr>
<tr>
<td>Hydrocortisone</td>
<td>10 µg/g</td>
<td>10.7 ± 0.8</td>
</tr>
<tr>
<td>Insulin + hydrocortisone</td>
<td>0.0024 i.u./g</td>
<td>5.75 ± 0.5</td>
</tr>
<tr>
<td>Hydrocortisone</td>
<td>10 µg/g</td>
<td>11.7 ± 0.6</td>
</tr>
<tr>
<td>Insulin + hydrocortisone</td>
<td>0.0024 i.u./g</td>
<td>6.2 ± 0.5</td>
</tr>
<tr>
<td>Hydrocortisone</td>
<td>10 µg/g</td>
<td>5.6 ± 0.5</td>
</tr>
</tbody>
</table>

For experimental details, see the legend for Fig. 1. The activity reported here represents the average value obtained from 10 animals.

### Table IV

**Effect of dibutyryl-cyclic adenosine 3',5'-monophosphate on insulin activation of glycogen synthetase in vitro**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Concentration</th>
<th>Activity (µmoles/g liver/10 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Insulin</td>
<td>0.24 i.u./ml</td>
<td>5.2 ± 0.5</td>
</tr>
<tr>
<td>Hydrocortisone</td>
<td>10 µg/ml</td>
<td>10.5 ± 0.8</td>
</tr>
<tr>
<td>Insulin + hydrocortisone (t = 0)*</td>
<td>0.24 i.u./ml + 1 µg/ml</td>
<td>6.1 ± 0.6</td>
</tr>
<tr>
<td>Insulin + hydrocortisone (t = 0)*</td>
<td>0.24 i.u./ml + 10 µg/ml</td>
<td>5.1 ± 0.5</td>
</tr>
<tr>
<td>Insulin + hydrocortisone (t = 0)*</td>
<td>0.24 i.u./ml + 20 µg/ml</td>
<td>5.1 ± 0.5</td>
</tr>
<tr>
<td>Insulin + hydrocortisone (t = 0)*</td>
<td>0.24 i.u./ml + 100 µg/ml</td>
<td>5.1 ± 0.5</td>
</tr>
<tr>
<td>Insulin + hydrocortisone (t = 0)*</td>
<td>0.24 i.u./ml + 10 µg/ml</td>
<td>5.3 ± 0.5</td>
</tr>
<tr>
<td>Insulin + hydrocortisone (t = 2)*</td>
<td>0.24 i.u./ml + 10 µg/ml</td>
<td>9.9 ± 0.8</td>
</tr>
</tbody>
</table>

*Time (in hours) during the 3-hour incubation period at which hydrocortisone was added to the medium. Insulin was added at the initiation of the incubation period (t = 0).*

The antagonistic effect of glucose-6-P on inhibition of glycogen synthetase can be excluded from the minced liver preparations, the possibility of metabolite control of glycogen synthetase in the absence of the hormonally induced enzyme conversion can be rigorously studied in this *in vitro* system. With the minced liver system, we have examined the possibility of metabolite control of glycogen synthesis. The data obtained with the partially purified enzyme show that glucose-6-P (a) lowers the Kₘ for UDP-glucose (22), (b) partially reverses inhibition of glycogen synthetase by ATP, and (c) changes the substrate saturation kinetics for UDP-glucose from sigmoidal to hyperbolic.

The effect of glucose-6-P on the Kₘ for UDP-glucose is shown in Table V. The Kₘ for UDP-glucose is reduced 6-fold when the glucose-6-P concentration is increased from 2.5 to 32 mM. A similar effect of glucose-6-P on the Kₘ for UDP-glucose of the rabbit muscle enzyme has been reported (23).

The antagonistic effect of glucose-6-P on inhibition of glycogen synthetase activity by ATP is shown in Fig. 2A. At a ratio of
TABLE V

Effect of glucose-6-P on apparent $K_m$ for UDP-glucose of tadpole liver glycogen synthetase

With partially purified enzyme from control tadpoles, the apparent $K_m$ for UDP-glucose was determined from the double reciprocal plots. The standard assay procedure was employed, except that the concentration of glucose-6-P for each $K_m$ determination was as listed below.

