A Comparison of the Effects of Diabetes Induced with either Alloxan or Streptozotocin and of Starvation on the Activities in Rat Liver of the Key Enzymes of Gluconeogenesis

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1. Measurements of the activities in rat liver of the four key enzymes involved in gluconeogenesis, i.e. pyruvate carboxylase (EC 6.4.1.1), phosphoenolpyruvate carboxykinase (EC 4.1.1.32), fructose 1,6-diphosphatase (EC 3.1.3.11) and glucose 6-phosphatase (EC 3.1.3.9), have been carried out, all four enzymes being measured in the same liver sample. Changes in activities resulting from starvation and diabetes have been studied. Changes in concentration (activity/unit wet weight of tissue) were compared with changes in the hepatic cellular content (activity/unit of DNA). 2. Each enzyme was found to increase in concentration during starvation for up to 3 days, but only glucose 6-phosphatase and phosphoenolpyruvate carboxykinase showed a significant rise in content. Fructose 1,6-diphosphatase appeared to decrease in content somewhat during the early stages of starvation. 3. There was a marked increase in the concentration of all four enzymes in non-starved rats made diabetic with alloxan or streptozotocin, for the most part similar responses being found for the two diabetogenic agents. On starvation, however, the enzyme contents in the diabetic animals tended to fall, often with streptozotocin-treated animals to values no greater than for the normal overnight-starved rat. Deprivation of food during the period after induction of diabetes with streptozotocin lessened the rise in enzyme activity. 4. The results are compared with other published values and factors such as substrate and activator concentrations likely to influence activity *in vivo* are considered. 5. Lack of correlation of change in fructose 1,6-diphosphatase with the other enzymes questions whether it should be included in any postulation of control of gluconeogenic enzymes by a single gene unit.

Numerous workers have reported enhanced activity of one or other of the four key enzymes in the pathway to glucose from lactate or pyruvate that catalyse essentially irreversible steps, namely pyruvate carboxylase (EC 6.4.1.1) and phosphoenolpyruvate carboxykinase (EC 4.1.1.32), fructose 1,6-diphosphatase (EC 3.1.3.11) and glucose 6-phosphatase (EC 3.1.3.9). These enzymes appear to be present in lower activity than the other freely reversible enzymes of the glycolytic–gluconeogenic pathway (Scrutton & Utter, 1968) and are thus likely to be associated with rate-limiting steps in the synthesis of glucose. Although their activities are generally assumed to change in roughly parallel fashion in response to similar stimuli, there are only a few studies (e.g. Eisenstein & Strack, 1968; Filsell, Jarret, Taylor & Keech, 1969) in which the four enzymes have been measured simultaneously in any one liver sample. This seems to be a prerequisite before making any adequate assessment of the relative response of each enzyme, and in

speculation as to whether the changes in their activities are co-ordinately controlled (Weber, Singhal & Srivastava, 1965).

In this study we have measured the activity of each of the four enzymes in the same liver in response to (a) starvation and (b) diabetes induced either with alloxan or with streptozotocin, which is claimed to have certain advantages (greater specificity and lower toxicity) over alloxan (Junod *et al.* 1967; Mansford & Opie, 1968). Since the weight of the liver changes in different metabolic conditions, it is also necessary to distinguish between changes in concentration of enzymes as judged by activities per unit wet weight and the total hepatic content. We have therefore compared activities per unit weight of tissue with those found per unit of DNA as an indication of the cell number. We have also compared the activities observed under optimum assay conditions with published rates of gluconeogenesis and tried on the basis of the available kinetic parameters to estimate the possible

activities of the enzymes *in vivo* at the substrate concentrations prevailing.

MATERIALS AND METHODS

Animals. Rats used were male albino Wistars, weight range 150–250 g. Diabetes was induced either by the intraperitoneal injection of alloxan, 200 mg/kg body weight, or intravenous injection of streptozotocin, 60 mg/kg body weight. Neither group was starved before injection. Unless stated, all animals were fed *ad libitum* and had access to water at all times. Onset of diabetes was indicated by the condition of the animals and their wasting. It was confirmed by positive urine sugar as indicated by Clinistix and high blood sugar at death tested with Dextrostix. Clinistix and Dextrostix were obtained from Ames Co., Slough, Bucks., U.K.

