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Glycogen Synthesis in the Perfused Liver of Streptozotocin-Diabetic Rats

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1. Net glycogen accumulation was measured in sequentially removed samples during perfusion of the liver of starved streptozotocin-diabetic rats, and shown to be significantly impaired, compared with rates in normal (starved) rats. 2. In perfusions of normal livers with glucose plus C_3 substrates, there was an increase in the proportion of glycogen synthetase '*a*', compared with that in the absence of substrates. This response to substrates, followed in sequential samples, was less marked in diabetic rats. 3. The impairments in net glycogen synthesis and enzymic sensitivity in the perfused liver of diabetic rats were reversed by pretreatment *in vivo* with glucose plus fructose, or insulin. Glucose alone did not produce this effect. 4. Glucose, fructose, insulin or cortisol added to the perfusion medium (in the absence of pretreatment *in vivo*) did not stimulate glycogen synthesis in diabetic rats. 5. In intact diabetic rats, there was a decline in rates of net hepatic glycogen accumulation, and the response of glycogen synthetase to substrates. The most rapid rates of synthesis were obtained after fructose administration. 6. These results demonstrate that there is a marked inherent impairment in hepatic glycogen synthesis in starved diabetic rats, which can be rapidly reversed *in vivo* but not in perfusion. Thus hepatic glycogen synthesis does not appear to be sensitive to either the short-term direct action of insulin (added alone to perfusions) or to long-term insulin deprivation *in vivo*. The regulatory roles of substrates, insulin and glycogen synthetase in hepatic glycogen accumulation are discussed.

In animals with diabetes caused by insulin deficiency, impairment of glucose tolerance is partially attributable to alterations in hepatic metabolism, including excessive production of glucose by gluconeogenesis (Renold *et al.*, 1953; Haft, 1968; Exton *et al.*, 1972, 1973*a*,*b*; for reviews see Renold *et al.*, 1956; Steiner, 1966; Exton *et al.*, 1970; Exton, 1971). The high blood glucose concentration in diabetes is commonly associated with a diminished hepatic glycogen content, (compared with that in matched fed normal animals) implying that there is a defect in the accumulation or retention of glycogen in the liver. Thus, in diabetes, hexose phosphate products of gluconeogenesis tend to form free glucose (in blood) rather than glycogen (Friedmann *et al.*, 1970; Exton *et al.*, 1972). After insulin injection, the glycogen content of the liver is replenished (Steiner, 1964). Many experiments with ^{14}C-labelled precursors have demonstrated impaired glycogen synthesis in diabetes (for references see Renold *et al.*, 1956; Steiner, 1966; Friedmann *et al.*, 1970; Hornbrook, 1970).

The significance of some of the above studies of glycogen metabolism is not always clear. For example, experiments with fed diabetic animals may not distinguish between a defect in the capacity for storage of hepatic glycogen [which may be limited in diabetes (Hornbrook, 1970; Friedmann *et al.*, 1967)], and a defect in the rate of glycogen synthesis. Also, it is not established which aspects of the altered hepatic glycogen metabolism in diabetes reflect inherent changes within the organ, rather than changes in circulating constituents which can control hepatic metabolism. Finally, there is uncertainty about whether the changes in hepatic glycogen metabolism result from a decline in direct (short-term or long-term) insulin actions on the liver.

Insight into the impairment of hepatic glycogen metabolism in diabetes may be gained from experiments in which net hepatic glycogen accumulation is followed in the isolated liver, in conditions where glycogen synthesis in normal rats is known to occur at rates similar to those in the intact animal (Hems *et al.*, 1972). Such experiments, with the perfused liver of the rat, are reported in the present paper. In particular a marked impairment in the net synthesis of glycogen in the perfused liver of diabetic rats is described and shown to be rapidly reversible by carbohydrate (including fructose) or insulin, if administered *in vivo*, but not if added to the perfusion medium. The relationship between hepatic glycogen synthesis and the activities of glycogen synthetase and phosphorylase has also been investigated; again, the role of insulin in regulating these enzymes is not clear (see reviews by Pilkis & Park, 1974; Ryman & Whelan,

1971, sections VID and VIIIB). A part of the study has been reported in preliminary form (Whitton & Hems, 1974).

Materials and Methods

Animals

Albino male Sprague–Dawley rats, weighing about 200g, were fed on a standard (Thompson's) supplemented cereal diet. Diabetes was induced with streptozotocin [75mg/kg (see Junod *et al.*, 1969; Hoftiezer & Carpenter, 1973)] dissolved in 0.01M-sodium citrate buffer, pH4.5, given intravenously under diethyl ether anaesthesia to fed rats, weighing about 220g. The blood glucose concentration was determined 4 days after the injection, after bleeding from a tail vein, under ether anaesthesia. Rats which had lost weight (during this 4 days), and had a blood glucose concentration exceeding 12mM, were selected for the experiments. Diabetic rats were not insulin-maintained; occasional deaths occurred, especially during starvation. All diabetic rats were used within 11 days after the injection. Both normal and diabetic rats were starved for 48h before use, from about 10:00h. In occasional (untreated) rats (normal or diabetic) the initial glycogen concentration in the perfused liver was unusually high (>50μmol of glucose/g); such livers exhibited high apparent rates of further net glycogen synthesis (1.0–2.0μmol of glucose/min per g). We have no explanation for this phenomenon (which could reflect coprophagy): results from such livers have not been included in the data.

Chemicals

Chemicals were the highest grade commercially available (for sources see Hems & Whitton, 1973; Hems *et al.*, 1972). Amino acids, D-fructose and cortisol (hydrocortisone) 21-sodium succinate were from Sigma Chemical Co. Ltd. (London S.W.6, U.K.). Streptozotocin from Upjohn Ltd. (Kalamazoo, Mich., U.S.A.) was recrystallized from ethanol. Enzymes and substrates for enzymic assays or analysis were from C. F. Boehringer Corp. (London) Ltd. (London W.5, U.K.). Radioactive isotopes were from The Radiochemical Centre (Amersham, Bucks., U.K.). Anti-insulin (and control) serum was prepared from guinea pigs (Mansford, 1967).

