The Effect of Streptozotocin-Induced Diabetes and of Insulin Supplementation on Glycogen Metabolism in Rat Liver

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The effects of streptozotocin-induced diabetes and of insulin supplementation to diabetic rats on glycogen-metabolizing enzymes in liver were determined. The results were compared with those from control animals. The activities of glycogenolytic enzymes, i.e. phosphorylase (both *a* and *b*), phosphorylase kinase and protein kinase (in the presence or in the absence of cyclic AMP), were significantly decreased in the diabetic animals. The enzyme activities were restored to control values by insulin therapy. Glycogen synthase (I-form) activity, similarly decreased in the diabetic animals, was also restored to control values after the administration of insulin. The increase in glycogen synthase (I-form) activity after insulin treatment was associated with a concomitant increase in phosphoprotein phosphatase activity. The increase in phosphatase activity was due to (i) a change in the activity of the enzyme itself and (ii) a decrease in a heat-stable protein inhibitor of the phosphatase activity.

Several studies have suggested that control of glycogen metabolism in liver is regulated by a cascade enzyme system that is similar to the one already established in skeletal muscle (Krebs, 1972; Hers, 1976). Glucagon and adrenaline stimulate the formation of active phosphorylase *a* (EC 2.4.1.1) (Sutherland & Cori, 1951; Sutherland, 1951) and convert glycogen synthase (EC 2.4.1.11) from a glucose 6-phosphate-independent (I) form into a glucose 6-phosphate-dependent (D) form (Larner & Villar-Palasi, 1971; Stalmans & Hers, 1973). Insulin, on the other hand, converts glycogen synthase from a glucose 6-phosphate-dependent form into a glucose 6-phosphate-independent form, both *in vivo* and *in vitro* (Steiner & King, 1964; Mersmann & Segal, 1967; Gold & Segal, 1967; Bishop & Larner, 1967; Kreutner & Goldberg, 1967; DeWulf & Hers, 1968). Bishop & Larner (1967) further showed that insulin not only activates liver glycogen synthase, but also inactivates phosphorylase. Gold (1970) and Tan & Nuttall (1976) suggested that insulin has a direct effect on the synthase-activating enzyme. Furthermore, it is still controversial whether the insulin effect is mediated by cyclic AMP or by some unknown 'second' messenger (Hers, 1976).

In the present study the effects of streptozotocin-induced diabetes and of insulin treatment of these diabetic rats on liver glycogen metabolism have been examined. The enzymes involved in the regulation of glycogen synthesis, as well as in its degradation, have been examined in three groups of animals, i.e. con-

trol, streptozotocin-induced-diabetic and insulin-treated diabetic animals. These studies also explore the mechanism involved in the activation of rat liver glycogen synthase by insulin.

Experimental

Materials

Crystalline rabbit skeletal-muscle phosphorylase *b* (EC 2.4.1.1) was isolated as described by Fischer & Krebs (1958). Rabbit skeletal-muscle phosphorylase kinase (EC 2.7.1.38) was prepared as described by Hayakawa *et al.* (1973) and was generously given by Dr. J. H. Wang & Mr. T. Singh (Department of Biochemistry, Faculty of Medicine, University of Manitoba, Winnipeg, Manitoba, Canada). ^{32}P-labelled phosphorylase *a* was prepared from phosphorylase *b* by using [γ-^{32}P]ATP, Mg^{2+} and phosphorylase kinase as described by Krebs *et al.* (1958). The preparation of rabbit liver phosphoprotein phosphatase (EC 3.1.3.16) and phosphorylated histone has been described previously (Khandelwal *et al.*, 1976). UDP-[^{14}C]glucose was obtained from New England Nuclear (Lachine, Que., Canada), and [γ-^{32}P]ATP was from ICN, Irvine, CA, U.S.A. [^{14}C]Glucose 1-phosphate and PCS (phase-combining solvent) for liquid scintillation were obtained from Amersham/Searle, Oakville, Ont., Canada. UDP-glucose, glucose 1-phosphate, rabbit liver glycogen, AMP, Mes,† type II-A histone and cyclic AMP were

† Abbreviation: Mes, 4-morpholine-ethanesulphonic acid.

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from Sigma Chemical Co., St. Louis, MO, U.S.A. Sephadex G-25 was obtained from Pharmacia, Dorval, Que., Canada. All other chemicals were of reagent grade.