Materials. ATP (disodium salt), ITP (disodium salt), glucose 6-phosphate (sodium salt), fructose 1,6-diphosphate (sodium salt), GSH, sodium pyruvate, CoA, NADP, potassium lithium acetyl phosphate, oxaloacetate, phosphotransacetylase, citrate synthase, phosphoglucose isomerase and glucose 6-phosphate dehydrogenase were all purchased from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. Tris (Trizma base) was purchased from Sigma (London) Chemical Co., London S.W.6, U.K. and alloxan from British Drug Houses Ltd., Poole, Dorset, U.K. Bovine plasma albumin (fraction V) was obtained from Armour Pharmaceutical Co., Eastbourne, Sussex, U.K. and sodium [^{14}C]bicarbonate (44 mCi/mmol) from The Radiochemical Centre, Amersham, Bucks., U.K. Streptozotocin was a gift from the Upjohn Co., Kalamazoo, Mich., U.S.A.

Assays. DNA and RNA were measured by the method of Fleck & Munro (1962) as adapted by Manchester & Harris (1968). Protein was assayed by the biuret method (Layne, 1957) after extraction of lipid by shaking with diethyl ether. Glycogen was measured by the method of Good, Kramer & Somogyi (1933), the glucose formed on hydrolysis being assayed with glucose oxidase (Huggett & Nixon, 1957). Total liver amino acids were measured in zinc hydroxide filtrates (Hagedorn & Jensen, 1923) with ninhydrin by the procedure of Cocking & Yemm (1954).

Enzyme assays. All four enzymes were assayed at 30°C: except for pyruvate carboxylase, they were stable in intact liver stored at –30°C and additionally, both fructose 1,6-diphosphatase and phosphoenolpyruvate carboxykinase were stable as deep-frozen prepared extracts. All assays were performed at optimum substrate concentrations as determined in this laboratory. Rats were killed by decapitation followed by bleeding. The livers were removed and frozen at –80°C as rapidly as possible.

(a) Pyruvate carboxylase. This was assayed within 24 h of death, the intact liver being kept at –80°C until required; after longer periods, loss of activity occurred. A portion of the frozen tissue was homogenized in 9 vol. of acetone cooled on solid CO_2 , filtered and the resulting powder dried for 2 h *in vacuo*. The acetone-dried powder was then taken up in 9 vol. of warm (30°C) 125 mM-tris-HCl buffer, pH 7.4, for immediate assay. Pyruvate carboxylase was measured by following the incorporation of ^{14}C from $\text{NaH}^{14}\text{CO}_3$ into oxaloacetate, the latter being trapped as citrate. The medium used was adapted from those of Henning & Seubert (1964) and Felicioli, Gabrielli & Rossi (1967) and contained (final concns.) 125 mM-tris-

HCl buffer, 7.5 mM-sodium pyruvate, 8.3 mM- MgSO_4 , 2.2 mM-ATP, 0.83 mM-CoA, 0.83 mM-acetyl phosphate, 10 units of phosphotransacetylase, 0.5 unit of citrate synthase and 100 mM- $\text{NaH}^{14}\text{CO}_3$ (approx. 20000 c.p.m./mol); final pH 7.2 at 30°C. The reaction was started by the addition of the liver extract, which was diluted two- or three-fold immediately before use, to 0.2 ml of the above medium. The reaction was terminated after 2, 4 or 6 min incubation by addition of 0.5 ml of 10% (w/v) trichloroacetic acid. The $^{14}\text{CO}_2$ was removed by gassing for 10 min with non-radioactive CO_2 and the radioactivity incorporated was counted in a Nuclear-Chicago liquid-scintillation counter, 0.1 ml portions of the sample being taken up in 10 ml of a scintillation fluid containing 10 g of 2,5-bis-(5-*tert*-butylbenzoxazol-2-yl)thiophen (BBOT), 200 g of naphthalene, 1 litre of methylCellosolve and 1.5 litres of toluene.