Perfusion of the liver

Liver perfusion was carried out with 50ml of bicarbonate-buffered saline containing albumin and erythrocytes, essentially as described by Hems *et al.* (1972) except that the gas phase was air+CO_2 (95:5) after a period of 5min with O_2+CO_2 (95:5). Glucose was added to the medium so that the initial concentration was about 28mM, except in cases where glycogen accumulation was not being studied. If present, lactate, glycerol and pyruvate were added to the perfusion medium (5.0, 3.33 and 1.67mM respectively) and then infused at 3ml/h from 15min after the start of perfusion in a mixture containing 0.50M-sodium lactate, 0.33M-glycerol and 0.17M-sodium pyruvate. In other perfusions, fructose was present (initially 5mM, and then infused at 0.5M, 3ml/h). In some perfusions a supplemented medium was used [essentially as described by John & Miller (1969)], in which were present initially, in addition to carbohydrate substrates: insulin (500munits), cortisol 21-sodium succinate (1mg) and a mixture of all the normal plasma amino acids, except cystine, proline and hydroxyproline [glutamine 2.5mM initially, and other values, each initially at about four times the normal concentrations in fed rats, taken from Scharff & Wool (1966)]. These constituents were maintained by infusion (1.5 or 3ml/h respectively) of separate solutions (in water) containing insulin (330munits/ml) plus cortisol (330μg/ml), or amino acids (at approximately 100 times the normal plasma concentrations: see above) except for tyrosine which was not sufficiently soluble, and was therefore added as a solid to each perfusion (8mg).

Liver samples (in sequence during perfusion) and $HClO_4$ extracts of perfusion medium, were obtained as described (Hems *et al.*, 1972).

In some experiments, rats were pretreated for 50min *in vivo*, followed by perfusion. Glucose (1.25M) plus fructose (0.25M) was given subcutaneously (2ml). When either hexose was given singly, 2ml of 1.5M-glucose or 2ml of 0.25M-fructose was administered. Guinea-pig control serum (0.2ml) or anti-insulin serum (0.2ml) were given intravenously without anaesthesia. Insulin (1 unit) was injected subcutaneously in 1ml of water.

Measurement of glycogen accumulation in intact rats

Glycogen synthesis in intact rats was followed by analysis of sequential liver samples during infusion of substrates into a tail vein (Hems *et al.*, 1972). Rats were anaesthetized with Nembutal, and glucose (0.5–1.5M) was infused by means of a Delta pump (Watson–Marlow Ltd., Falmouth, Cornwall, U.K.) at 3ml/h. In some infusions the glucose was supplemented by other substrates: either 0.25M-fructose, or glycerol, serine and pyruvate (each 0.33M). Some rats received glucose, fructose or C_3 substrates intragastrically (under light ether anaesthesia) or subcutaneously before Nembutal.

Preparation of liver samples

Liver samples obtained after perfusion or cervical dislocation (*in vivo*) were immediately frozen in liquid

N_2, and were stored (–20°C) for up to 2 weeks as required. This did not significantly affect enzyme activities (compared with those in fresh homogenates). Pieces of frozen liver were ground in liquid N_2, followed by isolation of glycogen (Good *et al.*, 1933), or extraction in 4vol. of $HClO_4$ (6%, w/v) before analysis of intermediate contents (Hems & Brosnan, 1970), or homogenization in buffers as required for enzyme assays.

Analytical methods

Glycogen synthetase, in the presence and the absence of glucose 6-phosphate (i.e. total and synthetase '*a*' activities respectively) and phosphorylase were assayed essentially as described by Das & Hems (1974). In the usual synthetase assay (method 1), centrifuged homogenates were incubated with 6.7mM-UDP-glucose. In one other group of experiments (method 2) homogenates were not centrifuged, and details of the assay were altered as follows: final UDP-glucose concentration, 12mM (see Blatt & Kim, 1971*b*); incubation time 10min; for assay of 'a' form, 10mM-Na_2SO_4 was present (De Wulf *et al.*, 1968). Phosphorylase was extracted in 35mM-α-glycerophosphate buffer, pH6.1, containing 1mM-EDTA and 20mM-NaF, and assayed in the presence of AMP (1mM) to systematize the contribution of phosphorylase '*b*' to the activity recorded. UDP-glucose and glucose 6-phosphate were determined as described by Mills & Smith (1963) and Hohorst (1963) respectively. The analyses were carried out in sequence in the cuvette. The second reaction, for UDP-glucose measurement, was initiated with 20μl of UDP-glucose pyrophosphorylase [EC 2.7.7.9; C. F. Boehringer Corp. (London) Ltd.] after the addition of phosphoglucomutase and potassium pyrophosphate, which did not itself produce any changes in E_{340}; recovery of UDP-glucose was 85%. Glycogen and glucose were determined as described elsewhere (Hems *et al.*, 1972).

Results

Glycogen accumulation in the perfused liver of starved diabetic rats

In livers of starved diabetic rats, there was a marked decrease in rates of net glycogen accumulation, compared with rates in starved normal rats (Fig. 1; cf. Tables 1 and 2), in which a linear time-course of net glycogen accumulation in the perfused liver was maintained for at least 80min (Fig. 1). The defect in net glycogen synthesis in livers of diabetic rats, observed in perfusions with glucose plus either fructose or a mixture of C_3 substrates (lactate, glycerol and pyruvate), was completely reversed by the administration of a mixture of glucose plus fructose

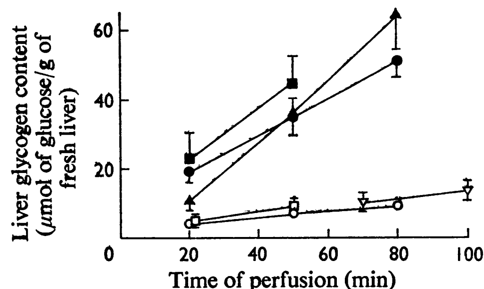

Fig. 1. *Time-course of net glycogen accumulation in normal and diabetic rats*

Livers were perfused with bicarbonate-buffered saline containing erythrocytes, and additions as indicated. Two samples [median followed by left lateral lobe (see Hems *et al.*, 1972)] or three samples (above two preceded by right lobe) were removed in sequence. ▲, ■, ●, Normal (starved) rat livers; ▽, □, ○, diabetic rat livers. Additions were as follows: (i) glucose (30mM) plus C_3 substrates (▲, □, six perfusions; ■, 12 perfusions); (ii) supplemented medium, including insulin, cortisol and amino acids, plus glucose, 30mM (●, five perfusions; ○, two perfusions), or glucose (20mM initially) and fructose, 5mM (▽, four perfusions). Other details are given in the text. Results are mean values, and bars indicate the S.E.M.