Treatment of animals

For the experiments 36 3-month-old male Sprague–Dawley rats from the Faculty of Dentistry, University of Manitoba, breeding colony were used. The animals were maintained in individual meta-bolism cages in a light (12h light/12h dark)- and temperature (22–24°C)-controlled room and were allowed free access to a commercial pelleted diet (Wayne Lab-Blox F6 obtained from Allied Mills, Chicago, IL, U.S.A.) and to water. The animals were allowed 7 days to adapt to their individual housing before the start of the experiment.

Diabetes was induced in 24 rats with streptozo-tocin (2-deoxy-2-{[(methylnitrosamino)carbonyl]-amino}-D-glucopyranose; a gift from Upjohn Drug Co., Kalamazoo, MI, U.S.A.), which was adminis-tered intravenously in 0.1 M-sodium citrate buffer, pH 4.5, into a tail vein at a dose of 70 mg/kg body wt. The criteria used to assess the severity of the diabetic state have been described in detail (Howland & Zebrowski, 1974). Urine glucose concentrations were measured daily with Tes Tape (Eli Lilly and Co., Indianapolis, IN, U.S.A.). Within 24 h of streptozo-tocin treatment, all animals showed at least 2% (w/v) glucose in urine, and, with the exception of the 12 rats that received insulin therapy, all of the treated rats maintained this extent of glycosuria for the duration of the 40-day experiment. Protamine zinc insulin (Connaught Laboratories, Toronto, Ont., Canada) was administered by intraperitoneal injection each morning at the same time for only the last 7 days of the experiment to 12 randomly selected diabetic rats in an amount (5–28 i.u.) that was adequate to maintain their urine glucose concentrations below 0.1%. Blood samples were drawn by heart puncture under ether anaesthesia on the last day of the experi-ment for serum glucose (non-starved) determination. Livers were removed immediately and quickly frozen by being placed between two slabs of solid CO_2. These livers were stored at −70°C until further processed.

Preparation of liver homogenates

For all of the enzyme assays in this study except phosphatase, frozen rat livers were homogenized with 4 vol. of 20 mM-Tris/HCl (pH 7.4) containing 0.25 M-sucrose, 4 mM-EDTA, 0.5 mM-dithiothreitol and 50 mM-NaF. For the phosphatase assay, rat livers were homogenized in the same buffer, but without NaF. The homogenates were centrifuged at 8000 g for 30 min and the supernatants collected after filtration through glass-wool and four layers of cheesecloth. The supernatants were then either extensively diluted (up to 200-fold) or passed through a small column (1.2 cm × 15 cm) of Sephadex G-25 before being used in the described analyses.

Preparations of samples for the determination of phosphoprotein phosphatase inhibitor activity

The 8000 g supernatant as obtained for the phos-phatase assay was heated to 95°C in a water bath. The resultant boiled suspension was cooled in an ice bath and then centrifuged at 3000 g for 10 min. The clear supernatant was adjusted to 15% (w/v) with respect to trichloroacetic acid by adding a cold solution of 100% acid. The precipitate, which contained the inhibitor protein, was separated by centrifugation at 10000 g for 20 min, then dissolved in 20 mM-Tris/HCl, pH 7.0, containing 0.25 M-sucrose and 0.5 mM-dithiothreitol and used for the experi-ments involving the inhibitor protein.

Enzyme assays

Phosphorylase a and total phosphorylase (a plus b) activities were determined by the method of Tan & Nuttall (1975). The reaction mixture for the assay of phosphorylase a contained 33 mM-Mes buffer, pH 6.3, 15 mM-glucose 1-phosphate with tracer amounts of [^{14}C]glucose 1-phosphate (pH 6.3), 3.4 mg of rabbit liver glycogen/ml, 5 mM-AMP, 150 mM-NaF and properly diluted liver extract in a total volume of 0.1 ml. For the measurement of total phosphorylase activity, the assay mixture was the same as above except that glucose 1-phosphate and glycogen con-centrations were raised to 273 mM and 13.4 mg/ml respectively. The incubation period was 10 min at 30°C. The radioactive glycogen was precipitated on a Whatman 31ET paper, washed and counted for radio-activity as described by Reimann et al. (1971) for the protein kinase assay, except that 66% (v/v) ethanol was used in place of trichloroacetic acid solutions. One unit of phosphorylase activity was defined as the amount of enzyme that incorporated 1 nmol of [^{14}C]glucose from [^{14}C]glucose 1-phosphate into glycogen/min.