(b) Phosphoenolpyruvate carboxykinase. This was measured by an assay based on that described by Foster, Lardy, Ray & Johnston (1967) and the incubation medium contained 62 mM-tris-HCl buffer, 1 mM-glutathione, 6 mM-ITP, 6 mM- MgSO_4 , 13 mM-NaF, 4.5 mM-oxaloacetate and 0.1 mM- MnCl_2 ; final pH 7.0 at 30°C. Liver was homogenized in 9 or 14 vol. of 0.25 M-sucrose at 0°C and the reaction was started by the addition of 0.2 ml of the supernatant after centrifugation for 1 h at 100000g to 0.55 ml of the incubation medium. Both enzyme extract and reaction medium were preincubated for 5 min separately to bring to 30°C before mixing. The reaction was stopped after 0, 3, 6, or 9 min by addition of 3 ml of 10% (w/v) trichloroacetic acid at 0°C. The P_i formed was then measured by the method of Lowry & Lopez (1957) before and after the hydrolysis of the phosphoenolpyruvate formed by the enzyme. Phosphoenolpyruvate was cleaved in 1 ml samples after neutralization by the addition of 0.5 ml of 1% HgCl_2 (Lohmann & Meyerhof, 1934).

The assay differs from that generally used by Foster *et al.* (1967) in that the concentration of MgSO_4 was lowered from 20 to 6 mM but 0.1 mM- MnCl_2 was added instead; the resulting activity is at least twice that measured in the presence of magnesium alone (Foster *et al.* 1967).

(c) Fructose 1,6-diphosphatase. This was assayed in extracts prepared by the method of Underwood & Newsholme (1965), i.e. fresh or frozen liver was homogenized in 19 vol. of 0.15 M-KCl containing 5 mM-cysteine-HCl and 2 mg of bovine plasma albumin/ml. The pH of the homogenate was adjusted to 5.2 with HCl, and the extract incubated at 37°C for 10 min. After cooling on ice to 0°C, the homogenate was centrifuged at 35000g for 45 min. The soluble fraction was neutralized with 1 M-KOH and frozen until required. The activity of the enzyme was followed spectrophotometrically in medium containing 50 mM-tris-HCl buffer, pH 7.5, 20 mM- β -mercaptoethanol, 5 mM- MgSO_4 , 0.05 mM-fructose 1,6-diphosphate, 0.2 mM-NADP, 0.05 mM-EDTA and 0.7 and 2 units each of glucose 6-phosphate dehydrogenase and phosphoglucose isomerase/ml. The final volume was 3 ml. The reaction was started by the addition of 0.05 ml of the enzyme extract, and the reduction of NADP was followed on a Unicam SP.800 recording spectrophotometer. As noted by Underwood & Newsholme (1965), higher concentrations of fructose 1,6-diphosphate or MgSO_4 inhibited the enzyme.

(d) Glucose 6-phosphatase. This was assayed in extracts of either fresh tissue or material stored at -30°C . Liver was homogenized in 99 or 149 vol. of 40mM-sodium cacodylate-HCl buffer (British Drug Houses Ltd., Poole, Dorset, U.K.), pH 6.5; the assay technique was based on those of Freedland & Harper (1957) and Arion & Nordlie (1967). The incubation medium contained 40mM-sodium cacodylate-HCl buffer, pH 6.5, and 30mM-glucose 6-phosphate. The final volume after initiation of the reaction by the addition of 0.2ml of homogenate was 0.4ml. The reaction was terminated after 15min by the addition of 2ml of 10% (w/v) trichloroacetic acid, and the P_i released was measured (Lowry & Lopez, 1957).