<table>
<thead>
<tr>
<th>Glucose-6-P (mM)</th>
<th>$K_m \times 10^{-3}$M</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5</td>
<td>9.2</td>
</tr>
<tr>
<td>5</td>
<td>8.6</td>
</tr>
<tr>
<td>8</td>
<td>3.8</td>
</tr>
<tr>
<td>16</td>
<td>2.0</td>
</tr>
<tr>
<td>32</td>
<td>1.4</td>
</tr>
</tbody>
</table>

Fig. 2. A, glucose 6-phosphate concentration dependence and double reciprocal plot for glycogen synthetase activity. Activity at varying glucose-6-P (G-6-P) concentrations with (O---O) or without (●--●) ATP (1.5 mM). Percentage of activity (□-□) is that obtained at a given concentration of glucose-6-P with 1.5 mM ATP compared to the activity in the absence of ATP with partially purified enzyme. The concentration of UDP-glucose (UDPG) was 1.34 mM. B, UDP-glucose concentration dependence and double reciprocal plot for glycogen synthetase activity at a constant level of ATP and glucose-6-P. The concentration of ATP was 6 mM; glucose-6-P concentration was either 3 mM (O---O) or 12 mM (●--●).

The effect of glucose-6-P on the kinetics for saturation of glycogen synthetase by UDP-glucose is shown in Fig. 2B. These results, which show that glucose-6-P lowers the $K_m$ for UDP-glucose, reverses inhibition of the enzyme by anions, and changes the kinetics for UDP-glucose from cooperative to hyperbolic, suggest the possibility of metabolite control of glycogen synthetase independent of transformation of the less active to the more active form. In order to further examine this possibility we have studied the effect of metabolites on the synthesis of glycogen in preparations of minced liver.

Effect of Glucose on Glycogen Synthesis and on Cellular Metabolites—The effect of glucose on glycogen synthesis in minced liver preparations is shown in Fig. 3, A and B. Minced tadpole livers were incubated in 5-ml portions of either unsupplemented Amphibian medium, which is 4.4 mM glucose (O---O) or glucose-supplemented Amphibian medium, which is 280 mM glucose (●--●). Uniformly labeled $^{14}$C-glucose was added to the minced liver preparations as follows: to 0.1 uCi per 5 ml of control medium, 4.4 mM glucose and to 0.3 uCi per 5 ml of glucose supplemented medium, 280 mM glucose. Samples were taken at the time intervals indicated and carefully washed with isotonic saline to remove traces of the radioactive medium. Glycogen was isolated as described in the text and the incorporation of $^{14}$C-glucose into glycogen was measured. B, the rate of glucose incorporation into glycogen was determined with different concentrations of glucose in the medium. The glucose concentrations are indicated in the figure. Other experimental conditions are the same as described for Fig. 3A.
Fig. 5. Effect of ammonium ions on the incorporation of glucose into glycogen in vitro. Minced tadpole livers were incubated in 5 ml of Amphibian medium (4.4 mM glucose) containing 0.1 μCi of uniformly labeled 14C-glucose. Samples were taken at the time intervals indicated and the incorporation of 14C into glycogen was measured. Control samples contained no NH₄⁺ (O). A second set of samples was incubated with NH₄⁺ present during the entire experimental period (■). A third set of samples was incubated for 1 hour in medium containing NH₄⁺ and then carefully washed and placed in fresh medium containing no NH₄⁺ (●).

Fig. 6. Effect of ammonium ion on glucose-6-P (G-6-P) levels in tadpole liver in vitro. Minced tadpole livers were incubated in 5 ml of Amphibian medium. Samples were taken at the time intervals indicated, and glucose-6-P was assayed. The following conditions were used: O—O, no NH₄⁺, 4.4 mM glucose; ■—■, no NH₄⁺, 280 mM glucose; ▼—▼, NH₄⁺ (50 mM) added after 1 hour of incubation in medium containing 4.4 mM glucose; ▲—▲, NH₄⁺ (50 mM) added after 1 hour of incubation in medium containing 280 mM glucose.

glycogen synthesis reflects direct activation of hepatic glycogen synthetase (9, 24).

In order to clarify the mechanism by which glucose activates glycogen synthesis in vitro, we have studied the response of key metabolites to glucose supplementation of minced liver preparations. Fig. 4 shows the time course for changes in ATP and glucose-6-P levels in minced liver preparations containing 4.4 mM and 280 mM glucose. As noted in this graph and in others, there was a sharp decrease in these metabolites during the initial phase of incubation in vitro. Following the initial decrease in concentration of glucose-6-P to 0.01 μmole per g of liver during the first 15 min of incubation, this metabolite increased to 0.06 μmole per g of liver in the preparation with 4.4 mM glucose medium and to 0.24 μmole per g of liver in the preparation with medium containing 280 mM glucose. Following the initial decrease in ATP concentration the level of ATP was maintained at a relatively constant level during the remainder of the 3-hour incubation. However, the samples incubated in 280 mM glucose contained a lower level of ATP. This may reflect the difference in glucose-6-P synthesis between the two conditions. During the 2-hour incubation the glucose-6-P to ATP ratio increased from 0.07 to 0.17 and from 0.17 to 1.0 in medium containing 4.4 mM and 280 mM glucose, respectively. The concentration of UDP-glucose fell from about 20 mmoles per g to about 10 mmoles per g during the first hour of incubation with either high or low glucose medium. The level of UDP-glucose thereafter was relatively constant.

