Synthesis of Liver Glycogen in Starved Alloxan Diabetic Rats

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SUMMARY

Alloxan diabetic rats are considered unable to synthesize liver glycogen because of a low activity of glycogen synthetase. The present report shows, however, that livers of alloxan diabetic rats have activities of the I-form of glycogen synthetase intermediate between those of starved normal or adrenalectomized rats, and can synthesize substantial amounts of liver glycogen after refeeding of a chow diet or administration of hydrocortisone. Neither glycogenic procedure increased the activity of glycogen synthetase in livers of starved alloxan diabetic rats. In contrast, both normal and adrenalectomized rats had increased activities of the I-form of glycogen synthetase three hours after refeeding.

After twenty-four hours of access to food, normal and adrenalectomized rats had liver glycogen contents similar to those found in nonstarved rats, and a markedly decreased activity of synthetase I. Alloxan diabetic rats refed for twenty-four hours also had lowered activity of synthetase I, but the concentration of glycogen was not higher than that found after three hours of refeeding.

These results show that livers of alloxan diabetic rats have sufficient glycogen synthetase activity to synthesize glycogen rapidly, but the ability to accumulate larger amounts of glycogen is limited by an alteration in the control of glycogen synthetase activity by the content of liver glycogen. Also, the effect of hydrocortisone on the activity of glycogen synthetase in normal or adrenalectomized rats is the result of insulin release; however, not all the changes in glycogen metabolism produced by adrenalectomy can be explained by insulin deficiency. DIABETES 19:916-23, December, 1970.

Livers of alloxan diabetic rats have been reported to incorporate less C-14-glucose into glycogen than those of normal rats. It is generally accepted that glycogen synthetase is the enzyme affected, since administration of insulin to diabetic rats produces an increase in glycogen associated with increased activity of glycogen synthetase. Although the activity of glucokinase is low in livers of diabetic rats, the increase in activity of this enzyme produced by insulin occurs later than the rise in glycogen.

Administration of adrenal glucocorticoids also increases the activity of the I-form of glycogen synthetase in livers of rats and mice. Kreutner and Goldberg found that hydrocortisone did not increase the activity of glycogen synthetase in livers of adrenalectomized-alloxan diabetic rats. They concluded that the glucocorticoid effect on hepatic glycogen metabolism was the result of insulin release, a finding that is confirmed in the present report. Mersmann and Segal reported that livers of starved adrenalectomized rats have a low activity of the glycogen synthetase activating system, an enzyme which converts synthetase D to synthetase I in concentrated liver homogenates. This activity could be restored by administration of hydrocortisone. Homogenates of livers from nonfasted adrenalectomized rats, however, rapidly converted synthetase D to synthetase I. This observation suggests that some of the effects of adrenalectomy on glycogen metabolism are the result of changes in release of insulin by the pancreas.

Although insulin appears necessary for the deposition of liver glycogen by its effects on glycogen synthetase, several studies suggest that synthesis of glycogen can occur in the absence of insulin. Friedman et al. used glucagon to deplete glycogen from livers of alloxan diabetic rats and found that glycogen was resynthesized from glucose at a rate similar to that of normal rats. Administration of insulin increased the final concentration of glycogen, but not the rate of synthesis. Also, Tarnowski et al. and Long and Smith found that administration of hydrocortisone to starved adrenalectomized-alloxan diabetic rats resulted in increased liver glycogen.

The experiments reported here show that livers of starved alloxan diabetic rats have sufficient glycogen synthetase activity to accumulate glycogen rapidly after refeeding or the administration of hydrocortisone. It is
suggested that insulin deficiency does not affect primarily the activity of glycogen synthetase, but lowers the maximal amount of glycogen stored. This results from an alteration in the relationship between the tissue glycogen content and the activity of glycogen synthetase.