RESULTS

Activities of the four enzymes are presented in two ways; first, activity per unit wet weight of liver; secondly, to minimize the effect of changing hepatic weight, as per unit DNA, a parameter which together with the cell number is probably fairly constant during the period of our experiments on starvation and diabetes (Thomson, Heagy, Hutchison & Davidson, 1953 and Table 1). Activities were measured at 30°C ; at 37°C they might be expected to be approx. 1.5 times as great (Scrutton & Utter, 1968). Of the four enzymes, under our conditions of assay, pyruvate carboxylase and phosphoenolpyruvate carboxykinase have less activity in the fed rat than either fructose 1,6-diphosphatase or glucose 6-phosphatase by a factor of about 2.

Starvation. Activities were measured after starvation for one day (overnight), two and three days. All rose on starvation but to varying extents; the activities per unit of wet weight are clearly enhanced by the loss of liver weight as is seen by comparison with results expressed per unit of DNA (Table 2). Thus whereas the activity per g of tissue of pyruvate carboxylase doubles after overnight starvation, the activity per liver is not significantly greater (though suggestively up). The activity of pyruvate carboxylase changes little on further starvation. This initial increase is in contrast with the results of Krebs (1965) and Reshef,

Hanson & Ballard (1969) where no significant change was noted. Filsell *et al.* (1969) found an initial but very small increase on starvation, but Freedman & Kohn (1964) and Henning, Stumpf, Ohly & Seubert (1966) have found increases of two- and three-fold over the value in the fed animal, depending on the length of time the rat was starved.

By contrast with pyruvate carboxylase, phosphoenolpyruvate carboxykinase activity increases significantly during the first period of starvation both in terms of content and concentration. Activity on either basis appeared to rise as starvation progressed, to 2.5 and 4.5 times the values for fed animals for content and concentration respectively after 3 days without food. Young, Shrago & Lardy (1964) found no further increase in activity relative to liver protein content after an initial doubling during the first 24h; Filsell *et al.* (1969) also found that the activity only rose during the initial day of starvation, but they recorded a four-fold increase during this period.

The results with fructose 1,6-diphosphatase also exemplify the importance of the mode of expression of the activity. Activity of the enzyme expressed as a concentration rises on starvation overnight at least partly because of loss of weight of the liver; activity per cell actually shows a decrease, apparently regained by the second day of starvation. Thus content of enzyme per liver is barely changed from the value in the fed animal after 3 days of fasting, whereas concentration shows a rise of 80%. Weber & Cantero (1959) and Weber (1963) found a steady fall in fructose 1,6-diphosphatase activity per cell assayed during starvation for up to 6 days with 72% of the original activity remaining at 3 days; however, relative to the fall in supernatant nitrogen, fructose 1,6-diphosphatase was deemed to be conserved against general loss of protein.

Again by contrast, glucose 6-phosphatase exhibits an increase both in terms of DNA and wet weight. The rise continues as starvation progresses to give increases of approximately two-fold and three-fold respectively after 3 days. The relative

Table 1. *Total liver DNA phosphorus under different metabolic conditions*

Each value represents the mean $\pm$ S.E.M. of the number of observations in parentheses in the first column. Diabetic rats were killed on day 3 after the administration of alloxan or streptozotocin. The value of P is expressed relative to the values with the normal fed rat.

Condition of rat		Liver weight (g)	DNA per g (μg of DNA P/g)	DNA per total liver (μg of DNA P)
Fed	(6)	7.5 ± 0.13	189 ± 8.1	1423 ± 48
Starved overnight	(6)	4.9 ± 0.20	270 ± 14	1331 ± 101
P			<0.001	
Alloxan-diabetic, fed	(5)	6.2 ± 0.20	254 ± 4.9	1548 ± 40
P			<0.001	
Streptozotocin-diabetic fed	(5)	8.8 ± 0.40	186 ± 5.6	1620 ± 70

Table 2. *Effect of starvation on the activities of the four key gluconeogenic enzymes of rat liver*

Each value, expressed as μmol of product formed at $30^\circ\text{C}/\text{min}$ per g of wet liver or per $100\mu\text{g}$ of DNA P, is the mean $\pm$ s.e.m. of the number of observations in parentheses in the first column. Values of P are indicated by comparison with the 1-day (overnight)-starved value.