Glycogen synthase activity was determined by the incorporation of [^{14}C]glucose from UDP-[^{14}C]-glucose into glycogen (Thomas et al., 1973). Total synthase (I- plus D-form) and synthase (I-form) activities were determined in the presence of 10 mM-glucose 6-phosphate and 14 mM-Na_2SO_4 respectively. One unit of glycogen synthase activity was defined as the amount of enzyme that incorporated 1 nmol of [^{14}C]glucose from UDP-[^{14}C]glucose into glycogen/min. Protein kinase activity was assayed by following the incorporation of ^{32}P from [γ-^{32}P]ATP into type II-A histone as described by Reimann et al. (1971).

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One unit of protein kinase (EC 2.7.1.37) activity was defined as the amount of enzyme that incorporated 1 nmol of $^{32}P_i$ from [γ-^{32}P]ATP into histone/min. Phosphorylase kinase activity was determined by measuring the amount of phosphorylase a formed from phosphorylase b by a modification (Brostrom et al., 1971) of the method originally described by Krebs et al. (1964). The activity of this phosphorylase a was measured in the direction of glycogen synthesis by the method of Cori et al. (1943). One unit of phosphorylase kinase activity was defined as the amount of enzyme that converted 1 unit of phosphorylase b into phosphorylase a/min. Phosphoprotein phosphatase activity was determined by using ^{32}P-labelled phosphorylase a and phosphorylated histone as substrates, as described previously (Khandelwal et al., 1976). One unit of phosphoprotein phosphatase activity was defined as the amount of enzyme that released 1 nmol of $^{32}P_i$ from ^{32}P-labelled substrates/min.

Phosphoprotein phosphatase inhibitor activity was determined by its ability to inhibit the dephosphorylation of ^{32}P-labelled phosphorylase a. The reaction mixture contained 50 mM-Tris/HCl buffer, pH 7.5, 0.5 mM-dithiothreitol, 0.4 mg of bovine serum albumin, 1 mM-caffeine, 0.5 mg of ^{32}P-labelled phosphorylase a/ml, phosphoprotein phosphatase and inhibitor preparations in a total volume of 50 μl. The amount of phosphatase used per assay was sufficient to give an activity of 1 pmol of $^{32}P_i$ released from ^{32}P-labelled phosphorylase a/min without inhibitor. Inhibitor activity was linear with time up to at least 40% inhibition of the phosphatase activity. In all assays, the incubation was carried out at 30°C for 30 min and the released $^{32}P_i$ was separated and counted for radioactivity as described previously (Khandelwal et al., 1976). One unit of inhibitor activity was defined as the amount that would inhibit 20% dephosphorylation of ^{32}P-labelled phosphorylase a under the stated conditions.

Analytical methods

Serum glucose was determined by the glucose oxidase method (Glucostat; Worthington Biochemical Corp., Freehold, NJ, U.S.A.). Liver glycogen was determined by the anthrone method as described by Sandham & Kleinberg (1969). Protein was determined by the method of Lowry et al. (1951), with bovine serum albumin as the standard. P_i was measured by the method of Fiske & SubbaRow (1925). The method for preparing tissue extracts and for analyses of cations was similar to that described by Reynafarje & Lehninger (1969).

Results and Discussion

Blood glucose and liver glycogen concentrations

The blood glucose and liver glycogen concentrations in control, streptozotocin-induced-diabetic and insulin-treated diabetic animals are shown in Table 1. Diabetic animals showed about a 4-fold higher blood glucose concentration and about a 3-fold decrease in liver glycogen concentration compared with control or insulin-treated diabetic animals. No significant difference was observed between control and insulin-treated diabetic animals.

Phosphorylase activities

The decrease in liver glycogen content in diabetic animals could be due to either the inhibition of glycogenesis or the activation of glycogenolysis.

Table 1. *Blood glucose and liver glycogen concentrations in control, streptozotocin-induced-diabetic and insulin-treated diabetic rats*
Details are given in the Experimental section. Blood glucose values are the mean±S.E.M. for 12 animals in each group, and liver glycogen values are for four animals in each group.

