Liver Glycogen Synthesis in Intact Alloxan Diabetic Rats*

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It is now clearly established that insulin controls the utilization of glucose by muscle and adipose tissue (1-4), presumably by stimulating glucose transport across an otherwise impermeable cell membrane. Although a similar role of insulin in hepatic glucose uptake seems unlikely, since the liver cell is reported to be freely permeable to both inflow and outflow of glucose (5), there is nevertheless a considerable body of evidence pointing to marked impairment of hepatic glucose utilization in diabetes (6-10). The most impressive of this evidence stems from studies with rat liver slices in vitro (11-14), which demonstrated an almost complete suppression of the incorporation of C14-labeled glucose into the liver glycogen of alloxan diabetic rats. These results are presumed to be due to a block in glucose phosphorylation, since comparable effects are not observed with fructose-C14.

Our recent finding that hepatic glucokinase activity is low in alloxan diabetic rats (15) also argues in favor of a rate-limiting role of this enzyme in liver glycogen synthesis. However, hepatic glucokinase is also low in fasting (15), yet hepatic glycogen repletion occurs rapidly when carbohydrate is administered (16).

The suggestion has also been offered recently that a block in liver glycogen synthesis in diabetes may occur at the enzyme uridine diphosphate glucose-glycogen glucosyltransferase (17-20). In accord with an impairment of hepatic glycogen synthesis in diabetes, it is generally assumed that liver glycogen is low in this condition. However, a review of the available clinical and experimental data reveals that low hepatic glycogen levels in diabetes are the exception rather than the rule. Whereas liver glycogen levels may be somewhat low in the diabetic when compared with normal, fed animals, they are appreciably higher when compared in the fasted condition (21-27).

These conflicting views concerning the role of insulin lack in influencing liver glycogen deposition prompted us to make a further study of glycogen synthesis in intact, alloxan diabetic rats, and the results of this investigation are reported in this communication.

EXPERIMENTS AND RESULTS

By measuring rates of hepatic glycogen synthesis under conditions in which synthesis occurs rapidly in the normal animal, it should be possible to determine whether or not this process is limited in the diabetic. Cori and Cori showed many years ago (16) that the injection of glucose by stomach tube to previously fasted rats leads to prompt synthesis of liver glycogen, with reproducible yields of levels of 2 to 3% in about 4 hours. A search of the literature uncovered only one study in which intact normal and diabetic rats were compared with respect to the rate of synthesis of liver glycogen in response to glucose feeding. According to Longley, Bortnick, and Roe (28), depancreatized rats synthesized liver glycogen as rapidly as did normal rats over a 3- to 4-hour period after glucose feeding. In attempting to interpret results of this type of experiment one is faced with the fact that there are wide initial differences between normal and diabetic rats. Firstly, the already high blood glucose level in the latter would doubtlessly minimize any effect of exogenous glucose administration. Secondly, the already high initial hepatic glycogen level would further obscure comparisons based on differences between initial and final glycogen levels. The situation is set forth in the experiments summarized in Fig. 1. By means of the same procedure used in previous experiments (29) designed to measure rates of liver glycogen repletion in fasted rats, groups of normal and alloxan diabetic rats, matched for age at about 10 weeks and weight at about 200 g, were fasted for 24 hours and then given intragastrically an injection of 5 mmols of uniformly labeled glucose-C14 dissolved in 3 ml of water. Pairs of rats were killed at hourly intervals up to 4 hours, and liver glycogen was isolated as described previously (29).

The fasted normal rats, with initial blood glucose levels of about 70 mg per 100 ml, had an average liver glycogen level of 0.25% initially. Four hours after glucose feeding the average glycogen level was 3.4%, and on the average about 10% of the administered uniformly labeled glucose-C14 was incorporated therein. In contrast, the diabetic rats whose blood glucose levels were from 150 to 500 mg per 100 ml had an average initial liver glycogen level of 3.0%, which remained essentially unchanged 4 hours after glucose feeding, and only 0.4% of the administered dose of labeled glucose was incorporated.

