Rat liver glycogen synthase shows almost a 2-fold increase in activity 8 days after onset of alloxan diabetes. Immunological and catalytic criteria indicate that the increase in activity is associated with an increase in the amount of enzyme in the diabetic. Apparent rates of degradation were determined for isolated glycogen synthase and phosphorylase from the livers of 2-, 5-, and 8-day diabetic, insulin-treated diabetic and normal rats using the double isotope \(^{[3]H}\)leucine and \(^{[14}C\)leucine labeling method (Arias, I. M., Doyle, D., and Schimke, R. T. (1969) J. Biol. Chem. 244, 3303-3315). Relative rates of enzyme synthesis and degradation were determined by comparing the \(^{3}H\)/\(^{14}C\) ratios of the isolated enzymes to the isotope labeling of a liver fraction representing the average of liver proteins. Glycogen synthase showed a gradual increase in the rate of degradation through the course of diabetes with the average relative rate of degradation in the 8-day diabetic 1.8 times greater than the normal. The relative rate of synthesis for glycogen synthase in the diabetic was 2.2- to 2.5-fold greater than the normal. Phosphorylase from 5- and 8-day diabetic rats had relative rates of degradation 4.0-5.3 times greater than that of the normal. In the diabetic, the relative rate of degradation of phosphorylase was greater than for synthase while the opposite was observed in the normal rat. The relative rate of synthesis for phosphorylase from diabetic rats was approximately 4.5-fold greater than normal. The increased concentration of glycogen synthase in the diabetic liver is because of an increased rate of synthesis and not a decreased rate of enzyme degradation.

The activity of rat liver glycogen synthase can be controlled by hormonal factors that change the phosphorylation state of the enzyme (1, 2). The liver from diabetic rats shows that total glycogen synthase activity is greater than normal; however, the enzyme exists almost completely in the physiologically inactive \(b\) form (3-7), suggesting that the level of synthase \(b\) as substrate for the interconverting enzymes is under hormonal control as well. Glycogen synthase from the diabetic rat showed identical immunochemical properties as normal enzyme (8, 9) and purified synthase \(b\) from the diabetic showed physical and catalytic characteristics similar to enzyme isolated from the normal animal (8). These results suggest that the increase in enzyme activity in the diabetic was due to changes in enzyme amount and not a diabetes-induced abnormal synthase. Also, it has been recently shown that an insulin-induced increase in liver glycogen synthase amount occurs in conjunction with a hormone-induced increase in total liver protein (9).

Since insulin deficiency causes variable changes in turnover of liver proteins in rats and mice (10-14), it is unknown if the increase in glycogen synthase is due to either an altered rate of enzyme synthesis or degradation. In the present study, we determined relative rates of turnover for glycogen synthase from normal and diabetic rats using the double isotope method of Arias et al. (15). The results show that the rate of turnover of glycogen synthase was altered in the diabetic state relative to other liver proteins and suggest that an increase in enzyme synthesis primarily accounts for the changes in total glycogen synthase levels found in the diabetic liver. Liver phosphorylase from diabetic animals showed altered turnover values with a large increase in relative rate of degradation.

**EXPERIMENTAL PROCEDURES**

**Treatment of Animals** - Diabetes was induced in male Sprague-Dawley rats (200-250 g) by a single subcutaneous injection of alloxan (150 mg/kg body weight in 0.1 M NaCl) following an overnight fast. Rats were then injected intraperitoneally with 100 mg/kg streptomycin and acetylsalicylic acid (ASA) 1 h before use.

**Material** - Radiochemical isotopes \(^{1}H\), \(^{3}H\), \(^{13}C\), \(^{14}C\) were obtained from New England Nuclear. Enzyme substrates, \(^{1}H\), \(^{13}C\) and \(^{14}C\) U-\(^{13}C\) glycylglycine, \(^{1}H\), \(^{13}C\) and \(^{14}C\) glycylglycine, \(0.1 M\) NaHCO\(_3\), \(0.1 M\) glycylglycine, pH 7.4, \(0.2 M\) sucrose, \(0.1 M\) Tris and \(20 \mu M\) \(2^{-}\)-mercaptoethanol were used in this study.