	Blood glucose concentration (mM)	Liver glycogen concentration (mg/g of tissue)
Control	7.9 ± 0.4	36.1 ± 3.0
Diabetic	32.3 ± 1.2	10.0 ± 1.0
Insulin-treated diabetic	6.6 ± 1.6	29.2 ± 2.5

Table 2. *Glycogen phosphorylase activities in control, streptozotocin-induced-diabetic and insulin-treated diabetic rat livers*
Details are given in the Experimental section. Values are the means±S.E.M. for eight animals. P values relative to the control values were obtained by Student's t test: *$P<0.05$; **$P<0.01$; ****$P<0.001$.

Enzyme	Control (units/mg of protein)	Control (units/mg of liver)	Diabetic (units/mg of protein)	Diabetic (units/mg of liver)	Insulin-treated diabetic (units/mg of protein)	Insulin-treated diabetic (units/mg of liver)
Phosphorylase a	69.3 ± 5.4	8.3 ± 0.6	30.5 ± 2.9****	4.2 ± 0.5****	80.7 ± 3.4*	8.7 ± 0.8
Phosphorylase $a + b$	192.9 ± 5.0	23.1 ± 1.5	91.4 ± 8.9****	12.5 ± 1.2**	196.5 ± 8.8	21.5 ± 1.3
% of phosphorylase a	36 ± 2		35 ± 3		41 ± 3	

The enzymes that regulate the above processes are glycogen synthase for glycogenesis and phosphorylase for glycogenolysis. The activity of phosphorylase in the livers of the three groups of animals is shown in Table 2. Phosphorylase *a* activity as well as total phosphorylase (*a* plus *b*) activity was significantly lower in diabetic animals than in the control or insulin-treated diabetic animals. No significant difference between the control and the insulin-treated diabetic groups was found. Phosphorylase *a* as a percentage of total phosphorylase was not altered by any of the experimental treatments. These results on phosphorylase are not in agreement with the reported observations in adipose tissue (Jungas, 1966) or in liver (Bishop & Larner, 1967), in which insulin actually inactivated phosphorylase activity. However, there were two basic differences between our experimental conditions and those used by these earlier workers. First, they were determining the effects of insulin in normal animals, whereas in the present study diabetic animals were utilized. Secondly, these other workers determined only the short-term (in minutes) effects of insulin. In comparison we have determined the long-term (in days) effects of insulin. The possibility also exists that differences could be due to some direct effect of streptozotocin on the liver, which would not have been seen in the previous studies in normal rats. Torres *et al.* (1968) did not observe any effect of insulin on skeletal-muscle phosphorylase in normal rats.

Protein kinase and phosphorylase kinase activities

To determine whether the decreased activities of liver phosphorylase in diabetic animals were due to a decrease in the activities of phosphorylase kinase and/or of protein kinase, the activities of these two latter enzymes were determined in the three groups of animals. The activities of protein kinase, whether measured in the presence or in the absence of cyclic AMP, and the activity of phosphorylase kinase, were both significantly lower in diabetic animals than in the other two groups (Table 3). No significant

differences in protein kinase and phosphorylase kinase activities were found between the control and the insulin-treated groups. This general trend of decline in glycogenolytic enzymes in diabetic animals is perhaps due to either the inhibition of glycogenolysis by the hyperglycaemic state of the animals or the retardation of glycogenolysis by some endogenous control that may be related to the markedly lower amounts of liver glycogen in the diabetic animals.

Regulation of glycogen synthase

Several observations suggest that insulin plays a direct role in the regulation of glycogenesis by regulating the conversion of a glucose 6-phosphate-dependent form into a glucose 6-phosphate-independent form of glycogen synthase (Steiner & King, 1964; Mersmann & Segal, 1967; Gold & Segal, 1967; Bishop & Larner, 1967; Kreutner & Goldberg, 1967; DeWulf & Hers, 1968; Gold, 1970; Larner & Villar-Palasi, 1971; Stalmans & Hers, 1973; Lawrence *et al.*, 1977). The activities of the glucose 6-phosphate-independent form and of total glycogen synthase were also determined in the present study, and are reported in Table 4. The activity of the glucose 6-phosphate-independent form of glycogen synthase was lower in the diabetic animals than in either the control or insulin-treated animals. Total glycogen synthase activity, on the other hand, was significantly higher in diabetic animals when the results are expressed per mg of tissue. Insulin-treated animals showed significant increases in glycogen synthase (I-form) activity and in total glycogen synthase activity compared with the control animals. Glycogen synthase (I-form) activity, as a percentage of total glycogen synthase activity, was also significantly lower in diabetic animals.