METHODS

Male Sprague-Dawley rats (Holtzman strain) were given alloxan monohydrate (40 mg./kg., I.V., Nutritional Biochemical Corp.) twenty-four hours after withdrawal of food. Alloxan-treated rats were used either two days or three weeks after the injection of alloxan. Rats used two days after alloxan were not given food prior to the experiment to avoid a fast-feed-fast protocol. Rats kept for three weeks after injection of alloxan were divided into three groups: 1. Rats not treated further prior to starvation. More than 50 per cent of this group died and surviving rats had a negligible increase in body weight. 2. Rats given daily injections of Protamine Zinc Insulin (4 units per day, S.C.). These rats gained weight normally until insulin injections were stopped seven days prior to use. At this time a rapid weight loss of 20 to 30 gm. occurred. 3. Rats adrenalectomized on the day of the last injection of insulin. Adrenalectomies were done on both normal and insulin-maintained alloxan-treated rats anesthetized with ether. These rats were given 1 per cent NaCl for drinking water and used five to seven days after removal of adrenal glands.

Two glycogenic procedures were used: 1. Refeeding of a chow diet (Ralston-Purina Co.) after a prolonged fast. 2. Injection of hydrocortisone (5 mg./kg., S.C.) (Solu-Cortef, Upjohn Co.). With the exception of adrenalectomized-alloxan diabetic rats, rats refed the chow diet for three hours were starved for the previous sixty-nine hours. Animals refed for twelve or twenty-four hours had appropriately shorter periods of starvation. Adrenalectomized-alloxan diabetic rats were starved only forty-five to forty-eight hours because they did not survive longer periods of food withdrawal.

Hydrocortisone was injected three hours prior to excision of the liver sample. Rats given alloxan two days previously were starved for sixty-nine hours as in the refeeding procedure. All other rats given hydrocortisone were fasted for twenty-one hours.

Rats were anesthetized with Na phenobarbital (200 mg./kg., I.P.). Body temperatures of anesthetized rats were maintained by thermostatically controlled heating lamps. Samples of the ventral medial lobe of the liver were excised and frozen in Freon-12 cooled to its melting point with liquid nitrogen. Tissue samples were stored at —80°, and weighed at —20° prior to extractions.

Activity of glycogen synthetase was measured with (total activity) and without (1-form) added glucose-6-P (10 mM). The production of UDP from UDP-glucose was determined by the two-step procedure described previously with the following modifications. Glycylglycine-NaOH buffer (pH 8.0) was used in both substrate mixtures, and the concentration of glycogen acceptor was increased from 10 mM to 50 mM (glucose residues). The incubation temperature was 37°. Sodium sulfite was added to the substrate mixture without glucose-6-P to protect glycogen synthetase against heat inactivation. This addition was not necessary when glucose-6-P was present.

Activity of glycogen phosphorylase was determined in the direction of glucose-1-P formation with incubation at 37°. The reaction mixture contained AMP (0.5 mM), glycogen (50 mM) and orthophosphate...
LIVER GLYCOGEN IN DIABETIC RATS

(50 mM) in a volume of 0.1 ml. with extract equivalent to 0.1 mg. of liver. The reaction was stopped with HCl and diluted 10-fold with 50 mM tris acetate buffer (pH 7.6) containing NADP (0.01 mM) prior to addition of phosphoglucomutase (5 μg.) and glucose-6-P dehydrogenase (1 μg.).

The concentrations of glucose-6-P, glucose and UDP-glucose in the liver samples were estimated by established fluorometric methods.25,26 Glycogen was isolated from samples of liver by the procedure of Hassid and Abraham.27 After hydrolysis in HCl, glucose was determined in a spectrophotometer with hexokinase (2 units/ml.) and glucose-6-P dehydrogenase (1 unit/ml.).

Rabbit liver glycogen was obtained from Mann Research Labs. UDP-glucose was obtained from Calbiochem. UDP-glucose dehydrogenase was supplied by the Sigma Chemical Co. and tested for limitations noted previously.15 Other substrates and enzymes were obtained from the Boehringer-Mannheim Corp.