Period of starvation (days)	DNA (μg of DNA P per g of liver)	Pyruvate carboxylase		Phosphoenolpyruvate carboxykinase		Fructose 1,6-diphosphatase		Glucose 6-phosphatase	
		per g of liver	per unit of DNA	per g of liver	per unit of DNA	per g of liver	per unit of DNA	per g of liver	per unit of DNA
0 (4) P	176 ± 6 <0.001	3.34 ± 0.36 <0.001	1.90 ± 0.19	3.62 ± 0.32 <0.001	2.06 ± 0.12 <0.01	7.00 ± 0.59 <0.01	4.00 ± 0.40 <0.05	6.42 ± 0.62 <0.001	3.67 ± 0.35 <0.05
1 (8) P	309 ± 15 314 ± 4	7.36 ± 0.46 6.29 ± 0.46	2.42 ± 0.18 2.00 ± 0.18	10.22 ± 0.25 14.15 ± 0.56 <0.001	3.33 ± 0.18 4.52 ± 0.18 <0.01	9.14 ± 0.24 14.10 ± 0.57 <0.001	3.00 ± 0.15 4.50 ± 0.25 <0.001	16.58 ± 0.55 18.60 ± 1.31	5.47 ± 0.40 6.00 ± 0.45
3 (6) P	310 ± 6	7.00 ± 0.50	2.27 ± 0.15	16.00 ± 0.33 <0.001	5.17 ± 0.17 <0.001	12.80 ± 0.60 <0.001	4.10 ± 0.17 <0.001	21.15 ± 1.00 <0.001	6.80 ± 0.25 <0.05

increases were greater than those indicated by Weber (1963). In contrast, Filsell *et al.* (1969) found no further increase in activity after an initial doubling overnight when rats were starved for up to 3 days.

Diabetes. Table 3 shows the activities of the four enzymes measured both in alloxan- and streptozotocin-diabetic rats. Diabetes had in this instance been induced 3 days previously and may therefore be considered 'acute'. Except for one group of streptozotocin-treated animals, the rats had continual access to food, and the logical comparison is with normal fed animals. Values for normal starved animals measured at the same time are, however, also included. The last line in the table is for a group of streptozotocin-induced diabetic animals that were deprived of food for 24h between days 1 and 2 to see whether starvation during the induction of the enzyme influenced the activities attained.

Alloxan and streptozotocin both produced a roughly threefold rise in the activity of pyruvate carboxylase expressed relative to DNA. This is proportionally far greater than the rise on starvation. The increase in activity was significantly less ($P < 0.02$) when food was temporarily withdrawn during induction, though still greater than the effect of overnight starvation. The rise in pyruvate carboxylase in alloxan-diabetes is in qualitative agreement with most published results (Wagle, 1964, 1966; Freedman & Kohn, 1964; Prinz & Seubert, 1964; Henning *et al.* 1966), but quantitatively greater, as in general a twofold rise over the value for the fed animal is reported; this, however, is in conflict with the results of Krebs (1965, 1966) and Brech, Shrago & Wilken (1970) where no rise in activity was found.

With alloxan, there was a fivefold increase in concentration of phosphoenolpyruvate carboxykinase, content being increased about 3.5 times. These changes are similar to those reported by others (Young *et al.* 1964; Lardy, Foster, Shrago & Ray, 1964; Henning *et al.* 1966; Shrago & Lardy, 1966; Wagle, 1966), though again greater than found by Krebs (1965, 1966). The enhancement in activity was nearly threefold after streptozotocin, but significantly less than the enhancement after alloxan. A rise of similar proportions to that produced by streptozotocin was found within hours by Shrago, Lardy, Nordlie & Foster (1963) in the liver of rats made diabetic by the administration of mannoheptulose. As was noted with pyruvate carboxylase, temporary withdrawal of food during the period of treatment produces a smaller increase in the activity of phosphoenolpyruvate carboxykinase.