The decrease in the glycogen synthase (I-form) activity in diabetic livers could be due to either an increase in the phosphorylation of the enzyme by protein kinase, or a decreased rate of dephosphorylation of the enzyme by protein phosphatase. Bishop *et al.* (1971) have observed that insulin caused an increase in glycogen synthase activity in dog livers without any change in either cyclic AMP concen-

Table 3. *Protein kinase and phosphorylase kinase activities in control, streptozotocin-induced-diabetic and insulin-treated diabetic rat livers*

Details are given in the Experimental section. Values are the means±s.e.m. for four animals. *P* values relative to the control values were obtained by Student's *t* test: *P<0.05; **P<0.01; ***P<0.005.

Enzyme	Control		Diabetic		Insulin-treated diabetic	
	(munits/mg of protein)	(munits/mg of liver)	(munits/mg of protein)	(munits/mg of liver)	(munits/mg of protein)	(munits/mg of liver)
Protein kinase						
Without cyclic AMP	140.3± 5.7	16.1±1.2	87.2± 2.0***	12.3±0.9**	132.8± 9.0	15.8±0.3
With cyclic AMP	397.1±15.2	45.2±0.5	243.7±18.9***	34.4±4.2**	358.0±13.9	42.4±1.8
Phosphorylase kinase	316.0±14.0	39.3±2.6	204.0±22.0**	30.7±2.4*	319.0±22.0	35.1±3.2

Table 4. *Glycogen synthase activities in control, streptozotocin-induced-diabetic and insulin-treated diabetic rat livers*
Details are given in the Experimental section. Values are the means$\pm$s.e.m. for eight animals. P values relative to the control values were obtained by Student's t test: ***$P < 0.005$; ****$P < 0.001$.

Enzyme	Control		Diabetic		Insulin-treated diabetic	
	(unit/mg of protein)	(unit/mg of liver)	(unit/mg of protein)	(unit/mg of liver)	(unit/mg of protein)	(unit/mg of liver)
Glycogen synthase (I-form)	0.160 ± 0.007	0.019 ± 0.002	0.076 ± 0.005****	0.011 ± 0.001****	0.223 ± 0.023***	0.036 ± 0.003***
Glycogen synthase (I-+D-form)	0.424 ± 0.019	0.051 ± 0.004	0.474 ± 0.033	0.065 ± 0.004	0.477 ± 0.038	0.074 ± 0.007***
% of glycogen synthase (I-form)	38 ± 2		16 ± 1		47 ± 3	

trations or cyclic AMP-dependent protein kinase activity. Similar results were also obtained by Glinsmann & Mortimore (1968), who showed that insulin antagonizes the effect of glucagon metabolism in perfused livers without any significant change in cyclic AMP concentration. Subsequently, Miller & Larner (1973) concluded, after examining the effect of insulin on hepatic glycogen synthesis by use of either diabetic or glucagon-stimulated normal rats, that insulin increases the formation of glycogen synthase (I-form) with a concomitant decrease in cyclic AMP-dependent glycogen synthase (protein) kinase activity. In the present study, the injection of insulin in diabetic rats caused an increase in the activity of protein kinase whether measured in the presence or in the absence of cyclic AMP (Table 3). This increase might not be related to the activation of glycogen synthase, because under those circumstances one would expect a decrease in kinase activity. These results therefore would not indicate a direct role for protein kinase in the activation of glycogen synthase by insulin. However, one cannot rule out the possibility that a specific glycogen synthase (protein) kinase is involved in this process. The presence of such a kinase has been reported in kidney (Reimann & Schlender, 1976) and in skeletal muscle (Huang et al., 1975).