RESULTS

Activities of glycogen synthetase and phosphorylase and concentrations of associated metabolites in livers of diabetic rats: Rats given alloxan two days previously and adrenalectomized-allowan diabetic rats had activities of the I-form of glycogen synthetase one half those found in livers of starved normal rats (tables 1 and 2), but higher than the activity of synthetase I found in livers of adrenalectomized rats (11 ± 2 mmoles/kg/hr., figure 1). Total glycogen synthetase activity and the activity of glycogen phosphorylase were similar to values for normal rats. The concentration of UDP-glucose was elevated in livers of both two-day-alloxan diabetic and adrenalectomized-alloxan diabetic rats (tables 1 and 2), and was comparable to that found for adrenalectomized rats8,15,28 (figure 2).

The two groups of diabetic rats had markedly different concentrations of glucose and glycogen (table 2). Rats used two days after alloxan treatment had elevated tissue glucose, but a normal concentration of glycogen even though they had been fasted for seventy-two hours. In contrast, adrenalectomized-alloxan diabetic rats were hypoglycemic and had concentrations of glycogen similar to those of adrenalectomized rats (figure 2).16

The insulin-maintained alloxan diabetic rats were used seven days after insulin injections were stopped.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Glycogen Synthetase</th>
<th>Phosphorylase</th>
<th>Glucose</th>
<th>Glycogen</th>
<th>UDP-glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>without G-6-P</td>
<td>with G-6-P</td>
<td>+AMP</td>
<td>mmoles/kg. * hr⁻¹</td>
<td>mmoles/kg. * hr⁻¹</td>
</tr>
<tr>
<td>Alloxan—2 days</td>
<td>37± 6*</td>
<td>170±11</td>
<td>24±5* (20)</td>
<td>97±15 (6)</td>
<td>13.5 ±1.5* (16)</td>
</tr>
<tr>
<td>72 hours starved</td>
<td>39± 8</td>
<td>148±17</td>
<td>33±8 (11)</td>
<td>—</td>
<td>15.7 ±1.2 (11)</td>
</tr>
<tr>
<td>+ 3 hours chow refed</td>
<td>52± 8</td>
<td>159±19</td>
<td>32±3 (8)</td>
<td>54± 8f (11)</td>
<td>11.2 ±2.4 (8)</td>
</tr>
<tr>
<td>Alloxan—3 weeks</td>
<td>63± 5</td>
<td>307±63*</td>
<td>24±4* (5)</td>
<td>95±10 (5)</td>
<td>11.5 ±2.3* (5)</td>
</tr>
<tr>
<td>Insulin maintained</td>
<td>85±16</td>
<td>239±26</td>
<td>39±9 (5)</td>
<td>80±15 (5)</td>
<td>16.4 ±1.4 (5)</td>
</tr>
<tr>
<td>24-72 hours starved</td>
<td>109± 9f</td>
<td>241±13</td>
<td>50±5f (11)</td>
<td>47± 4f (11)</td>
<td>16.4 ± .8 (6)</td>
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<tr>
<td>+ Hydrocortisone</td>
<td>36± 6*</td>
<td>145±9</td>
<td>25±5* (11)</td>
<td>120±10 (11)</td>
<td>2.95 ±1.1* (11)</td>
</tr>
<tr>
<td>5 mg./kg., 3 hours</td>
<td>39± 7</td>
<td>150±11</td>
<td>27±6 (8)</td>
<td>84±14t (8)</td>
<td>10.7 ±1.8t (8)</td>
</tr>
<tr>
<td>+ Adrenalectomy</td>
<td>45±10</td>
<td>189±30</td>
<td>24±4 (8)</td>
<td>72±12t (8)</td>
<td>3.27 ± .38 (4)</td>
</tr>
</tbody>
</table>

Values are averages ± S.E.M. The numbers of rats per group are in parentheses. *p < .01 for difference from the values of starved normal rats. 1p < .05 for difference from the appropriate control value. 2p < .01 for difference from the appropriate control value.
It is probable that this group of animals was less diabetic than the two previous groups since they were selected by survival without insulin but with intact adrenal glands. Insulin-maintained alloxan diabetic rats differed from two-day-alloxan diabetic rats and adrenalectomized-alloxan diabetic rats in the activity of both I-form and total glycogen synthetase (table 2). The activity of synthetase I was similar to that found in livers of normal rats, whereas the total glycogen synthetase activity was higher. The concentration of UDP-glucose was not elevated in livers of insulin-maintained alloxan diabetic rats; however, the concentration of glycogen was higher than that found in livers of starved normal rats (table 2).