With fructose 1,6-diphosphatase, proportionally smaller rises were found in the diabetic state than for the other enzymes. The alloxan-diabetic animals

Table 3. *Effects of diabetes induced by alloxan or streptozotocin on the activities of the four key hepatic gluconeogenic enzymes of the rat*

Each result, expressed as μmol of product formed at $30^\circ\text{C}/\text{min}$ per g of wet liver or per $100\ \mu\text{g}$ of DNA P, represents the mean $\pm$ S.E.M. of the number of observations in parentheses in the first column. All three groups of diabetic animals were killed 3 days after the administration of alloxan or streptozotocin. The value of P is expressed relative to the normal fed control.

Condition of rat	DNA (μg of DNA P per g of liver)	Pyruvate carboxylase		Phosphoenolpyruvate carboxykinase		Fructose 1,6-diphosphatase		Glucose 6-phosphatase	
		per g of liver	per unit of DNA	per g of liver	per unit of DNA	per g of liver	per unit of DNA	per g of liver	per unit of DNA
Normal, fed	189 ± 8	3.45 ± 0.15	1.85 ± 0.10	4.46 ± 0.33	2.38 ± 0.15	10.85 ± 0.91	5.74 ± 0.40	8.70 ± 0.56	4.62 ± 0.30
Normal, starved	270 ± 14	6.95 ± 0.42	2.60 ± 0.17	13.62 ± 0.35	4.86 ± 0.20	11.70 ± 0.53	4.11 ± 0.20	24.75 ± 0.98	9.20 ± 0.53
P	<0.001	<0.001	<0.01	<0.001	<0.001	<0.001	<0.01	<0.001	<0.001
Alloxan-diabetic, fed	254 ± 5	13.05 ± 0.31	5.14 ± 0.15	20.95 ± 0.69	8.23 ± 0.22	14.25 ± 1.03	5.56 ± 0.50	30.15 ± 1.85	11.92 ± 0.78
P	<0.001	<0.001	<0.001	<0.001	<0.001	<0.05	<0.01	<0.001	<0.001
Streptozotocin-diabetic, fed	186 ± 6	10.25 ± 0.66	5.49 ± 0.48	11.71 ± 0.60	6.35 ± 0.23	17.28 ± 0.83	9.35 ± 0.63	21.60 ± 1.95	11.65 ± 1.12
P		<0.001	<0.001	<0.001	<0.001	<0.01	<0.01	<0.001	<0.001
Streptozotocin-diabetic, re-fed*	196 ± 4	7.42 ± 0.35	3.79 ± 0.20	8.35 ± 0.70	4.30 ± 0.33	16.77 ± 0.80	8.59 ± 0.42	17.88 ± 0.92	9.05 ± 0.50
P		<0.001	<0.001	<0.001	<0.001	<0.01	<0.01	<0.001	<0.001

* This group were fed for 1 day after the injection of streptozotocin, starved overnight and re-fed.

actually showed no rise in terms of organ content, reminiscent of the results on starvation. With streptozotocin, however, content was elevated by about 60%, very similar to the results of Weber (1963) for alloxan-diabetes.

A marked similarity exists between the response of glucose 6-phosphatase and of phosphoenolpyruvate carboxykinase to either agent. Both alloxan and streptozotocin increase the hepatic content (Table 3), the concentration of glucose 6-phosphatase being three to four times higher than in the normal fed rat, compared with only twice the concentration recorded previously (Freedland & Barnes, 1963; Langdon & Weakley, 1955; Nordlie & Arion, 1965). The cell content reached similar values in both alloxan- and streptozotocin-diabetes.

Effects of starvation and diabetes. If the diabetic state induces these enzymes, as also does starvation, the question arises as to whether the changes are additive. This seems probable, if only on the grounds of the loss in weight of the liver during starvation leading to elevation of the concentrations of the remaining constituents. However, Table 4 suggests that this is not necessarily so. Both streptozotocin- and alloxan-treated animals were studied at an acute and chronic stage of diabetes and the hepatic enzyme activities measured after animals had been starved overnight, were compared with normal starved animals. Unfortunately some differences can be seen in the activities of the two sets of normal animals in Table 4. These and other results suggest there may be both seasonal and strain differences in the actual values of the activities obtainable. The second series in Table 4 employed animals brought into the Department, as opposed to the stock normally used.