in vivo, 50min before perfusion (Table 1). Glucose alone was not effective in correcting impaired synthesis (in a dose equal to that of glucose and fructose together, i.e. 3mmol), and neither was fructose (0.5mmol) in the absence of glucose (Table 1). Insulin treatment for similar periods (50 or 75min) largely restored the rate of glycogen synthesis (Table 1). The possibility that insulin was implicated in the response to hexose pretreatment *in vivo* was investigated by administering anti-insulin serum in conjunction with glucose plus fructose; this decreased the rate of eventual glycogen synthesis, compared with that in perfusion after treatment *in vivo* with glucose, fructose and control serum (Table 1). Insulin in the medium, during perfusion of diabetic rat livers (in the absence of pretreatment *in vivo*) did not increase the rate of glycogen synthesis from glucose plus fructose or other substrates (results not shown in detail; see also next section).

In all conditions, there was net output of glucose during perfusion of livers of diabetic rats (Table 1), as in livers of normal starved rats perfused in these conditions [even during maximum glycogen deposition (Hems *et al.*, 1972)]. If glucose was not added in perfusions of diabetic rat livers, no glycogen accumulated, and the rate of net gluconeogenesis (from the C_3-substrate mixture) was $1.11\pm0.20(3)\mu$mol of glucose/min per g of fresh liver (mean $\pm$ S.E.M.). Thus the capacity for glucose synthesis was sufficient to

Table 1. *Glycogen accumulation during perfusion of livers from streptozotocin-diabetic rats*

Livers from 48h-starved diabetic rats were perfused for 50min with bicarbonate–albumin–saline containing washed erythrocytes, as described by Hems *et al.* (1972). Substrates were added after 15min. Glucose was initially 28mM, and the C_3 substrates, lactate, glycerol and pyruvate, were initially 5, 3.3 and 1.3mM (respectively), and then infused (see Hems & Whitton, 1973). Fructose was initially 5mM, and then infused (0.5M, 3ml/h). Pretreatments were administered subcutaneously 50min before perfusion: glucose plus fructose in 2ml (1.25 and 0.25M respectively), fructose (2ml, 0.25M) or glucose (2ml, 1.5M). Other details are given in the text. Results are means±S.E.M.; mean rates of glycogen accumulation were calculated from rates in individual perfusions (see Hems *et al.*, 1972).

		Average glycogen content (μmol of glucose per g of fresh liver)		Calculated rate of glycogen accumulation (μmol of glucose/min per g)	Glucose concentration (mM)	
		20min	50min		20min	50min
Perfusions with glucose, lactate, glycerol and pyruvate						
Pretreatment	No. of perfusions					
None	6	3.5	9.3	0.19±0.04	27.1±0.9	28.7±1.3
Glucose, fructose	6	48.1	72.3	0.81±0.12	29.4±1.2	32.0±1.4
Glucose, fructose, anti-insulin serum	4	6.9	18.6	0.39±0.08	29.3±1.0	31.5±1.1
Glucose, fructose, control serum	4	18.6	39.1	0.68±0.18	28.2±1.3	29.9±1.6
Insulin (50min)	5	11.3	26.6	0.51±0.15	27.5±0.8	29.0±0.9
Insulin (75min)	3	13.0	31.6	0.62±0.24	28.7±0.9	30.9±0.6
Perfusions with glucose plus fructose						
Pretreatment						
None	7	7.1	17.7	0.35±0.07	25.6±0.9	28.5±1.1
Fructose	3	3.5	9.6	0.20±0.06	30.4±1.6	33.7±1.3
Glucose	3	42.9	54.3	0.38±0.05	30.6±0.9	32.7±0.9
Glucose, fructose	9	25.6	50.8	0.84±0.13	31.0±0.8	33.6±1.2

support normal net glycogen accumulation [0.68–0.82μmol/min per g; see Fig. 1 and Hems *et al.* (1972)].

Perfusions of livers with supplemented medium, including insulin

In an attempt to increase glycogen synthesis rates during perfusion of livers from diabetic rats, the standard medium, containing carbohydrate substrates, was supplemented with further substrates and hormones. Two groups of perfusions were carried out. In one group, events during pretreatment with glucose plus fructose were simulated in the initial phase (about 1h) of perfusion: the medium contained fructose (initially 5mM, not subsequently infused), glucose (20mM) and insulin, cortisol and amino acids [all infused essentially as described by John & Miller (1969); see the Materials and Methods section]. After 50min, more glucose was added to bring the concentration to about 30mM (optimum for glycogen synthesis (Hems *et al.*, 1972)], and C_3 substrates were added, as in standard perfusions. Net glycogen accumulation, determined between 70 and 100min (Fig. 1) was no faster than in perfused livers of other non-pretreated diabetic rats. Thus fructose, glucose and insulin were not effective in restoring glycogen synthesis if added to the perfusate (in the absence of pretreatment *in vivo*).

In a second group of perfusions of diabetic rat livers, the medium was supplemented with insulin, cortisol and amino acids (as above, but without fructose) in addition to the standard conditions of glucose (30mM) plus C_3 substrates. Liver samples were removed after 20 and 50min. Again, there was no improvement in the low rate of glycogen accumulation (Fig. 1). In control experiments, livers from normal (starved) rats were perfused with this supplemented medium; net glycogen synthesis (Fig. 1) occurred at a rate similar to that in perfusions with glucose plus C_3 substrates (Fig. 1) or fructose (Table 2). Thus supplementation did not inhibit synthesis in normal (starved) livers.