Glycogen Synthesis after Glucagon Injection—These data thus confirmed in vivo the previous observations made in vitro by Spiro, Ashmore, and Hastings (14), Osborn, Felts, and Chaikoff (12), and others (see 6) pointing to a severely limited incorporation of glucose carbon into liver glycogen of alloxanized rats. At the same time, the high initial hepatic glycogen levels of these diabetic rats led us to consider the possibility that this in itself might be inhibitory to further glycogen synthesis. To test this hypothesis, means were sought to lower artificially the high liver glycogen level of the diabetic rats. Preliminary experiments indicated this could be done easily and conveniently by injection of small doses of glucagon (30). We were surprised to observe that whenever the liver glycogen level was lowered to essentially normal fasting levels by glucagon injection, the liver glycogen subsequently returned to its initial level, even

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Fig. 1. Changes in liver glycogen in 24-hour fasted normal and alloxan diabetic rats after the intragastric injection of 5 mmoles of uniformly labeled glucose-C<sup>14</sup> in 3 ml of water. Shaded bars depict data for normal, and clear bars, for diabetic rats. Ranges are indicated by vertical lines and the number of animals is given by the number in parentheses over each bar.

Fig. 2. Changes in liver glycogen of 24-hour fasted normal and diabetic rats during and after glucagon injection. Data are presented as in Fig. 1.

Fig. 3. Changes in liver glycogen of 24-hour fasted normal and diabetic rats. Conditions were exactly as in Fig. 2, except that 1 hour after the last glucagon injection, each rat was given intragastrically 5 mmoles of glucose in 3 ml of water.

without additional glucose administration, and this occurred about as rapidly as it did in fasted normal rats given glucose.

The results of a series of experiments of this type are summarized in Fig. 2. Groups of normal and diabetic rats were fasted for 24 hours; then each rat was given an intraperitoneal injection of glucagon in a dosage of 150 µg per kg, divided into four equal hourly doses. At intervals of 2 hours during, and at hourly intervals for 5 hours after glucagon injection, two or three rats of each group were killed and liver glycogen was determined. As reported previously by Cahill, Zottu, and Earle (30), the glycogen content decreased rapidly under this treatment. In the diabetic rats the level fell precipitously, from an average of 3% down to <0.1% in 4 hours; in the normal rats, from 0.25% down to 0.02%. In 1 hour after the last glucagon injection there was a strikingly rapid recovery in the hepatic glycogen of the diabetic rats, which continued until by the 5th hour the level reached 2.5%. These results thus leave no doubt of the capability of the diabetic rat liver to replete its liver glycogen rapidly when it is previously depleted to normal fasting levels. Some “rebound” in the liver glycogen of the normal rats also followed depletion with glucagon, presumably owing to the transient increase of blood glucose which follows glucagon treatment, but it never reached the levels observed in the diabetic rats.

To compare the normal and diabetic rats under conditions of optimal glycogen repletion in normal rats, the same experiment was performed, but 5 mmoles of glucose were given to all rats at the 4th hour of glucagon treatment. As seen in Fig. 3, the rise in liver glycogen in the diabetic rats kept pace with that of the normal animals for at least 3 hours, and even after 5 hours the liver glycogen levels in the normal animals, at 2.5 to 3%, were not markedly higher than those in the diabetic animals, at approximately 2%.

Liver Glycogen Synthesis from Glucose-1-C<sup>14</sup>—To probe somewhat more deeply into the origin of the liver glycogen under the conditions described, glucose-1-C<sup>14</sup> was utilized in similar experiments. With the injection of this material, not only could information be obtained on the extent of total glucose incorporation into liver glycogen, but also, by measuring the degree of transformation of glucose carbon 1 to carbon 6, an idea could be had of the extent of incorporation into blood glucose and liver glycogen of glucose “resynthesized” from 3 carbon breakdown products.