The concentration of glucose-6-P in livers of all three groups of diabetic rats was not different from that of normal rats (30-40 μmoles/kg).

Glycogen synthesis after refeeding: Three hours after refeeding of a chow diet, the concentration of glycogen in livers of normal rats was increased. The activity of synthetase I was not elevated in these livers, but the per cent of glycogen synthetase in the I-form was increased due to a variable fall in the total glycogen synthetase activity (table 1 and figure 1). Whether this change is indicative of a physiological increase in glycogen synthetase activity is difficult to determine. Refeeding did not consistently decrease the concentration of UDP-glucose in livers of normal rats (table 1 and figure 2).

All three groups of alloxan diabetic rats had higher concentrations of liver glycogen three hours after refeeding the chow diet. This increase was not associated with a change in activity of synthetase I or total glycogen synthetase, or the concentration of UDP-glucose (table 2). The amount of glycogen accumulated was related to the initial activity of synthetase I. Two-day-alloxan diabetic rats and adrenalectomized alloxan diabetic rats synthesized one half the amount of liver glycogen made by normal rats or the insulin-maintained alloxan diabetic rats (table 2).

Glycogen synthesis after the administration of hydrocortisone: Administration of hydrocortisone to normal rats increased the concentration of liver glycogen. This change was associated with an increase in per cent synthetase I and a decrease in UDP-glucose (table 1). The increase in glycogen produced by hydrocortisone in two-day-alloxan diabetic rats was equivalent to that found in liver of normal rats (table 2). Although the concentration of UDP-glucose was decreased, there was no change in the activity of synthetase I. The decrease in UDP-glucose would suggest that glycogen synthetase
was activated in situ; however, the concentration of glucose-6-P, a known activator of glycogen synthetase, was not increased after hydrocortisone (data not shown).

In contrast, insulin-maintained alloxan diabetic rats had an increased activity of synthetase I and a decreased concentration of UDP-glucose, but the hepatic glycogen content was not elevated significantly (table 2). Adrenalectomized-alloxan diabetic rats did not have an increased concentration of glucose or glycogen after administration of hydrocortisone in the three-hour period studied (table 2).

Administration of hydrocortisone lowered the activity of glycogen phosphorylase in livers of all three groups of alloxan-treated rats. It is apparent that the activity of this enzyme does not correlate well with the ability of the liver to accumulate glycogen.

Activity of glycogen synthetase and concentration of associated metabolites at several times after refeeding: Determinations were done on livers of normal rats, adrenalectomized rats, and rats kept for three weeks after alloxan treatment at several times up to twenty-four hours after refeeding of a chow diet (figures 1, 2 and 3). Three hours after refeeding, normal rats had an increased per cent synthetase I, elevated glycogen and decreased UDP-glucose. Glucose-6-P was elevated slightly (figures 1 and 2). After twelve and twenty-four hours of refeeding, the concentration of liver glycogen was similar to, or higher than, the concentration of glycogen found in livers of nonstarved rats (300 mmol/kg). At both times, the activity of synthetase I was decreased markedly and UDP-glucose was higher than values found for starved normal rats (figures 1 and 2).

Starved adrenalectomized rats had less liver glycogen than normal rats, a low activity of synthetase I and elevated UDP-glucose as reported previously. Three hours after refeeding, the activity of synthetase I was increased and the concentration of UDP-glucose decreased (figures 1 and 2). Liver glycogen was elevated, but the increase was less than that found in normal rats. The changes in glycogen synthetase and UDP-glucose also were found twelve hours after refeeding adrenalectomized rats, possibly because the concentration of glycogen was not as high as that in livers of normal rats at the same time after refeeding. No further rise in the glycogen content was found after twenty-four hours of refeeding, and the activity of synthetase I and concentration of UDP-glucose were similar to initial values.

Three hours after starved three-week-alloxan diabetic rats were refed, the concentration of liver glycogen was increased without a change in activity of glycogen synthetase or concentration of UDP-glucose (figure 3). The glycogen content was only slightly higher after twenty-four hours of refeeding even though the concentration of glucose remained elevated. At this time, the activity of the I-form of glycogen synthetase was decreased markedly; but, in contrast to the livers of refed normal rats, UDP-glucose was not increased.