**Enzyme Isolation** - Livers were removed from rats after decapitation and homogenized (15 w/v) in ice cold (0°C) buffer composed of 0.1 M glycylglycine, pH 7.4, 0.1 M sucrose, 0.1 M NaCl and 0.1% Triton X-100. The homogenate was centrifuged (5,000 x g for 10 min). The supernatant was used as the starting material for the isolation of liver glycogen synthase and phosphorylase. The isolation of glycogen synthase from normal and diabetic rat liver was performed earlier (10) and is the same procedure used for the present study. The enzyme was isolated from the DEAE-cellulose column; it was purified by gel filtration on a Sephadex G-75 column (16). The final enzyme preparations, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and ultracentrifugation, contained between 80 and 100% of the total activity present in the isolated enzyme.

**Enzyme Activity** - Rat liver glycogen synthase from normal and diabetic rats was assayed using the hexokinase method (17). The reaction was started by the addition of a 1-ml mixture containing 60 mM Tricine (pH 7.4), 10 mM MgCl\(_2\), 0.1 mM 14C-glucose, and 5 mM ATP in a final volume of 0.5 ml. The reaction was allowed to proceed for 30 min. After precipitation with 5% trichloroacetic acid, the reaction mixture was filtered through Whatman no. 3MM paper. The filtration apparatus was washed with 5% trichloroacetic acid and the filters were then counted in a liquid scintillation counter. Enzyme activity was expressed in terms of the amount of 14C-glucose incorporated per hour per mg of protein.

**Radiochemicals** - \(^{1}H\) and \(^{13}C\) glycylglycine, \(^{14}C\) glycylglycine, \(^{1}H\)-leucine (10 mCi/ml), \(^{13}C\)-leucine (10 mCi/ml) and \(^{14}C\)-leucine (30 mCi/ml) were used as substrates for the isolated enzymes.

**RESULTS**

Results show that the rate of turnover of glycogen synthase from normal and diabetic rats using the double isotope method of Arias et al. (15). The results show that the rate of turnover of glycogen synthase was altered in the diabetic state relative to other liver proteins and suggest that an increase in enzyme synthesis primarily accounts for the changes in total glycogen synthase levels found in the diabetic liver.

Liver phosphorylase from diabetic animals showed altered turnover values with a large increase in relative rate of degradation.
Turnover of Liver Glycogen Synthase and Phosphorylase in Diabetic Rats

The amount of radioactivity in the isolated fractions labeled in vivo was determined by adding 100-300 μl of each to 7 ml Aquasol II (New England Nuclear), the suspension was neutralized with HCl, and water was added to a final volume of 10 ml. Proteins from the 1320-250 μl were precipitated in an equal volume of 30% trichloroacetic acid, washed three times in 2 volumes 10% trichloroacetic acid, and dissolved in two volumes of 0.5 N NaOH with 0.1% deoxycholate. Aliquots of the trichloroacetic acid precipitates, 100 to 150 μl, were prepared for counting as described above. The integrations per min (dpm) were determined with a Scinti Delta 300 counter by the channel ratio and the external standard method.

**Results**

**Turnover of Synthase and Phosphorylase** — The apparent rates of protein degradation were estimated by the double-isotope labeling technique (13). The first isotope, 13C-glucosamine (31 μCi/g body weight), was injected intravenously 48 hours before the injection of the second isotope, 13C-glucose (150 μCi/g body weight). The second isotope was allowed to incorporate into protein for one hour, the animals were sacrificed and glycogen synthase and phosphorylase were isolated (13). The time between isotope injections was based on a previously estimated half-life for normal glycogen synthesis of 3-9 hours (9) and the theoretical optimal number of days required between injections of labeled glucosamine for short half-life proteins using the double-isotope labeling technique (13).

A ratio of 13C/13C was calculated for each protein and enzyme fraction and is a reflection of the apparent degradation rate of proteins (13). A "relaxation" rate of degradation was calculated by comparing the 13C/13C ratio in each liver fraction to the 13C/13C ratio in the 8000 x g supernatant. The relative rate of degradation was determined by dividing the apparent half-life of the 8000 x g supernatant by the isotope labeling time in the 13C/13C ratio. A relative rate of synthesis was determined by comparing the isotope incorporation into enzyme activity to the 13C/13C ratio in the 8000 x g supernatant.