Effect of Ammonium Ion on Glycogen Synthesis and Metabolites—If metabolic control of hepatic glycogen synthetase involves glucose-6-P and ATP, the level of these metabolites should regulate the rate of glycogen synthesis. Since NH₄⁺ stimulates phosphofructokinase from various sources (25-27), this ion could reduce the intracellular concentration of glucose-6-P. Therefore, the effect of ammonium ion on glycogen synthesis and on the concentration of metabolites in minced liver preparations was examined. Ammonium acetate, at the concentrations used, had no effect on the activity of partially purified hepatic glycogen synthetase.

The data in Fig. 5 show that NH₄⁺ did indeed regulate the synthesis of glycogen in minced liver preparations. It is apparent that NH₄⁺ caused complete inhibition of glycogen synthesis when added at the start of the incubation. This inhibition was accompanied by a drastic decrease in glucose-6-P concentration (Fig. 6). ATP levels also decreased in liver tissue incubated with NH₄⁺ (Fig. 7) as would be expected for a cond-
UDP-glucose was 1.34 mM. Velocity (v) is measured in μmoles per mg of protein per min.

The concentration of fructose 1,6-diphosphate used were: A—A, 0 μM; O—O, 10 μM; □—□, 20 μM. The concentration of UDP-glucose was 1.34 mM. Velocity (v) is measured in μmoles per mg of protein per min.

Since fructose 1,6-diphosphate inhibits glycogen synthetase competitively with glucose-6-P (Fig. 8), the effect of NH₄⁺ on glycogen synthesis could very well be compounded; by stimulating phosphofructokinase, NH₄⁺ could (a) decrease the cellular concentration of glucose-6-P, an activating metabolite, and (b) increase the concentration of fructose 1,6-diphosphate, an inhibitory metabolite. Further work now in progress should help clarify this hypothesis.

The fact that the removal of ammonium ion at different stages of incubation of minced livers in vitro results in the resumption of glycogen synthesis (Fig. 5), shows that the effect of ammonium ion is not the result of any permanent cellular damage.

**Effect of Glucose on Phosphorylase**—Our studies of the effect of glucose on glycogen synthetase and phosphorylase show that glucose does not activate the synthetase in minced liver preparations. However, phosphorylase activity in liver preparations incubated in Amphibian medium containing 280 mM glucose was inhibited 90 to 95% as compared to control preparations incubated with 4.4 mM glucose for 1 hour. These studies are consistent with the theory that hepatic glycogen synthesis can be controlled by the modulation of cellular metabolites in the absence of the hormonally controlled transformation of glycogen synthetase.

**DISCUSSION**

The tadpole system described in this communication constitutes the first such in vitro system in which glycogen synthetase activation, in response to insulin, has been demonstrated. The transformation of glycogen synthetase in response to insulin has been observed with rat diaphragm and fat pad in vitro (27). However, insulin activation of the enzyme has never been duplicated with liver preparations in vitro. Other effects of insulin in vitro, such as increased glycogen retention or glucose incorporation into glycogen, have been observed in liver preparations (28). Although we are still far from a complete understanding of the mechanism by which insulin activates glycogen synthetase, the in vitro liver system at least allows us to conclude that activation is a direct effect of the hormone on liver, as the target tissue, and not an indirect effect by other factors from extrahepatic tissues. Since the time course from stimulus, insulin administration, to response, activation of glycogen synthetase, in the isolated liver system is similar to that observed in vivo (either intact or pancreatectomized tadpoles) this system should be useful in determining the steps interposed between stimulus and response.

With this in vitro system, we have directed our attention to two important questions relevant to control mechanisms of hepatic glycogen metabolism. First, can liver glycogen synthetase be activated in vitro by both insulin and hydrocortisone, hormones with opposing physiological action on carbohydrate metabolism? The reports on the actions of these hormones in the control of glycogen synthetase are confusing (10, 11, 29). However, our studies on the mode of hydrocortisone action, with both the in vitro liver system and pancreatocentomized tadpoles, indicate that hydrocortisone counteracts the activation of glycogen synthetase by insulin.

The second question we hoped to answer with our in vitro system concerned the role of glucose-6-P and other metabolites in the control of glycogen synthetase and, consequently, the synthesis of hepatic glycogen in the absence of the hormonally induced conversion between the two forms of the enzyme.

That the concentration of glucose exerts an effect on the rate of its conversion into liver glycogen is shown by studies both in vivo and in vitro (23). A simple explanation of this observation is that glucose exerts a mass action effect by way of glucokinase. The resultant increase in glucose-6-P then has a stimulatory effect on the activity of glycogen synthetase. However, the lack of universal glucokinase occurrence and the constancy of the glucose-6-P concentration during different physiological conditions made such an explanation, based on a mass action effect of glucose, more doubtful.