**DISCUSSION**

The findings indicate that livers of starved alloxan diabetic rats have sufficient activity of the I-form of glycogen synthetase to synthesize glycogen rapidly when substrate is made available by refeeding or by administration of hydrocortisone. It is apparent also that livers of diabetic rats are limited in the accumulation of glycogen by their inability to increase synthetase I activity in response to a glycogenic stimulus.

The differences between groups of alloxan diabetic rats in activity of the I-form of glycogen synthetase and concentration of UDP-glucose may be related to the severity of the diabetic states. Two-day-alloxan diabetic rats and adrenalectomized-alloxan diabetic rats are...
probably more deficient in insulin than the alloxantreated rats maintained on insulin until seven days before use. Rats made severely diabetic by pancreatectomy,10 by administration of anti-insulin serum,31 or by treatment with alloxan22 do not live more than two or three days without insulin treatment or other procedures9 (i.e., adrenalectomy) which alleviate the marked changes in metabolism of carbohydrate and fat. Thus, decreased synthetase I activity and an elevated UDP-glucose are associated with marked insulin deficiency, whereas an increased total glycogen synthetase activity4 may indicate a milder diabetic state. It has been suggested that increased total enzyme activity represents synthesis of enzyme to compensate for the lack of insulin,8 but this interpretation does not seem likely.

The present data confirm reports of Friedmann et al.12,22 that livers of alloxan diabetic rats can rapidly resynthesize glycogen if the initial concentration of glycogen is decreased. In their studies, glycogen was lowered by administration of glucagon, and, in this report, by fasting. The increase in glycogen produced three hours after refeeding starved alloxan diabetic rats occurred without an increase in activity of synthetase I or concentration of glucose-6-P. However, the amount of liver glycogen accumulated after refeeding was less in the livers of two-day-alloxan diabetic rats and those of adrenalectomized-alloxan diabetic rats, and was related to the initial activity of synthetase I. Dewulf and Hers37 have suggested that glucose, and not insulin is the positive effector of increases in glycogen synthetase activity. This suggestion would not appear likely since refeeding produced a marked hyperglycemia in all three diabetic states studied without an increase in synthetase I.

Kreutner and Goldberg8 administered glucose to adrenalectomized-alloxan diabetic rats, fasted for only four hours, and did not find an increase in the content of liver glycogen. A possible explanation for the difference between their observations and the present ones could involve the dietary state of the animals used. It is apparent from the results of Friedmann et al.12 and those in figure 3 that diabetes produced by alloxan affects primarily the total amount of glycogen accumulated, and not so much the rate at which the accumulation occurs. Thus, Kreutner and Goldberg8 may not have observed an increase in glycogen in their alloxan diabetic rats because the glycogen content was maximal for that particular diabetic state due to the short period of fasting.

The concentration of glycogen in the liver appears to control its rate of synthesis by changing the activity of the I-form of glycogen synthetase. Dewulf and Hers20 have reported that high concentrations of glycogen inhibit the conversion of inactive glycogen synthetase to the active form in concentrated homogenates of liver. Control of the activity of glycogen synthetase by the content of tissue glycogen has been reported for brain,33 skeletal muscle,34 heart,35 and ascites tumor cells.36 Twenty-four hours after refeeding, the activity of synthetase I was lower than control values in livers of both normal rats and alloxan diabetic rats. This decrease occurred, however, at substantially lower glycogen concentrations in alloxan diabetic rats. This finding suggests that a primary action of insulin on the activity of glycogen synthetase is a change in the inhibitory capacity of glycogen for the glycogen synthetase activating system. That is, insulin allows higher activities of synthetase I for a given concentration of liver glycogen. This action is similar to that described by Danforth44 for the effect of insulin on the activity of glycogen synthetase in skeletal muscle. This hypothesis for insulin action would not exclude a separate effect of insulin to increase the activity of the glycogen synthetase activating system by increasing its concentration.5-37