Control of glycogen synthesis in the perfused liver of normal rats

To gain insight into the impairment of net glycogen accumulation in the perfused liver of diabetic rats the activities of the enzymes glycogen synthetase and phosphorylase were measured (see the next two sections). In a preliminary study, the behaviour of these

Table 2. *Concentrations of enzymes and pathway intermediates during glycogen synthesis in the perfused liver of starved rats*

Livers from 48h-starved rats were perfused as described in Table 1, for 50min (except those with glucose alone: 60min). Enzymes, glycogen and metabolites were assayed in frozen samples of the left lateral lobe. An initial sample (medium lobe) was removed after 20min to measure glycogen, and the rate of glycogen accumulation in the left lateral lobe was calculated from the difference between lobe contents [as in Table 1, and see Hems *et al.* (1972)]. Other details are given in the text. Results are means±S.E.M. of the no. of observations indicated; mean percentages of synthetase '*a*' were calculated from % '*a*' values in individual samples.

Additions to medium	No. of perfusions	Enzyme assays (μmol/min per g of fresh liver): Glycogen synthetase (method 1) '*a*'	Glycogen synthetase (method 1) Total	Glycogen synthetase (method 1) '*a*' (%)	Glycogen phosphorylase	Metabolite content (nmol/g): UDP-glucose	Glucose 6-phosphate	Rate of net glycogen accumulation (μmol of glucose/min per g)
None	3	0.14±0.01	0.38±0.04	38±3	5.6±1.0	28± 7 (3)	<20 (3)	0
Glucose	3	0.18±0.03	0.37±0.05	48±3	3.6±0.3	96±12 (4)	48± 8 (4)	0.21±0.08
C_3 substrates	4	0.17±0.02	0.42±0.11	46±8	7.3±1.1	89±13 (5)	51± 6 (5)	0†
Glucose plus C_3 substrates	6	0.20±0.01	0.34±0.02	61±3	3.4±0.5	52±11 (10)	71±11 (10)	0.75±0.10
Glucose plus fructose	3	0.16±0.02	0.21±0.03	80±4	1.4±0.4	—	—	0.84±0.07
Glucose plus C_3 substrates	4*	0.17±0.06	0.20±0.05	83±7	2.3±0.5	—	—	0.54±0.19

* Supplemented medium (see Fig. 1).
† Net rate of gluconeogenesis 1.22±0.10 (5).

enzymes was characterized in the perfused liver of normal (starved) rats, in which maximum glycogen synthesis occurs during perfusion with glucose plus gluconeogenic substrates (Hems *et al.*, 1972). In these conditions, the hepatic concentrations of UDP-glucose and glucose 6-phosphate were measured (Table 2); the latter value was of the same order as that reported for perfused livers of starved rats (Ross *et al.*, 1967). During maximum glycogen synthesis, the concentration of UDP-glucose was decreased, compared with that (in the presence of substrates) where glycogen accumulation was slower (Table 2), suggesting that the glycogen synthetase reaction was accelerated. This inference was confirmed, since during maximum glycogen accumulation in the presence of glucose plus either fructose or a mixture of gluconeogenic C_3 substrates, an increased proportion (60–80%) of glycogen synthetase was present as the '*a*' form [also known as 'I', an active form *in vivo* (Hers *et al.*, 1970)]. This suggests that the percentage of synthetase in the '*a*' form may reflect the functional state of activation of synthetase, i.e. its capacity to bring about net glycogen deposition. There was decreased activity of glycogen phosphorylase in perfusions with glucose (Table 2), which was in itself not a sufficient condition for maximum net glycogen synthesis, as shown by the relatively low rate of synthesis in perfusions with glucose alone (Table 2). These effects of substrates and glucose on glycogen synthetase and phosphorylase during perfusion of normal (starved) rat livers generally agree with the reported effects of glucose (Glinsmann *et al.*, 1970; Buschiazzo *et al.*, 1970; Miller *et al.*, 1973) and fructose (Walli *et al.*, 1974).

Glycogen synthetase and phosphorylase activities in vivo

The activities of glycogen synthetase and phosphorylase in intact normal and streptozotocin-diabetic rats were compared (Table 3). In starved diabetic rats, compared with normal starved rats, there was a moderate decrease in the proportion of synthetase '*a*' and an associated slight increase in phosphorylase (expressed per g). These enzymes were assayed simultaneously in different lobes in the liver, as a prerequisite to the interpretation of their activities in lobes removed in sequence during perfusion (see the next section). There were no major differences between lobes in the activities of synthetase and phosphorylase (Table 3).

Glycogen synthetase and phosphorylase in perfused livers of diabetic rats

After 50min perfusion with glucose plus either fructose or a mixture of gluconeogenic C_3 substrates (unsupplemented medium) the proportion of glycogen synthetase in the '*a*' form (method 1) was 51–

Table 3. *Activities of glycogen synthetase and phosphorylase in intact starved normal and starved streptozotocin-diabetic rats*

Enzyme activities in simultaneously sampled lobes of livers from 48h-starved rats were assayed as described in the text. Results are means±S.E.M. from three normal and four diabetic rats.

		Glycogen synthetase (μmol/min per g; method 1)			
Rat	Lobe	'*a*'	Total	'*a*' (%)	Glycogen phosphorylase (μmol/min per g)
Normal	Median	0.10±0.02	0.22±0.06	49±6	5.8±1.4
Normal	Left lateral	0.13±0.01	0.32±0.05	45±7	5.1±1.2
Normal	Right	0.09±0.02	0.20±0.04	43±3	4.5±0.7
Diabetic	Median	0.14±0.02	0.37±0.01	39±5	6.1±0.8
Diabetic	Left lateral	0.11±0.02	0.33±0.02	34±8	6.4±0.6
Diabetic	Right	0.13±0.04	0.35±0.07	38±6	6.5±0.3

68%. In contrast with the results in normal (starved) rat livers, there was no correlation between synthetase '*a*' activity (at 50min) and the net rate of glycogen accumulation. In most conditions the percentage of synthetase in the '*a*' form in the initial sample (removed after 20min perfusion) was 23–48%, which resembled that in intact diabetic rats (Table 3). In two groups of perfusions, where the liver was in contact with fructose for about 100min (*in vivo* and in perfusion) the percentage of synthetase '*a*' was higher (Table 4): (i) pretreatment and perfusion with glucose plus fructose (65 and 64% '*a*'), (ii) no pretreatment *in vivo*, but prolonged perfusion with glucose and fructose in supplemented medium (68 and 78% '*a*'). These high values for synthetase '*a*' in perfusion were associated with rapid glycogen accumulation in the former situation, but not the latter (Table 4; see also Table 1 and Fig. 1).