We have examined the role of metabolites, particularly glucose-6-P and ATP, under different physiological conditions. Our studies indicate that the altered concentration of glucose-6-P, caused by increasing the concentration of glucose, results in the stimulation of glycogen synthesis in the absence of glycogen synthetase activation. The kinetic properties of the tadpole enzyme suggest that an increased glucose-6-P level not only contributes to the pool size of precursor, but also counteracts inhibition of glycogen synthetase by anions (such as ATP). If just two metabolites, namely glucose-6-P and ATP are considered under normal physiological conditions (glucose-6-P, 0.15 × 10⁻⁴ M, and ATP, 0.5 × 10⁻⁴ M, from Fig. 4), one would expect glycogen synthetase to be in the inhibited state, as indeed shown by the sigmoidal kinetics. Thus, increasing glucose-6-P relieves the inhibition to a certain degree and makes the enzyme more functional. Since the increase in glucose concentration used in our liver system was so large, the actual effect under more physiological circumstances is still uncertain. However, since the liver cell membrane is uniquely permeable to glucose entry, changes in blood glucose concentration will certainly be reflected in the liver glucose content and can then affect glycogen synthetase activity by means of the steps outlined above.

What is significant is that modulations of intracellular metabolites caused by manipulation of the incubation medium, by the addition of glucose or ammonium ion, can regulate the activity of glycogen synthetase in the absence of hormonal influences. Ammonium ion inhibition of glycogen synthetase, by way of modification of metabolite concentration, is particularly interesting and worthy of further comment. If phosphofructokinase is
activated by ammonium ion, we may predict that the net result may be a compound one since not only will glucose-6-P decrease, but in addition, the concentration of fructose 1,6-diphosphate, which is a competitive inhibitor of glucose-6-P, may increase. Fructose 1,6-diphosphate alone can serve as an activator but which is a competitive inhibitor of glucose-6-P, may increase.

In short, the results of our experiments with glucose and ammonium ion addition to the incubation medium support the hypothesis that, in the absence of hormonally induced enzyme transformation, the level of cellular metabolites may regulate the activity of glycogen synthetase. Although the experimental conditions may seem extreme, modulations in the cell under normal conditions may very well regulate glycogen synthetase activity via changes in the relative levels of many more activators and inhibitors then we have as yet studied, e.g. Mg++, inorganic phosphate, and nucleotides other than ATP. The ratio of activators to inhibitors during different physiological states is likely to be a more important control factor than the absolute concentration of any particular metabolite.

Ammonia inhibition of glycogen synthesis in the tadpole liver presents us with an attractive hypothesis for explaining the hepatic glycogen deposition during metamorphosis. During spontaneous or thyroxine induced metamorphosis, the tadpole undergoes a transition from amnonateolism to ureotelm (30). It has been observed that hepatic glycogen deposition coincides with the time that the transition in nitrogen excretion occurs. Liver ammonia concentration, relatively high during the larval stage, is drastically reduced as urea cycle enzymes become operative. Since ammonia has such a profound effect on hepatic glycogen synthesis, it appears that the heavy deposition of liver glycogen may be the result of the switch in nitrogen metabolism rather than activation of glycogen synthetase by way of the pancreatic hormone. This possibility is being studied further. Moreover, the time that hepatic glycogen deposition occurs coincides with both the stage of maximum inertness of the pancreas, due to the developmental regression caused by thyroxine (20), and with the time when the urea cycle enzymes become functional.

To summarize, with the in vitro liver system we can discriminate between two mechanisms for the control of glycogen synthetase activity and study them in some detail. Insulin stimulates glycogen synthetase activity via the conversion of the less active enzyme form to the more active form, which has a lower K_m for the substrate, UDP-glucose, as well as other altered kinetic properties. This conversion facilitates the synthesis of glycogen. The second regulatory mechanism, metabolite control, does not involve the hormonally induced enzyme transformation. Modulations of the concentration of key metabolites and the ratio of activators to inhibitors may facilitate the synthesis of glycogen by the naturally occurring form of glycogen synthetase.

REFERENCES
Regulation of Hepatic Glycogen Synthetase of *Rana catesbeiana*: EFFECT OF INSULIN AND HYDROCORTISONE ON GLYCOGEN SYNTHETASE IN A LIVER SYSTEM IN VITRO, AND REGULATION OF GLYCOGEN SYNTHETASE BY CELLULAR METABOLITES

Lois M. Blatt, J. Sanders Sevall and Ki-Han Kim


Access the most updated version of this article at http://www.jbc.org/content/246/4/873

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/246/4/873.full.html#ref-list-1