The length of diabetes refers to the day the second isotope was given to the animal to the time of the isotope injection 15°C. Synthetic activity was determined by determining the denet (5) incorporated into soluble liver protein one hour after injection of 31C-glucosamine as described previously. A relative rate of synthesis was determined by dividing the difference in the 13C/13C ratio in the 8000 x g supernatant by the 13C/13C ratio in the 8000 x g supernatant.

**RESULTS**

**Liver Characteristics**—The liver from the diabetic rat showed an increase in total glycogen synthase activity whether values are expressed as units/g of liver, wet weight, or as specific enzyme activity (units/mg of protein; Table I, see Miniprint). The increase in synthase activity with the 8000 x g supernatant and phosphorylase in the normal and 8-day diabetic rats shows the increase in activity associated with diabetes was because of an increase in the amount of immunotitratable enzyme (Fig. 1). The antibody neutralized the synthase activity in approximately twice the amount of the 8000 x g supernatant from the normal than from the 8-day diabetic and this difference remained through a 3- to 4-fold change in the protein concentration of the 8000 x g supernatant. In the insulin-treated 8-day diabetic, enzyme activity remained elevated relative to the normal but was decreased relative to the untreated diabetic. Synthase activity as units/g of liver, wet weight, in the insulin-treated diabetic was slightly elevated relative to normal and was because of an increase in liver weight (Table I). The protein content and weight of the liver of the 8-day diabetic did not differ from those in the normal animal, indicating that the increase in synthase activity was not because of an increase in total liver protein.

The 2- to 3-day diabetic rat showed a significant (17%) decrease in liver wet weight and a 14% increase in the amount of protein per g of liver, wet weight (Table I). The modest increase in protein content is not sufficient to account for the 58% increase in synthase activity at this stage of diabetes. The insulin-treated diabetic showed an increase (30% relative to normal) in liver size with a slight but not significant decrease in the amount of liver protein. Table I shows that diabetes resulted in the expected decrease in liver glycogen and increase in blood glucose which are reversed upon insulin treatment. It is interesting to note that liver glycogen content (percentage of liver wet weight) in the 8-day diabetic is 40-50% of the normal. Synthase in the 8-day diabetic is, by catalytic criteria, all 6-form enzyme (8) which, presumably, is inactive in vivo. However, the near normal level of liver glycogen in the 8-day diabetic (3-5% of liver wet weight as glycogen in the normal) would suggest that the increased synthase is partly active in vivo and is capable of synthesizing glycogen without b to a conversion.
The indistinguishable physical properties of isolated glycogen synthase from normal and diabetic rats have been reported previously (8). Both synthase and phosphorylase show a single protein-stained band on SDS-polyacrylamide gel electrophoresis (results not shown); therefore, the isolated enzymes were used for protein turnover determination because both enzymes could be conveniently recovered using the same preparative procedures for the normal and diabetic rats (8).

Turnover of Liver Glycogen Synthase and Phosphorylase—The 8000 × g supernatant fraction of liver was used as the basis of comparison for relative rates of enzyme degradation and synthesis and showed little change in \(^{1}H/^{13}C\) ratio between control and early diabetic animals with no consistent trend apparent through the course of diabetes studied here (Table II). The 8-day diabetic shows a slightly higher \(^{1}H/^{13}C\) ratio but the range overlapped the normal. There was a transient increase in the disintegrations/min/mg of protein incorporated into the 8000 × g supernatant fraction (Table II) from the short term diabetic compared to the normal; but, by 8 days of diabetes, the amount of isotope incorporated into protein was less than normal (30% decreased).