The activities of glycogen phosphorylase in liver samples removed during perfusion were lower than those in intact diabetic rats. The activities in different conditions, at either 20 or 50min, did not conform to any pattern (results not shown).

Changes in glycogen synthetase and phosphorylase during perfusion were assessed from the activities in sequential liver samples. This was valid because of the lack of any difference in activities in lobes sampled simultaneously (Table 3). In the livers of diabetic rats in which pretreatment had restored net glycogen accumulation, there was an increase in the proportion of glycogen synthetase '*a*' (Table 4) which presumably reflected the activating effect of substrates on synthetase, as observed in perfused livers of normal (starved) rats (Table 2). This response was much less marked in perfusions of diabetic rat livers when glycogen synthesis was low (Table 4). In perfusions with fructose there was no such relationship; thus in the absence of pretreatment, in perfusions with or without supplemented medium, glycogen synthesis was not rapid, despite the extensive conversion of synthetase into the '*a*' form during perfusion (Table 4), which resembled that in normal rats (Table 2). Conversely, when pretreatment was followed by rapid net glycogen synthesis in the perfused liver, synthetase '*a*' did not increase during perfusion, being already high initially (65%; Table 4).

In the experiments described above, the alteration in the percentage of synthetase '*a*' during perfusion was manifested as a fall in the total synthetase (Table 4) rather than in the absolute value of synthetase '*a*'. This may have reflected alteration in the distribution of synthetase in centrifuged homogenates, or could have been a subtle consequence of the action of modifiers in the homogenates, if UDP-glucose was not at saturating concentrations (see discussions by Bishop & Larner, 1967; Blatt & Kim, 1971*b*). To distinguish between these possibilities, key groups of perfusions were repeated, and details of the synthetase assay were altered (method 2). The results are shown in Table 5. In uncentrifuged homogenates, the decline in total synthetase during perfusion was no longer present. Also the activities obtained with method 2 were greater. These disparities could be due to particle-bound activity (lost during method 1) and the use of a shorter assay time and higher UDP-glucose concentration in method 2. The main findings from the experiments using method 1 were confirmed, i.e. that the proportion of synthetase '*a*' (also the absolute value) increased during perfusions of livers of diabetic pretreated rats, but not in livers of untreated rats, whether or not insulin was present in the perfusion medium (Table 5). The rates of net glycogen deposition (not shown) resembled those in Table 1; with insulin in the perfusate, the average rate was 0.26μmol of glucose/g (measured in three perfusions), confirming the lack of direct insulin action on net glycogen synthesis.

There was a decline in the activity of glycogen phosphorylase between 20 and 50min during perfusion of diabetic rat livers, as would be expected since glycogen synthetase and phosphorylase often exhibit reciprocal changes. The extents of the change in each enzyme were correlated. For example, there was relatively little decline in phosphorylase during standard perfusions with glucose plus C_3 substrates, of livers from diabetic rats [0.53±0.26(4)μmol/min

Table 4. *Glycogen synthetase activity in the perfused liver of starved streptozotocin-diabetic rats*

Livers were perfused as described in Table 1, with various substrates and after different pretreatments. Glycogen synthetase was assayed in liver samples removed after 20 and 50 min. Details are in the text, or Table 2. Other results from these perfusions are included in Table 1. Results are means ±S.E.M.

		Glycogen synthetase (μmol/min per g of fresh liver; method 1)						
		20min			50min			Calculated rate of glycogenaccumulation (μmol of glucose/min per g)
Pretreatment	No. of perfusions	'a'	Total	'a' (%)	'a'	Total	'a' (%)	
Perfusions with glucose, lactate, glycerol and pyruvate								
None	4	0.13±0.01	0.30±0.05	48±6	0.11±0.02	0.19±0.03	60±3	0.17±0.09
Glucose, fructose	4	0.13±0.02	0.38±0.07	35±6	0.16±0.01	0.27±0.03	64±9	0.86±0.17
Glucose, fructose, anti-insulin serum	4	0.14±0.15	0.32±0.05	46±5	0.14±0.02	0.25±0.06	61±9	0.39±0.08
Glucose, fructose, control serum	4	0.10±0.03	0.23±0.03	42±8	0.09±0.01	0.16±0.01	56±9	0.68±0.18
Insulin	4	0.13±0.01	0.29±0.02	46±2	0.15±0.04	0.23±0.05	64±6	0.59±0.16
None*	3	0.16±0.04	0.23±0.04	68±6	0.17±0.04	0.22±0.05	78±4	0.13±0.02
Perfusions with glucose plus fructose								
Pretreatment								
None	4	0.14±0.02	0.43±0.04	34±5	0.18±0.06	0.25±0.05	68±9	0.38±0.10
Fructose	2	0.09	0.33	28	0.09	0.18	51	0.19
Glucose	3	0.12±0.01	0.33±0.06	39±7	0.13±0.02	0.20±0.01	66±15	0.38±0.05
Glucose, fructose	4	0.16±0.04	0.25±0.04	65±9	0.12±0.01	0.19±0.02	64±5	0.86±0.21

* Supplemented medium, samples at 70 and 100min (see Fig. 1).

Table 5. *Glycogen synthetase activity in the perfused liver of starved streptozotocin-diabetic rats*

Livers were perfused as described in Tables 1 and 4, with glucose, lactate, glycerol and pyruvate, and glycogen synthetase was assayed (Method 2). In one group (*) insulin was infused into the perfusion medium: 1.5ml/h of a solution containing 330munits/ml, after an initial dose of 500munits. Other details are in the text or Table 2. Results are means ±S.E.M.

		Glycogen synthetase (μmol/min per g of fresh liver; method 2)					
		20min			50min		
Pretreatment (*in vivo*)	No. of perfusions	'*a*'	Total	'*a*' (%)	'*a*'	Total	'*a*' (%)
None	3	0.52±0.09	0.84±0.11	62±7	0.55±0.4	1.05±0.12	53±4
Glucose, fructose	3	0.64±0.05	0.98±0.09	66±6	0.83±0.10	1.11±0.08	75±6
Insulin (1 unit, 75min before perfusion)	4	0.60±0.16	1.12±0.23	52±3	0.78±0.15	1.12±0.18	69±5
None*	6	0.51±0.07	0.99±0.07	51±6	0.52±0.09	0.99±0.08	52±6

* Insulin added to perfusion medium.

per g of fresh liver; results expressed as mean±S.E.M.], compared with the greater decrease in perfusions after pretreatment *in vivo* [1.20±0.38(4) μmol/min per g], in which net glycogen synthesis was more rapid (Tables 2 and 4). Taking all groups, there was, however, no clear-cut (inverse) correction between the net rate of glycogen accumulation and the decline in phosphorylase activity during perfusions (with or without fructose).