It has been shown previously that when 3 carbon glucose precursors labeled in carbon 2 or 3 are administered to rats, the label becomes nearly completely randomized between carbons 1, 2, 5, and 6 of the newly synthesized liver glycogen. This is due to breakdown to 3 carbon compounds, followed by obligatory participation during gluconeogenesis of the 4 carbon acids of the citric acid cycle, owing to the physiological irreversibility in liver
of pyruvate kinase (31-33). Since glucose-1-C\textsuperscript{14} should yield pyruvate-3-C\textsuperscript{14}, by examining the liver glycogen for the presence of label in certain positions other than carbon 1, for example in carbon 6, one can approximate the extent of glucose resynthesis from glucose-1-C\textsuperscript{14}-derived 3 carbon compounds (33, 34). Normal and diabetic 24-hour fasted rats were given glucagon to deplete liver glycogen as in the experiments described above, and 4 hours later they were given "trace" doses of glucose-1-C\textsuperscript{14}. As before, pairs of rats were killed at hourly intervals, and the isolated liver glycogen was weighed, its total radioactivity was determined, and it was degraded chemically for determination of radioactivity in carbon 6. At the same time, samples of blood were assayed for glucose level and for total and C-6 radioactivity in the glucose in a similar manner.

The results of this experiment are shown in Table I, Experiment 1. In the normal fasted rats, the blood glucose level remained relatively constant at between 65 and 88 mg per 100 ml whereas the total radioactivity declined with an approximate half-time of about 1 hour. In accordance with the data in Fig. 2, the liver glycogen level rose moderately and the radioactivity incorporated increased regularly, from 2.4\% at 1 hour to 11.5\% of the administered dose at 5 hours. The degree of labeling in C-6 of both the blood glucose and the liver glycogen was surprisingly high. For the blood glucose the proportion of radioactivity in C-6 reached a relatively constant level in 2 hours of about 13\%, and the liver glycogen likewise had about 11 to 13\% of the total activity in C-6 throughout most of the experimental period.

In the diabetic animals, the blood glucose levels were in the range of 232 to 287 mg per 100 ml. Because of their larger glucose pool, the radioactivity levels were lower initially, and decreased at a lower rate. Also, the rate of replacement of C-1-labeled glucose by randomly labeled glucose was lower; the proportion of C-6-labeled glucose rose gradually from 2.9 to a maximum of about 10\% by the 4th hour. This behavior is to be expected from the larger pool size and consequent lower percentage turnover rate.

The net increase of total liver glycogen following depletion by glucagon in the diabetic animals followed a pattern similar to that seen in Fig. 2. By 2 hours the liver glycogen level formed a plateau at 1.7\%, at which point the incorporation of radioactivity also reached a plateau at about 11 to 13\% of the injected dose. The proportion of radioactivity in C-6 of the liver glycogen was similar to that in the liver glycogen of the normal animals, at about 10\%.

Experiment 2, Table I, was carried out exactly as Experiment 1 except that the glucose-1-C\textsuperscript{14} was administered in a total of 5 mmoles of glucose by stomach tube. As expected, this procedure chiefly affected the normal animals. The blood glucose levels were higher and specific activities lower than in those given the
trace dose, and the liver glycogen level reached 3% in 5 hours. However, the incorporation of label into total liver glycogen and the proportions in C-6 were not appreciably different. In the diabetic rats, the blood glucose levels were, of course, much higher than in those given the trace dose of glucose-1-C\textsuperscript{14}, and the specific activities were also much lower, thus resulting in a lowered incorporation of radioactivity in the liver glycogen. However, the levels of liver glycogen, as well as the proportions therein of labeled carbon in C-6, were not significantly different from those given the trace dose.

These data reveal that in both normal and diabetic rats there is an appreciable breakdown and resynthesis of glucose from smaller fragments. It is difficult to estimate the rate of this process from the data obtained,\textsuperscript{1} but it can be estimated roughly that in both the diabetic and the normal rats, approximately equivalent quantities of an injected glucose dose are deposited in the liver glycogen. The kinetics of the incorporation of radioactivity from C-1 into C-6 is far too complicated for accurate estimation of the resynthesis rate, but from previous considerations (33, 34) it can be assumed that an appreciable quantity, possibly 30 to 50%, of the liver glycogen has been derived from "resynthesized" glucose.\textsuperscript{2} The data give no indication of any important differences between the normal and diabetic animals either in the degree of incorporation of glucose into liver glycogen or in the proportions of directly introduced and resynthesized glucose in the liver glycogen.