The \(^{1}H/^{13}C\) ratio of isolated glycogen synthase increased throughout the 8 days of diabetes to where there was an almost 2-fold difference in the ratio between the control and the diabetic with no overlap in the range of values (Fig. 2A). Since the crude protein fraction in the 8-day diabetic showed increased \(^{1}H/^{13}C\) ratios as well, the differences between the diabetic and controls were reduced when the values were normalized relative to the 8000 × g supernatant and reported as the relative rate of degradation (Fig. 2A). The average relative rate of degradation varied from 1.3 to 1.8 times the control in all the diabetic groups and all values were outside the range of values for the normal animal. Therefore, it does not appear that a lower rate of enzyme degradation could account for the increase in synthase activity and, in fact, the rate of degradation of glycogen synthase appeared to be 30-80% greater than normal.

All of the diabetic groups showed a greater incorporation of isotope into synthase than did the control animals (Fig. 2B). The short term diabetic had 3.4 times the normal amount of isotope per mg of enzyme protein and the 8-day diabetic had 1.7 times the normal. There was no overlap between the diabetic and control animals’ individual values. The increase in the amount of isotope incorporated into glycogen synthase in the long term diabetic was opposite to the 8000 × g supernatant where the long term diabetic showed less isotope incorporated into protein than the normal (Table II). When the values for synthesis were normalized against the 8000 × g supernatant, the differences in the relative rate of synthesis of glycogen synthase were 2.2- to 2.5-fold greater than control (Fig. 2B). The values for the relative rates of synthesis were very similar in all the diabetic groups, indicating that the rate of synthesis of the enzyme increased early in diabetes and is maintained at the increased rate throughout the time period studied. The values for rates of degradation and synthesis determined for synthase suggest that liver glycogen synthase in diabetic animals turns over more rapidly than normal. Insulin treatment of diabetic rats (Fig. 2) resulted in a reduction of the rate of degradation, but the indices for enzyme

![Fig. 2. Turnover parameters for liver glycogen synthase in normal, diabetic, and insulin-treated diabetic rats. A. \(^{1}H/^{13}C\) ratio (cross-hatched bars) and relative rate of degradation (open bars). Rates of degradation were determined by the double isotope method (15) and described under "Experimental Procedures." Rats were injected with the first isotope, \(^{12}C\)leucine, 48 h before the injection of the second isotope, \(^{1}H\)leucine. The isotoles were allowed to incorporate into protein for an additional hour and then liver glycogen synthase was isolated. Relative rates of degradation were calculated by comparing the \(^{1}H/^{13}C\) ratio of glycogen synthase to the isotope ratio in an 8000 × g supernatant of the liver homogenate. Values are mean ± S.D. Sample size was 3 in all cases. B, disintegrations/min/mg of protein (cross-hatched bars) and relative rate of synthesis (open bars). Rates were injected with \(^{12}C\)leucine, and after 1 h, liver glycogen synthase was isolated. A relative rate of synthesis was calculated by comparing disintegrations/min/mg of protein in glycogen synthase to that in an 8000 × g supernatant of a liver homogenate. Values are mean ± S.D. Sample sizes are given in the open bars.](image-url)
synthesis remained elevated.

The $^3$H/$^14$C ratio for liver phosphorylase was elevated 2-fold in the 2- to 3-day diabetic and 4- to 5-fold in the 8-day diabetic animals relative to control values (Fig. 3A). These general relationships were maintained when the rate of degradation relative to the 8000 X g fraction was calculated. Although the diabetics showed more variability for phosphorylase turnover than synthase, there was no overlap in individual phosphorylase turnover values between control and diabetic rats. The change in the relationship of degradation for synthase and phosphorylase with the diabetic animals was very striking. In the normal animal, synthase showed an almost 2-fold greater $^3$H/$^14$C ratio and relative degradation rate than phosphorylase, while in the long term diabetic, phosphorylase showed a greater rate of degradation than did synthase. These data indicate the rate of degradation of phosphorylase in the diabetic animals was greater than synthase and presumably greater than the normal degradation rate of phosphorylase.