The above results demonstrated that the response of enzymes to glucose plus C_3 substrates during perfusion (measured in sequential samples) was impaired in diabetic rat livers, and restored after pretreatment with hexose or insulin *in vivo*.

Glycogen accumulation in intact normal and diabetic rats

For comparison with results in the perfused liver, glycogen accumulation was followed in anaesthetized intact streptozotocin-diabetic rats. There was an impairment in the capacity for net glycogen synthesis during infusion of either glucose or glucose plus a mixture of glycerol, serine and pyruvate (Table 6). As in the experiments with the perfused liver, fructose was required to bring about rapid glycogen deposition in diabetic rats (Table 6).

Influence of glucose and fructose on the activity of glycogen synthetase and phosphorylase in vivo

The effects of fructose and glucose *in vivo* on glycogen synthetase were measured (Table 7). In normal rats, glucose and fructose each induced conversion of synthetase into the '*a*' form. Glucose and fructose in combination brought about the greatest 'activation' of the enzyme (Table 7), in agreement with observations in the perfused liver (Table 2). In diabetic rats these effects were less marked (Table 7), in general agreement with Kreutner & Goldberg (1967) and Miller *et al.* (1973), and again confirming results in the perfused liver (Table 4). Administration of the hexoses, either alone or in combination, also produced a decline in phosphorylase activity in normal rats (to about 40% of the values in Table 3); the effect of fructose in combination with glucose was not greater than that of either hexose singly, and the response in diabetic rats was not impaired (results not shown in detail).

Discussion

Impairment of hepatic glycogen accumulation in diabetes

For a variety of reasons (see the introduction section) it has not been clear whether there is a significant and inherent alteration in maximum rates of net hepatic glycogen accumulation in diabetes. The streptozotocin-diabetic animal provides a suitable model for the clarification of this problem (Junod *et al.*, 1969). The changes in glycogen metabolism described in the present paper reflect the consequences of insulin-deficiency diabetes, rather than hepatic toxicity of streptozotocin, because they were reversible by insulin and hexoses *in vivo*.

The results show that there is a marked inherent impairment in the maximum rate of net glycogen accumulation in the liver in starved diabetic animals. In all groups of perfusions of the livers of diabetic rats (in the absence of pretreatment *in vivo*), rates of net glycogen synthesis were low compared with those in normal (starved) rats. This result confirms, by the measurement of net glycogen deposition in optimum conditions, the inherent impairment of hepatic glycogen synthesis in diabetes which had been inferred from measurements of incorporation of ^{14}C from ^{14}C-labelled precursors into glycogen in the perfused liver (Exton *et al.*, 1972, 1973*a*) or in liver slices (for reviews see Levine & Fritz, 1956; Renold *et al.*, 1956; Steiner, 1966).

Table 6. *Glycogen accumulation in intact starved normal and starved streptozotocin-diabetic rats*

Glycogen accumulation was followed in intact rats (see Hems *et al.*, 1972). Rats received various pretreatments (subcutaneously in two portions of 1 ml, unless indicated: see Table 1), were then anaesthetized with Nembutal, and received an infusion (3 ml/h) into a tail vein of glucose (1–1.25 M), fructose (0.25 M) or gluconeogenic C_3 substrates (a mixture of glycerol, serine and pyruvate, each 0.33 M, also used for pretreatment). The first liver sample was removed within about 15 min of anaesthesia, and the second 1 h later. The indicated time of pretreatment refers to the interval between injection and the first sample. Other details are given in the text or Table 1. Results are means ± S.E.M.

Rats	Pretreatment	Infusion	No. of experiments	Average glycogen content (μmol of glucose per g of fresh liver)		Calculated rate of glycogen accumulation (μmol of glucose/min per g)	Final blood glucose concentration (mM)
				Sample 1	Sample 2		
Normal	None	Glucose	6	35.2	72.3	0.62±0.09	32±4
Normal	Glucose plus fructose (60 min)	Glucose plus fructose	4	110.6	155.0	0.74±0.12	36±7
Diabetic	Glucose (intragastrically 12 min)	Glucose	2	5.2*	7.4*	0.04	25
Diabetic	Glucose (53 min)	Glucose	3	77.2	70.6	<0	37±5
Diabetic	Glucose plus C_3 substrates (intragastrically 40 min)	Glucose plus C_3 substrates	3	11.5*	17.1*	0.11±0.10	64±17
Diabetic	Glucose plus fructose (intragastrically 35 min)	Glucose plus fructose	4	26.6	63.2	0.61±0.18	61±9
Diabetic	Glucose plus fructose (60 min)	Glucose plus fructose	3	72.1	109.9	0.63±0.08	40±3

* Samples 53 min apart.

Table 7. *Influence of glucose and fructose on glycogen synthetase in intact starved normal and streptozotocin-diabetic rats*

Glycogen synthetase activities in simultaneously sampled lobes from 48 h-starved rat livers were assayed (method 1) as described in the text. Treatments were administered in 2 ml subcutaneously 50 min before sampling: glucose (2.5 M), fructose (0.25 M) or glucose plus fructose (1.25 and 0.25 M respectively). Other details are in the text. Results are means ± S.E.M. from three rats, in all groups except fructose-treated diabetic rats (five animals).

Rat	Treatment	Glycogen synthetase (% 'a'; method 1)	
		Median lobe	Left lateral lobe
Normal	—*	49±6	45±7
Normal	Glucose	77±2	70±4
Normal	Fructose	60±5	69±4
Normal	Glucose plus fructose	83±7	81±6
Diabetic	—*	39±5	34±8
Diabetic	Glucose	50±5	57±7
Diabetic	Fructose	46±7	46±6
Diabetic	Glucose plus fructose	47±6	48±3

* From Table 3, for comparison.