Involvement of phosphoprotein phosphatase

The idea for the involvement of phosphatase in the insulin effect in glycogen synthesis evolved from the work of Bishop (1970), who observed that the injection of insulin into dogs resulted in an activation of hepatic synthase phosphatase. The concentration of this synthase-activating enzyme was very low in alloxan-diabetic rat livers, and administration of insulin to these rats resulted in restoration of the enzyme to normal concentrations (Bishop & Larner, 1967; Gold, 1970). Nuttall et al. (1976) have also observed the activation of synthase phosphatase by insulin in the heart extracts of starved or diabetic

rats. In the present study, the activity of phosphoprotein phosphatase was determined by using exogenous as well as endogenous substrates. The activity of this enzyme with exogenous substrates phosphorylase a and phosphorylated histones is shown in Table 5. Diabetic rats showed a significant decrease in phosphatase activity with both of the substrates tested when expressed per mg of protein. When the results were expressed per mg of tissue, no difference was found between the control and diabetic groups. The reason for this discrepancy is not clear, but it might be indicative of a change in the specific activity rather than total activity of the enzyme. Insulin-treated diabetic animals, however, showed a significant increase in phosphatase activity with substrate phosphorylase a, whether the activity was expressed per mg of protein or per mg of tissue. No significant difference was found between the control and insulin-treated animals for phosphatase activity when phosphorylated histone was the substrate.

The activity of phosphoprotein phosphatase was further determined by measuring the time-dependent change in the activities of endogenous phosphorylase a and glycogen synthase (D-form) by incubating the liver extracts at 23°C. As shown in Fig. 1, the inactivation of phosphorylase a proceeded at a faster rate in liver tissue from the control and insulin-treated animals. However, it is noteworthy that the activity of phosphorylase a in these two groups never decreased at any time during incubation to the low value shown by the diabetic animals. The differences in the rate of inactivation of phosphorylase a in these groups are obvious when the results are expressed in terms of percentage phosphorylase a activity (Fig. 1, inset). Inactivation of phosphorylase a proceeded at a lower rate in diabetic animals than in control or insulin-treated diabetic animals. There was no difference between the latter two groups. The activation of glycogen synthase (D-form) is shown in Fig. 2. Like the inactivation of phosphorylase, the activation of glycogen synthase was also faster in

Table 5. *Phosphoprotein phosphatase activity in control, streptozotocin-induced-diabetic and insulin-treated diabetic rat livers with exogenous phosphorylase a and phosphorylated histone as substrates*

Details are given in the Experimental section. Values are the means±s.e.m. for eight animals. P values relative to the control values were obtained by Student's t test: **$P<0.01$; ***$P<0.005$.

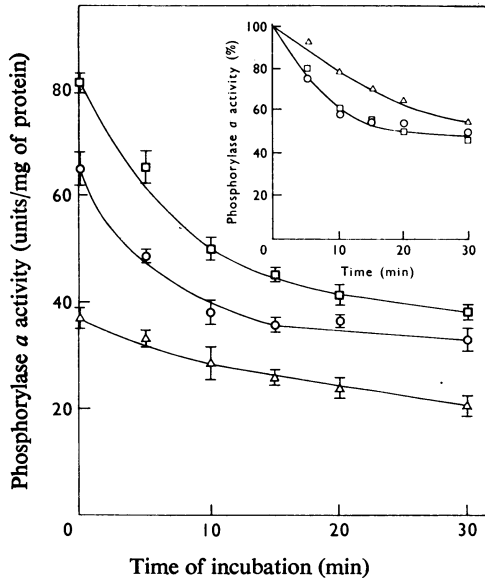| Substrate	Control (munits/mg of protein)	Control (munits/mg of liver)	Diabetic (munits/mg of protein)	Diabetic (munits/mg of liver)	Insulin-treated diabetic (munits/mg of protein)	Insulin-treated diabetic (munits/mg of liver)
Phosphorylase a	923.9 ± 55.7	106.7 ± 5.9	649.9 ± 59.6**	102.3 ± 12.5	1513 ± 205***	169.3 ± 5.1***
Phosphorylated histone	168.9 ± 15.0	18.3 ± 0.3	104.0 ± 12.8***	16.7 ± 0.6	158 ± 7.3	20.4 ± 0.9

Fig. 1. *Inactivation of glycogen phosphorylase in liver extracts of control (○), streptozotocin-induced-diabetic (△) and insulin-treated diabetic (□) rats in vitro*