The synthesis of phosphorylase (Fig. 3B) in the diabetic rats showed an increased rate of isotope incorporation at 2-3 days of diabetes that decreased somewhat but remained high at 8 days of diabetes. The relative rate of synthesis for phosphorylase was elevated 4.5 times normal by 2-3 days of diabetes and remained at this elevated rate throughout the experimental period. These large increases in the relative rates of degradation and synthesis for phosphorylase indicate that diabetes stimulates turnover of phosphorylase. In contrast to synthase, insulin treatment reduced the rate of synthesis for phosphorylase dramatically, suggesting that insulin affects the turnover parameters of these enzymes differently.

**DISCUSSION**

The increase in liver glycogen synthase activity observed in diabetic animals (3-7) is apparently due to an increase in amount of enzyme (Fig. 1) and is not an effect of diabetes on enzyme activity (8). This 2-fold difference in enzyme amount in the 8-day diabetic, relative to normal, was maintained over the range of volumes of 8000 X g supernatant tested with antibody, suggesting that there was no interference of enzyme activity by activators or inhibitors. In addition, we had previously shown that isolated diabetic glycogen synthase is almost completely in the $b$ form and does not show catalytic or physical differences that would account for increases in total enzyme activity (8). Therefore, changes in enzyme level during diabetes must be due to either changes in steady state rates of protein degradation or synthesis.

The turnover of isolated glycogen synthase and phosphorylase was estimated by the double isotope label method (15). There was little change in the apparent rate of degradation between normal and diabetic synthase as indicated by the $^3$H/$^14$C ratio until 8 days of diabetes when the ratio was almost 2-fold greater than the normal (Fig. 2). The re-utilization rate of leucine is probably different in normal and diabetic animals because of changes in amino acid precursor pool size. Therefore, conservative interpretation is required for comparisons between animals of in vivo degradation rates in vivo isotope. Diabetes is associated with significantly increased plasma and liver levels of branched chain amino acids in rats and humans (23-25), which results from not only increased muscle protein catabolism in early diabetes, but from hyperphagia and increased intestinal absorption of amino acids as the disease progresses (24-26). These characteristics of diabetic amino acid metabolism suggest that the specific radioactivity of the isotope in the amino acid precursor pool would be lower in the diabetic and, therefore, the $^3$H/$^14$C value would overestimate the degradation rate in the diabetic compared to the control. Similarly, the disintegrations/min incorporated per mg of enzyme protein would underestimate the synthetic rate. These changes can be partially compensated by calculating and comparing relative rates of degradation and synthesis of the specific enzyme protein to the average of proteins represented in the 8000 X g supernatant liver fraction. The relative rate of degradation for glycogen synthase in all of the 8-day diabetic animals tested indicated that the absolute rate of degradation of synthase may not be as great as the $^3$H/$^14$C ratio indicated but was still greater than the rate of degradation of the average liver proteins. In the normal, the rate of degradation of synthase appeared to be slightly less than that of the average liver proteins (Table II).

The results of this study showed that the relative rate of synthesis of glycogen synthase increased early after alloxan treatment and remained high and consistent throughout the 8 days of diabetes. These data suggest that the rate of turnover of liver glycogen synthase is increased in diabetes and the increased rate of synthesis accounts for the accumulation of glycogen synthase in the 8-day diabetic. There has not been a systematic study as to when glycogen synthase reaches a steady state level in the diabetic, but the degradation rate should increase if a new steady state is to be achieved. Fig. 2 suggests that a steady state relative rate of degradation occurs at 5-8 days after alloxan treatment. We use 8-day diabetic
animals because total synthase activity shows about a 2-fold increase 6-10 days after alloxan injection. Total glycogen synthase has been shown to remain significantly higher than normal 40 days after streptozotocin-induced diabetes (4).