In general, measurements of net glycogen synthesis in intact diabetic rats (Friedmann *et al.*, 1963, 1967; Hornbrook, 1970; Longley *et al.*, 1957), do not show such a marked impairment of synthesis as that revealed in the perfused liver. Thus the present perfusion experiments demonstrate the extent of the potential decrease in the maximum rate of glycogen synthesis in diabetes, that it is inherent to the liver, and its rapid reversibility (by treatment *in vivo*).

In experiments with intact diabetic animals, the decrease in the rate of hepatic glycogen synthesis can be shown by measurements of incorporation of ^{14}C from ^{14}C-labelled precursors into glycogen (Friedmann *et al.*, 1970; for reviews of earlier work see Steiner, 1966; Renold *et al.*, 1956). The general implication of these observations is that in diabetes, the hexose phosphate products of gluconeogenesis (produced at an increased rate) are directed towards free glucose formation, rather than glycogen [although the proportional contribution of gluconeogenesis to glycogen formation is increased (Stetten & Boxer, 1944)]. Results obtained in the perfused liver confirm this suggestion, in that during maximum glycogen synthesis, the net carbon sources of glycogen are gluconeogenic precursors, in either normal (starved) rat livers (Hems *et al.*, 1972) or in diabetic rat livers (present work). Net glucose uptake by the liver is not of significance in diabetes. However, glucose must be present during perfusion to permit glycogen deposition, even if net uptake of glucose does not occur. The mechanisms underlying this requirement for glucose are not clear (see also Seglen, 1974).

Measurements of net hepatic glycogen deposition in the liver of intact diabetic animals show that, as well as there being a defect in the rate of glycogen accumulation, there is a limitation in the amount of glycogen that can be stored (Hornbrook, 1970; Friedmann *et al.*, 1967). The present experiments did not relate to this aspect of hepatic glycogen metabolism in diabetes.

Restoration of hepatic glycogen accumulation in diabetes

Restoration of net glycogen synthesis in the perfused liver of starved diabetic rats was achieved by two procedures *in vivo*: (i) pretreatment with glucose plus fructose; (ii) insulin pretreatment (50–75min). This effect of insulin would be expected, as it has repeatedly been shown that the consequences of diabetes caused by pancreatic β-cell toxins may be reversed by insulin *in vivo*.

The results shed light on the origin of the inherent alteration in hepatic glycogen synthesis rates in diabetes. Since insulin produced no restoration of synthesis in perfusions lasting up to 100min, but induced restoration within about 75min, if administered *in vivo*, it follows that a decline in short-term direct actions of insulin on the liver cannot be a major contributory factor (Levine & Fritz, 1956). This conclusion agrees with the lack of action of insulin on net maximum glycogen synthesis in the perfused liver of normal (starved) rats (Hems *et al.*, 1972) and is not vitiated by the observation that insulin can suppress the breakdown of glycogen in the perfused liver (see Exton *et al.*, 1970) or in hepatocytes (Akpan *et al.*, 1974). Similarly, in studies with ^{14}C-labelled precursors, impairments in glycogen synthesis were not corrected by insulin in the perfused liver (Exton *et al.*, 1973*a*; Haft, 1968) or liver slices (Renold *et al.*, 1955).

Among possible factors (operating *in vivo*) by which insulin may affect the liver indirectly, the present experiments exclude any simple role for adrenal corticosteroids (since cortisol did not restore synthesis of glycogen), or for a fall in the blood concentration of glucagon or free fatty acids, since prolonged perfusion in the absence of these agents did not result in increased net glycogen accumulation.

The lack of a direct short-term action of insulin (added alone to perfusions of liver from diabetic or starved rats) on net glycogen accumulation, may be equated with its similar lack of action (when the sole hormone added) on glycogen breakdown during perfusion with undiluted blood (Mondon & Burton, 1971). However, if glucagon is also present in perfusion fluid, insulin can inhibit glucagon-induced glycogen breakdown (for reviews see Exton *et al.*, 1970; Pilkis & Park, 1974); it is probable that in conditions conducive to net glycogen accumulation (as in the present work), and at suitable hormone concentrations, insulin would counteract the inhibition of synthesis, which can be induced by glucagon (P. D. Whitton, unpublished experiments), and would 'stimulate' (i.e. de-inhibit) accumulation of glycogen.

The present experiments also show that the process of hepatic glycogen synthesis is not sensitive to a decline in direct long-term insulin action. This follows because the alterations in diabetic animals were reversible within 75min by procedures *in vivo* (tantamount to re-feeding), through mechanisms that did not include direct hepatic insulin action.

The restorative effect of hexoses on net glycogen synthesis (during eventual perfusion) appears to include a specific action of fructose. Thus the action of fructose plus glucose could not be reproduced by an equivalent quantity of glucose (3mmol) and anti-insulin serum did not completely prevent their action. Also, in the intact diabetic rat glucose alone or plus a mixture of C_3 substrates, did not restore net glycogen synthesis as effectively as did glucose plus fructose. This effect of fructose is reminiscent of its action in diabetic rats in restoring rates of fatty acid synthesis (Baker *et al.*, 1952). The mechanism of its action (yet to be fully explained) is likely to include production of glucose (and related metabolites) within the liver, since the liver is a main site of fructose utilization, and in diabetes this process is not impaired in the same way as that of glucose assimilation (Chernick & Chaikoff, 1951; Miller *et al.*, 1952). Additional factors may be implicated in the restorative effect of fructose and glucose *in vivo*. One such factor is likely to be insulin, which was effective over the same period (50min *in vivo*) in restoring near-maximum net glycogen synthesis (in eventual perfusion). Thus the restorative action of the hexoses was partially prevented by anti-insulin serum. Insulin secretion, although impaired, can be stimulated by administration of glucose to streptozotocin-diabetic rats (Junod *et al.*, 1969), and fructose may potentiate this action (Curry *et al.*, 1972).