Glycogen Synthesis in 48-Hour Fasted Rats—To rule out any remote possibility that the rapid synthesis of hepatic glycogen was somehow attributable to the glucagon used for depletion of liver glycogen, attempts were made to deplete the glycogen by other means before comparing glycogen repletion. Epinephrine was tried, but did not yield entirely consistent results. However, some success was achieved by starvation for 48 hours. In conformity with previous reports (38–40), we found that when alloxan diabetic rats were fasted 48 hours (the animals will not survive longer fasts) there was a dramatic drop in blood sugar to almost normal values, and urine glucose excretion virtually ceased. When a high carbohydrate diet was refed to such animals, the blood sugar rose rapidly and in 4 hours was back to the normal diabetic level of approximately 300 mg per 100 ml. Simultaneously there was a resumption of polyuria and glycosuria. The course of liver glycogen levels during periods of fasting for 48 hours and refeeding of normal and alloxan diabetic rats is depicted in Fig. 4.

The normal animals, on fasting, showed a progressive decrease in hepatic glycogen which was already noticeable within 1 to 8 hours and reached a minimal level in 24 hours. After resumption of food at 48 hours, the level quickly rose, and reached a maximum in 24 hours. In contrast, the diabetic rats did not lose much liver glycogen in 24 hours of fasting, but it did drop to near normal fasting values in 48 hours. In 4 to 8 hours after glucose feeding, the liver glycogen again rose steeply, almost keeping pace with that of the normal rats, and leveled off when the values were over 2%. These data confirm the previous experiments with glucagon treatment and again demonstrate the capability of the liver of the alloxan diabetic rat to synthesize glycogen when this substance is previously depleted.

Relation of Blood Glucose to Liver Glycogen—The high blood glucose level of the alloxan diabetic rat suggests that this may be a contributing factor in the high liver glycogen of these animals. Such a hypothesis would be in agreement with recently reported properties of hepatic glucokinase. This enzyme has a Michaelis constant for glucose of 0.01 to 0.02 M (41), a value of such magnitude that in the permeable liver cell, the activity of this enzyme would be highly dependent on the blood glucose levels. At the same time, the activity level of the enzyme is considerably lower than normal in diabetes (15). Under these conditions, the high blood glucose level might overcome a lower glucokinase activity as well as an elevated hepatic glucose 6-phosphatase activity (42) to maintain a high liver glycogen level. However, no relationship was found between blood glucose levels and liver glycogen levels. Fig. 5 shows values for blood glucose plotted against the corresponding liver glycogen levels in 37 24-
degradation. This low value is somewhat misleading, however, because most of the 3 carbon units resynthesized to glucose follow a pathway via 4 carbon acids, which results in redistribution of glucose carbon 1 essentially equally among carbons 1, 2, 5, and 6 (33). Thus the maximal incorporation in carbon 6 resulting from breakdown and resynthesis of glucose would only be about 25%. To obtain a valid estimate of the proportion of "recycled" glucose molecules in the liver glycogen, the percentage of the total activity found in carbon 6 should be multiplied by 4. It is on this basis that we assume that from 30 to 50% of the liver glycogen arose by resynthesis from 3 carbon precursors.
hour fasted rats. The lack of correlation is immediately evident, and may be further documented by pointing out that the four rats with lowest liver glycogens, <1.5%, had blood glucose values ranging all the way from 120 to 330 mg per 100 ml, and that the seven animals with lowest blood glucose, <120 mg per 100 ml, had liver glycogen levels ranging from 1% to 4%.