It is unlikely that the activity of phosphorylase contributes to the low concentration of liver glycogen found in refed alloxan diabetic rats.5,22 Nonfasted alloxan diabetic rats have a lower concentration of glycogen and less phosphorylase activity than that found in livers of normal rats (data not shown). However, the initial increase in glycogen produced in alloxan diabetic rats by refeeding or administration of hydrocortisone could be affected by the variable decrease in phosphorylase produced by these glycogenic procedures. Bishop and Larner9 have reported a reciprocal relationship between changes in activity of phosphorylase and glycogen synthetase in dog liver. This relationship was not found in livers of alloxan diabetic rats. These observations suggest that the two enzymes do not necessarily have overlapping control systems.

The results obtained with administration of hydrocortisone to alloxan diabetic rats are in general agreement with the suggestion of Kreutner and Goldberg8 that the effects of hydrocortisone are the result of insulin release. Hydrocortisone increased the activity of synthetase I only in the insulin-maintained alloxan diabetic rats, the group probably the least deficient in insulin. The increases in hepatic glycogen found after
hydrocortisone administration to starved alloxan diabetic rats were associated with increased tissue glucose and are probably the result of increased substrate availability through gluconeogenesis. Other investigators, who found an increase in liver glycogen in adrenalectomized-alloxan diabetic rats, also observed increases in blood glucose which I did not.

Although the effects of hydrocortisone on the activity of glycogen synthetase appear to depend on insulin, it is unlikely that the changes in glycogen synthetase found after adrenalectomy are only the result of a relative insulin deficiency. Starved adrenalectomized rats have a lower activity of synthetase I (I ± 2 mmoles/kg.h.) than those found in livers of starved alloxan diabetic rats (52 ± 8 mmoles/kg.h.) (figures 1 and 3). Also, adrenalectomized rats synthesized more glycogen in twenty-four hours after refeeding than that observed for alloxan diabetic rats.

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REFERENCES


Sugar and Coronary Heart Disease

Cigarette smokers use more sugared hot beverages than nonsmokers, an observation which confounds a superficial association between sugar and coronary heart disease.

Among the nutritional diversions of the last decade has been the hypothesis, promulgated mainly by J. Yudkin (Yudkin and J. Morland, Amer. J. Clin. Nutrition 20:503, 1967), that dietary sugar is a major factor in the contemporary epidemic of coronary heart disease (CHD). Historically, and even today, nutrition has been susceptible to anecdotal or tenuously founded claims in regard to special (either beneficial or deleterious) properties of particular foods or nutrients—claims easily raised and publicized but rather more difficult to test in a rigorous manner. When coupled with CHD, a disease with a strong nutritional component but with the additional complex involvement of other host and environmental factors, one is faced with a very formidable combination indeed, for there is the continuing temptation to look for simplistic, single-factor solutions which will at once explain away the many apparent mysteries of atherosclerosis and its clinical complications.

The basis for the sugar-CHD link derived from the association between the parallel secular trends both in sugar consumption and in mortality from CHD over the past fifty years and was supported by comparisons of sugar use among survivors of myocardial infarction and controls (Yudkin and J. Roddy, Lancet 2:56, 1964). Recently, two carefully designed population studies have cast some doubt on the concept of a direct link and have suggested that cigarette smoking may be the operative intermediary factor. It should be pointed out that there still are inconsistencies in the epidemiologic evidence linking smoking to CHD (C. C. Seltzer, J. Am. Med. Assn. 203:193, 1968; Arch. Environ. Health 29:418, 1970).

One of these two newer reports (P. C. Elwood et al., Lancet 1:1014, 1970) is based on two community surveys. Among women, 2,834 of 3,149 subjects twenty through sixty-four years of age in a defined population group were questioned about age, body weight, sugar consumption, the symptoms of angina pectoris, and cigarette smoking habits. Smokers used more sugar per day than nonsmokers and women with angina pectoris used about 10 gm. per day more sugar than those without. The latter difference was not, statistically significant, nor was sugar usage consistently related to the amount of cigarette smoking. As a check on the reliability of the estimates of sugar consumption, 100 wom-