The increase in the amount of physiologically inactive synthase, which is unable to be activated in liver from diabetic rats because of either insufficient levels or a defect in synthase phosphatase, appears to be a futile condition of diabetes. However, this observation does suggest that the level of synthase as substrate for synthase phosphatase is regulated, in some negative fashion, by insulin. Also, the increased level of inactive synthase is capable of overcoming physiological inhibition in vivo such that significant levels of liver glycogen can be synthesized. The increase in relative rate of synthesis for glycogen synthase was opposite that found for the rate of synthesis of the average of proteins in the 8000 x g supernatant and, generally, liver protein synthesis has been found to be slightly decreased in diabetes (11, 18, 28). The factor(s) involved in the induction of synthesis and the dosage of glycogen synthase are unknown; however, similar to glycogen synthase, there are gluconeogenic enzymes (pyruvate carboxylase, phosphoenolpyruvate (GTP) carboxykinase, and fructose 1,6-bisphosphatase) which have shown significant increases in activity during diabetes (13, 29-32). The increase in the activity of these gluconeogenic enzymes appeared to be due to increases in enzyme amount and results from de novo enzyme synthesis (13, 30-33). Glucocorticoids and glucagon or cAMP have been implicated in the induction of these gluconeogenic enzymes when the antagonism of insulin is suppressed or absent (33-35). Glucocorticoids also have a stimulating influence on glycogen synthesis in the liver of adrenalectomized and normal rats (3, 31, 36); but this increase in glycogen deposition depends on the presence of insulin (3, 31). These hormones alone have little effect on the levels of glycogen synthase or glycogen synthase in perfused livers of diabetic rats, suggesting that the defects in glycogen synthase were not a direct action of hormone (37-39). Isolated hepatocytes incubated with insulin showed no significant increase in total glycogen synthase activity above that produced by glucose alone in the medium (7, 40, 41); however, a combination of glucocorticoid, insulin, and glucose resulted in a significant increase in total synthase over that shown by glucose alone (40). The high levels of total glycogen synthase in the liver had been suggested as an attempt to compensate for the high levels of glucose in the liver (3) as may exist in the diabetic. Glucose has been found to stimulate glycogen synthesis in perfused livers from normal rats (5, 37) and total synthase activity was increased 10-25% (5). Glucose had little effect, however, on the synthase activity from diabetic rats (5, 37).

Glycogen synthase did not show complete reversal of changes in rate of enzyme synthesis when diabetes was treated with the dosage of insulin used here. Insulin treatment of diabetic rats has previously been shown to stimulate glycogen synthase activity above that found in the normal and may be dose-dependent (4). The insulin-treated diabetic rat in the present study still maintained a high relative rate of synthesis of synthase; therefore, the increased rate was not due to a general increase in protein synthesis, which is in contrast to the action of insulin in the normal rat (9) where the increase of synthase synthesis was attributed to a general increase in protein synthesis.

There is less known of the changes in phosphorylase levels during diabetes than of synthase because of the difficulties in directly measuring total phosphorylase activity (42). A decrease of 20 to 30% in phosphorylase activity in the diabetic is observed (data not shown) when assayed by the method of Stalmans and Hers (43). Similarly, total phosphorylase has shown significant decreases in activity after alloxan diabetes when assayed by the same method as above (44) and by the high substrate (Glc-1-P') assay (45) using liver homogenates from streptozotocin diabetic rats (4). These changes are usually accompanied by an increase in the percentage of total phosphorylase as the a form. The decrease in total phosphorylase activity in the diabetic animal could be due to the change in enzyme amount resulting from an increased rate of degradation. The changes in phosphorylase levels during diabetes may have been even more noticeable except that the rate of synthesis also increased.

The degradation patterns of phosphorylase and synthase showed a reversed relationship in the long term diabetic when compared to the normal animal, suggesting a more pronounced increase in the degradation rate of phosphorylase. Phosphorylase showed a large increase in both relative rates of degradation and synthase and synthase showed a large increase in the relative rate of synthesis and, to a lesser degree, in the rate of enzyme degradation. It might be expected that both turnover parameters would be altered if a new balance is to be achieved in the diabetic state. These data presented here do indicate that the general turnover of the glycogen-metabolizing enzymes, synthase and phosphorylase, is very rapid in the diabetic, involving both synthesis and degradation of new enzyme. Although the exact nature of the increase in glycogen synthase amount is not known, it is possible that a parallel increase in specific mRNA could be used as a probe for further studies of insulin's action at the molecular level. Since synthase and phosphorylase are substrates in a complex enzyme cascade system, it would be worthwhile to investigate the nature of the turnover of other elements of the system, especially in view of the reported loss of synthase phosphatase activity in the diabetic.

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