METHODS

Alloxan diabetes was produced in the rats by a single subcutaneous injection of recrystallized alloxan at a dosage of 140 mg per kg in 48-hour fasted young 100- to 120-g male rats of the CFN strain, obtained from Carworth Farms. They were used only after an interval of at least 4 weeks, when they displayed severe polyuria, glucosuria, and blood glucose levels of >400 mg per 100 ml. Animals were killed by decapitation and blood was collected in an open polyethylene beaker. Liver glycogen was isolated by the method of Boxer and Stetten (43) and was purified by two successive dissolutions in 5% trichloroacetic acid and precipitation with 2 volumes of ethanol. The final solution was made in water and, after precipitation with 2 volumes of alcohol, the pure white, granular material was dried with acetone and weighed. Blood and urine glucose were determined by the method of Seifter et al. (44). Separation of carbon 6 of the blood glucose from carbons 1 to 5 and determination of their radioactivities by means of periodate oxidation were carried out exactly as described previously (33, 45). To carry out the separate radioactivity determinations on carbons 1 to 5 and carbon 6 of liver glycogen, the procedure was modified as follows. Glycogen (10 mg) was hydrolyzed by heating at 100° for 2 hours in 3 ml of 0.3 M H2SO4 and ions were removed by passage of the cooled solution through a double bed ion exchange resin (MB-3 Dualite resin obtained from Rohm and Haas, Philadelphia). The resultant solution was used in the periodate procedure without further treatment.

Uniformly labeled glucose-C14 and glucose-1-C14 were purchased from the Volk Radiochemical Company, Chicago; and glucagon, Lot 25%234B-167-1, was generously provided by the Eli Lilly Laboratories.

DISCUSSION

In a brief communication, Longley et al. (28) reported that when fasted depancreatized rats were given an intraperitoneal injection of glucose at a dosage of 2 g per kg of body weight, the initial liver glycogen level of 0.8% was approximately doubled in 3 hours. Although it started at a lower initial level, the liver glycogen of normal rats rose at about the same rate as it did in the diabetic rats. Longley et al. concluded that the processes of liver glycogen synthesis are not directly impaired in diabetes. Allowing for differences in experimental approach, the results of the present study lead to essentially the same conclusion.

Although our present findings may appear at first glance to be at variance with the large body of liver slice data, they are, in fact, in complete accord in demonstrating that there is ordinarily very little exchange between the blood glucose and the liver glycogen of the alloxan diabetic rat. It is only when the hepatic glycogen stores are depleted that the unimpaired capability for glycogen synthesis is displayed by the liver of the alloxan diabetic rat. The essential difference between the normal and the diabetic animal evidently lies less in the intrinsic enzymatic equipment for glycogen synthesis than in the inability of the "diabetic" liver to alter its glycogen content either up or down once it has reached the characteristic intermediate level of approximately 2 to 3%. Under similar conditions of hyperglycemia the normal rat will readily incorporate glucose carbon into the liver glycogen (29) and may accumulate as much as 10% of its liver weight (Fig. 5). That this sluggishness of response to hyperglycemia is a specific feature of the diabetic state has been demonstrated strikingly by Steiner et al. (17, 18), who, on injection of insulin into alloxan diabetic rats, observed a sharp rise in liver glycogen from an initial level of 2% to 11% in 14 hours.

The marked impairment of glycogen synthesis in diabetic rat liver slices (11-14, 17-19) is associated with decreases in utilization of glucose for fatty acid formation and for oxidation to CO2, and for this reason is generally attributed to a lowered activity of hepatic glucokinase. A low level of this enzyme in diabetic rats has indeed been confirmed by means of direct assays, however, the activity is by no means negligible.

Studies from our laboratory (15, 41), supplemented by a recent preliminary communication by Vifuela, Salas, and Sols (46), demonstrated the presence of two glucokinases in rat liver: a relatively specific enzyme with a low affinity for glucose, which is normally preponderant but drops markedly during fasting and diabetes; and a less specific enzyme with a high affinity for glucose, which is normally present at low levels and which remains essentially unchanged in these conditions. These studies demonstrated the presence of this low Km glucokinase in diabetic rat liver in sufficient activity to account for the phosphorylation of at least 0.5 amole of glucose per g of tissue per minute at 37°. This activity would allow the synthesis of liver glycogen at a rate of approximately 0.5% per hour and thus can account for the accumulation of at least 2% in 4 hours. This is still somewhat below the observed accumulation of 2 to 3% in 4 hours. However, the data of Table I make it clear that a relatively large proportion, perhaps as much as 30 to 50%, of the glycogen

3 In a preliminary communication, D. G. Walker has reported the presence of what appear to be the same two glucokinases in guinea pig liver (47).
resynthesized after depletion with glucagon has been rebuilt from smaller fragments. Since resynthesis occurs predominantly in the liver, it seems likely that a large proportion of the resynthesized hexose molecules, in the form of glucose-6-P, goes directly to glycogen and thus bypasses the hepatic glucokinase.