Properties of glycogen synthetase in the perfused liver

There could be various origins for the impairment in the capacity for net glycogen synthesis in the liver of diabetic rats. The present experiments suggest that the explanation may reside at least partly in an inadequate responsiveness of the hepatic glycogen synthetase system to substrates. Thus during perfusion of livers from diabetic rats, in the absence of pretreatment, there was an impairment in the increase in glycogen synthetase '*a*' [an active form of the enzyme (Hers *et al.*, 1970)] which was observed in response to glucose plus C_3 substrates in normal rat livers. Similarly, if glucose alone is added to the medium perfusing the liver of diabetic rats (i.e. in conditions that are not conducive to maximum glycogen synthesis), impairment in the response of synthetase to glucose

may be observed (Miller *et al.*, 1973). In the present experiments, the impairment in the response of synthetase to substrates in diabetes was not complete, since fructose (in the presence of glucose) was able to activate synthetase in the perfused liver of diabetic rats (in the absence of pretreatment *in vivo*).

Effects of glucose on glycogen-metabolizing enzymes in the perfused liver have been reported (Buschiazzo *et al.*, 1970; Glinsmann *et al.*, 1970; Miller *et al.*, 1973). The present results show that gluconeogenic substrates can enhance this response, in the case of synthetase if not phosphorylase. Since optimum glycogen synthesis in the perfused liver of starved (normal or diabetic) rats involves gluconeogenic precursors as carbon sources, this action is relevant to the control of glycogen deposition.

An impaired activation of glycogen synthetase was also demonstrated in the intact diabetic rat, being most noticeable in the relative lack of response to glucose plus fructose, compared with that in normal (starved) rats. This is in general agreement with the results of Steiner (1964, 1966), Kreutner & Goldberg (1967) and Miller *et al.* (1973).

Insulin administration *in vivo* rapidly restored the sensitivity of glycogen synthetase and phosphorylase to glucose plus C_3 substrates in the diabetic liver (as it restored the associated low rates of net glycogen synthesis). This is unlikely to reflect a direct hepatic action of insulin, as shown by the results with method 2 in particular; thus the high proportions of synthetase '*a*' in the livers perfused with supplemented medium (including insulin) probably involved the action of fructose, observed in perfusions with fructose and glucose alone. Insulin effects on glycogen synthetase in isolated rat liver preparations [in contrast with frog liver (Blatt & Kim, 1971*a*)] are, in general, not extensive (Miller & Larner, 1973; Akpan *et al.*, 1974). An exception to this generalization is that insulin can counteract the rapid (inhibitory) actions of glucagon or adrenaline in the perfused liver on glycogen synthetase (Hostmark, 1973; Miller & Larner, 1973); this type of action may have a role in the rapid effect of insulin in intact diabetic animals, in which circulating glucagon concentrations are increased (Unger, 1972). However, it cannot be a major factor in the present results, as was explained above (second part of the Discussion section).

If direct short-term insulin action on hepatic glycogen synthesis (and synthetase) is not significant (in rats at least) then it follows that the effect of insulin on hepatic glycogen synthetase in intact animals (Bishop *et al.*, 1971; Blatt & Kim, 1971*b*; Nichols & Goldberg, 1972; Miller & Larner, 1973) does not involve direct action. This conclusion endorses in general that derived by Hers *et al.* (1970) from experiments with intact mice. Similar considerations also apply to the decrease in synthetase phosphatase activity in the liver of diabetic animals (Gold, 1970; Bishop, 1970); this activity can be restored by insulin *in vivo*, but perhaps not as a result of direct insulin action on the liver.

The behaviour of glycogen synthetase and phosphorylase *in vivo* and in perfusion suggest that fructose can exert specific effects on their activities (see Van den Berghe *et al.*, 1973) and in restoring their efficacy in the liver of diabetic rats. Thus fructose in combination with glucose in the perfusate produced the highest proportional activities of synthetase '*a*', in the liver of normal rats (*in vivo* or in perfusion). Also, in diabetic rats, fructose plus glucose *in vivo* resulted in the highest initial synthetase '*a*' in subsequent perfusions containing fructose. Anti-insulin serum did not completely prevent the action of fructose and glucose *in vivo* in increasing in the response of synthetase '*a*', during perfusion with C_3 substrates, suggesting that the effect of fructose (plus glucose) in correcting the impaired sensitivity of synthetase and phosphorylase to substrates in diabetes involves actions *in vivo* additional to the stimulation of insulin secretion. These are likely to include specific hepatic effects, such as a decline in the hepatic cyclic AMP content [which is raised in diabetes (Pilkis *et al.*, 1974)] and an increase in hepatic fructose 1-phosphate content; both these changes occur in response to fructose, being relevant to the associated decrease in glycogen phosphorylase activity (Van den Berghe *et al.*, 1973; Thurston *et al.*, 1974).

The attainment of a high proportion of synthetase '*a*' during perfusion with fructose shows that the enzymic apparatus for glycogen synthesis is not fundamentally lacking in the diabetic rat liver (see also Hornbrook, 1970). This also follows from the rapidity of the correction of impaired glycogen synthesis by pretreatment *in vivo*. Yet such activation by fructose was not a sufficient condition for maximum net glycogen accumulation. A possible explanation for this is the following. Maximum glycogen synthesis in the livers of normal (starved) animals involves glucose and other carbon sources acting in combination (Hems *et al.*, 1972). Glucose, although not providing carbon for glycogen synthesis, appears to exert an 'initiatory' effect on synthesis by influencing the fate of newly synthesized hexose phosphate, i.e. in directing this hexose to glycogen rather than free glucose. Presumably, in the diabetic rat liver, a component of this overall process is impaired. This deficiency cannot be in the process of gluconeogenesis, which is enhanced in diabetes. Also, the effect of fructose in the perfused liver suggests that the glycogen synthetase system is not defective. The defect which could not be corrected in the perfused liver (in the present study) may therefore involve the initiatory or cofactor role of glucose, which is implicated in net glycogen deposition in the normal liver (e.g. in the functioning of glycogen 'primer') and apparently in maintaining the full sensitivity of synthetase (to C_3

substrates as well as fructose). This would be in accord with the established impairment in hepatic glucose uptake in diabetes (see Steiner, 1966; Renold *et al.*, 1956), and would also explain why the hepatic glycogen deposition in diabetes can be corrected more rapidly than is accounted for by the restoration of glucokinase activity (Steiner, 1964), since this regulatory role of glucose could involve free glucose (Hers *et al.*, 1970) as well as metabolic products. The nature of the glucose effect on the liver is obscure; correction of this impaired glucose effect in diabetes also involves factors that are not clear, including insulin, but not solely (if at all) as a result of direct hepatic insulin action.

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