Liver extracts were prepared as described in the Experimental section. The extracts were incubated at 23°C and samples were taken for determining the phosphorylase activity at the indicated times. In the inset, phosphorylase *a* activity is expressed as percentage of the total. Values are the means±s.e.m. for four livers in each group. Standard error bars have been omitted from the inset for clarity.
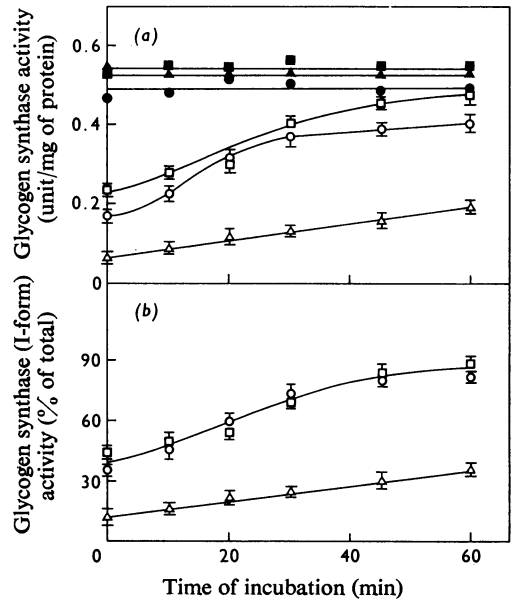

Fig. 2. *Activation of glycogen synthase in liver extracts of control, streptozotocin-induced-diabetic and insulin-treated diabetic rats in vitro*

Liver extracts were prepared as described in the Experimental section. The extracts were incubated at 23°C and samples were taken at the indicated times. Glycogen synthase activities are expressed in the presence (●, ▲, ■) or in the absence (○, △, □) of 10mm-glucose 6-phosphate in control (○, ●), streptozotocin-induced-diabetic (△, ▲) and insulin-treated (□, ■) animals (*a*). The percentage of total activity as activated enzyme is shown in Fig. 2(*b*). Enzyme assays are described in detail in the Experimental section. Values are the means±s.e.m. for four livers in each group. Standard-error bars have been omitted from the top three lines in Fig. 2(*a*) for clarity.

control and insulin-treated animals. After 1h of incubation, the glycogen synthase (I-form) activity reached 80% of the total synthase activity in the control and insulin-treated rats, but only 35% of the total synthase activity in the diabetic animals. The activities of phosphatase thus calculated are shown in Table 6. The phosphatase activity in diabetic animals was 25–37% of that in the other two groups. These results on phosphatase with exogenous and endogenous substrates would indicate a direct role of insulin in the activation of this enzyme.

Phosphoprotein phosphatase inhibitor activities

A comparison of the phosphatase activities reported in Tables 5 and 6 indicates that the percentage decrease in phosphatase activity in diabetic animals was greater when measured with

Table 6. *Phosphoprotein phosphatase activity in control, streptozotocin-induced-diabetic and insulin-treated diabetic rat livers with endogenous phosphorylase a and glycogen synthase (D-form) as substrates*

Liver extracts, prepared as described in the Experimental section, were incubated at 23°C and the activity of phosphatase was calculated by measuring the difference in phosphorylase a activity between 0 and 10 min and of glycogen synthase between 10 and 30 min. Assay conditions for phosphorylase a and glycogen synthase are given in the Experimental section. Values are the means ± S.E.M. for four livers in each group. Activity with phosphorylase a as substrate is expressed as units of phosphorylase a inactivated/min per mg of protein. The phosphorylase a unit is defined in the Experimental section. Activity with glycogen synthase as substrate is expressed as munits of glycogen synthase (D-form) converted into glycogen synthase (I-form)/min per mg of protein. The glycogen synthase unit is defined in the Experimental section. P values relative to the control values were obtained by Student's t test. ****$P < 0.001$.

Animals	Phosphatase activity with substrate	
	Phosphorylase a	Glycogen synthase (D-form)
Control	2.70 ± 0.13	7.2 ± 0.3
Diabetic	0.81 ± 0.05****	2.3 ± 0.1****
Insulin-treated diabetic	3.19 ± 0.18	6.2 ± 0.3

Table 7. *Phosphoprotein phosphatase inhibitor activities in control, streptozotocin-induced-diabetic and insulin-treated diabetic rat livers*

Details are given in the Experimental section. Values are the means ± S.E.M. for eight animals in each group. P values relative to the control values were obtained by Student's t test. ***$P < 0.005$.