Other recent data also point to possible enzyme defects in hepatic glycogen synthesis in diabetes. The observations of Leloir and Goldemberg (17) indicate that glucose-6-P is a coenzyme for UDP-glucose-glycogen glucosyltransferase and thus plays a special role in glycogen synthesis, aside from its role as a carbon source. Larner (20) suggested (a) that this enzyme exists in two forms, one of which requires glucose-6-P for activity, and (b) that this form is converted to the other by insulin. Steiner et al. (18) found a striking increase of both forms of this enzyme after insulin injection in alloxan diabetic rats, paralleling glycogen buildup (however, they failed to observe a clear-cut relationship between insulin action in diabetic rats and the hepatic glucose-6-P level (17, 18)). Despite these indications of enzymatic barriers to liver glycogen synthesis in diabetes, the fact that glycogen synthesis does proceed readily under the circumstances described discounts an enzymatic impairment as a rate-controlling factor.

It is not yet clear why the diabetic rat liver maintains a high glycogen level. As shown in Fig. 4, the liver glycogen of the alloxan diabetic rat does not drop as rapidly on fasting as that of the normal rat, although it will be nearly depleted eventually, after 48 hours of fasting. These observations point to a lowered activity of liver phosphorylase, probably owing to a deficiency of the a form since it is readily overcome by glucagon. Although there is no clear evidence of a lack of glucagon secretion in alloxan diabetes, Fox, Galansino, and Pozza (48) have suggested that hypoglycemia may be the necessary stimulus for glucagon secretion, a condition that would not occur in the alloxan diabetic rats. Another possibility may be envisioned by the finding of Tybergein, Tomizawa, and Williams (49) that a minute quantity of glucagon, which is far too small to elicit glycogenolysis in rabbit liver slices, will do so in the presence of insulin.

The present results, when considered in the light of the foregoing discussion, suggest that insulin lack does not directly affect rate-controlling enzymes of glycogen synthesis or break-down, but rather acts somehow on the mechanism of glycogen storage. This process undoubtedly is closely associated with the internal cell structure. It is now evident that the cell membrane is a continuum extending from the nucleus throughout the cytoplasm to the external surface. If one recognizes that insulin acts on the external membrane of the muscle cell, a similar action on internal membranes of the liver cell may be envisioned, somehow controlling the proper storage and mobilization of liver glycogen.

SUMMARY

When glucose-C¹⁴ is injected into intact, 24-hour fasted, alloxan diabetic rats, there is very little incorporation of labeled carbon into liver glycogen, and the glycogen level, already at 2 to 3%, does not increase. However, when hepatic glycogen was depleted by prior injection of glucagon, resynthesis occurred promptly, to reach levels of 2 to 3% in about 4 hours, even without external glucose administration. Whether or not glucose is administered, glycogen repletion occurs in alloxan diabetic rats, about as rapidly as in normal, 24-hour fasted rats given glucose. Similar glycogen repletion patterns are observed after glucose feeding to alloxan diabetic rats partially depleted of liver glycogen by prolonged fasting.

When glucose-¹⁴C is given to glucagon-treated alloxan diabetic rats, the liver glycogen level rises to about 1 to 2% in 2 hours, and as much as 13% of the total radioactivity is incorporated therein. In 5 hours, the C¹⁴ incorporation is 5 to >13% of the injected dose, of which approximately 10% is in carbon 6 of glycogen glucose. These data indicate that the alloxan diabetic rat has the same enzymatic capability for hepatic glycogen synthesis as normal, fasted rats, and that in both diabetic and fasted animals a large proportion, possibly as much as 40%, of the newly formed glycogen has been resynthesized from smaller fragments.

Previous failure to observe incorporation of glucose carbon into glycogen of liver slices is presumed to be due to the already high liver glycogen of alloxan diabetic rats.

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