Animals	Phosphoprotein phosphatase inhibitor activity (units/ml)
Control	13.3 ± 1.0
Diabetic	17.7 ± 0.7***
Insulin-treated diabetic	14.4 ± 0.5

endogenous substrates than with exogenous substrates. However, phosphatase activities with exogenous substrates were measured after sufficient dilution to avoid possible interfering effects of endogenous liver-extract factors. On the other hand, the effect of endogenous interfering factor(s) would be reflected under the conditions with endogenous substrates. The presence of a heat-stable inhibitor protein of phosphatase activity has previously been demonstrated in rabbit liver (Brandt *et al.*, 1975). To determine whether the quantitative differences in the decrease of phosphatase activity measured under these two sets of conditions is due to a change in the concentration of heat-stable inhibitor, the
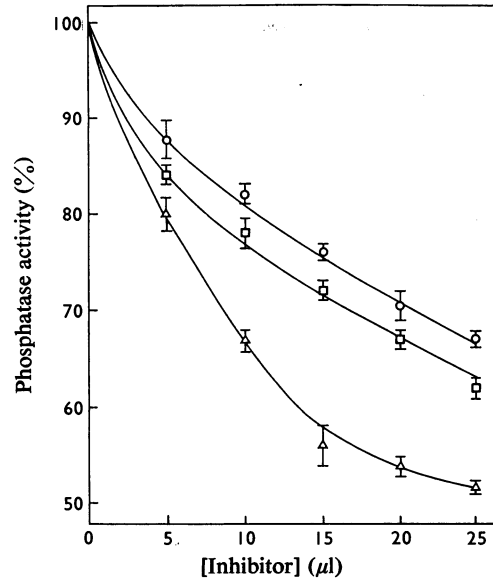

Fig. 3. *Inhibition of phosphoprotein phosphatase by various concentrations of heat-stable protein inhibitor of control (○), streptozotocin-induced-diabetic (△) and insulin-treated diabetic (□) rat livers*

The inhibitor preparations were prepared as described in the Experimental section. 100% activity represented phosphoprotein phosphatase activity in the absence of inhibitor. Values are the means ± S.E.M. for four animals.

inhibitor activities were also measured in these three groups (Table 7). A significant increase in inhibitor activity was observed in diabetic animals compared with the other two groups. The effect of different amounts of inhibitor prepared from all three groups on the inhibition of phosphatase activity is shown in Fig. 3. As expected, the inhibitor prepared from diabetic livers showed the maximum inhibition. These results, along with those obtained on phosphatase activity, further indicate that the activation of phosphatase by insulin is partly due to the effect on the enzyme itself and partly due to the change in heat-stable inhibitor of phosphoprotein phosphatase.

Other studies

It has been suggested that insulin effects might be mediated through cyclic GMP (Illiano *et al.*, 1973). The addition of this nucleotide to the liver extracts in the present study had no effect on either phosphatase or its inhibitor activity. The other probable messenger for this hormone could be a cation, as hypothesized by Hers (1976). The concentrations of four of the regulatory cations were therefore examined in the livers of control, diabetic and insulin-treated diabetic animals. Diabetic animals showed an approx. 20% decrease in total tissue Ca^{2+} (Table 8).

Table 8. *Cation concentrations in control, streptozotocin-induced-diabetic and insulin-treated diabetic rat livers*

The preparation of tissue extracts for cation analyses is described in the Experimental section. Concentrations of cations were calculated by assuming that tissue dry weight is 30% of tissue wet weight. Values are the means ± S.E.M. for four animals. P values relative to the control values were obtained by Student's t test: ***$P < 0.005$.

Animals	Concn. (μmol/g of liver)			
	Mg^{2+}	Ca^{2+}	Na^+	K^+
Control	7.78 ± 0.53	0.91 ± 0.06	20.5 ± 0.5	85.8 ± 2.2
Diabetic	7.43 ± 0.33	0.71 ± 0.05***	20.4 ± 0.6	84.5 ± 2.3
Insulin-treated diabetic	7.58 ± 0.52	0.85 ± 0.06	19.9 ± 0.5	91.1 ± 3.0

No differences in the concentrations of other cations were found among these three groups. Whether Ca^{2+} plays a role in the activation of phosphatase is not known, but, like cyclic GMP, cellular concentrations of Ca^{2+}, when added to liver extracts, also had no effect on phosphatase or the inhibitor activity. Whether the insulin effects are exerted by the alterations in the substrates for phosphoprotein phosphatase are also not known. Thus the mediator through which insulin (i) activates liver phospho-protein phosphatase and (ii) decreases the amount of inhibitor of phosphatase still remains unknown, and further studies are required to identify it.

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