On this basis pyruvate carboxylase was not elevated in either the acute or chronic streptozotocin-diabetic animal. With the chronic alloxan-diabetic animal some enhancement was observed. Since the rise produced in the fed diabetic animal was greater than the rise produced by starvation, the similarity of the results for three of the four conditions to the starved control values implies that the value for the diabetic animal falls rather than rises on starvation. This is shown further by direct comparison of the actual values (Tables 3 and 4) and is implicit in the results of starvation during induction (Table 3). By contrast, fructose 1,6-diphosphatase, which showed little change in content with starvation, remained persistently enhanced in the starved diabetic animal (Table 4). Phosphoenolpyruvate carboxykinase and glucose 6-phosphatase exhibit more nearly comparable changes than do the other enzymes, with little enhancement of activity in the starved streptozotocin-diabetic animal by comparison with the starved control, but there was a substantial rise in

Table 4. *Effects of diabetes induced by alloxan or streptozotocin on the activities of the four key gluconeogenic enzymes of rat liver*

Each value, expressed as μmol of product formed at $30^\circ\text{C}/\text{min}$ per g of wet liver or per unit of DNA P, is the mean $\pm$ s.e.m. of the number of observations in parentheses in the first column. Unless stated, all rats were starved overnight. The value of P is expressed relative to the values for the normal starved controls. Because the animal stock used for lines 3 and 4 was different from that used in the rest of the work separate controls (lines 1 and 2) have been included.

Condition of rat	DNA (μg of DNA P per g of liver)	Pyruvate carboxylase		Phosphoenolpyruvate carboxykinase		Fructose 1,6-diphosphatase		Glucose 6-phosphatase	
		per g of liver	per unit of DNA	per g of liver	per unit of DNA	per g of liver	per unit of DNA	per g of liver	per unit of DNA
Normal, fed P	(4) 176 $\pm$ 6 <0.001	3.34 $\pm$ 0.36 <0.001	1.90 $\pm$ 0.19	3.62 $\pm$ 0.32 <0.001	2.06 $\pm$ 0.12 <0.01	7.00 $\pm$ 0.59 <0.01	4.00 $\pm$ 0.40 <0.05	6.42 $\pm$ 0.62 <0.001	3.67 $\pm$ 0.35 <0.05
Normal, starved Alloxan-diabetic 2 days P	(8) 309 $\pm$ 15 224 $\pm$ 11 <0.01	7.36 $\pm$ 0.46 5.71 $\pm$ 0.33 <0.02	2.42 $\pm$ 0.18 2.54 $\pm$ 0.18	10.22 $\pm$ 0.25 16.37 $\pm$ 0.61 <0.001	3.33 $\pm$ 0.18 7.48 $\pm$ 0.55 <0.001	9.14 $\pm$ 0.24 8.45 $\pm$ 0.42	3.00 $\pm$ 0.15 3.82 $\pm$ 0.23 <0.05	16.58 $\pm$ 0.55 20.95 $\pm$ 0.94 21.00 $\pm$ 1.07	5.47 $\pm$ 0.40 9.38 $\pm$ 0.66 5.64 $\pm$ 0.60
Streptozotocin- diabetic 9 days P	(6) 372 $\pm$ 29	8.89 $\pm$ 0.50	2.46 $\pm$ 0.22	13.90 $\pm$ 0.61	3.82 $\pm$ 0.30	14.51 $\pm$ 0.52	3.96 $\pm$ 0.22	<0.01	<0.001
Normal, fed P	(6) 189 $\pm$ 8 <0.001	3.45 $\pm$ 0.15 <0.001	1.85 $\pm$ 0.10 <0.01	4.46 $\pm$ 0.33 <0.001	2.38 $\pm$ 0.15 <0.001	<0.001 10.85 $\pm$ 0.91	<0.01 5.74 $\pm$ 0.40	<0.01 8.70 $\pm$ 0.56 <0.001	<0.01 4.62 $\pm$ 0.30 <0.001
Normal, starved Alloxan-diabetic 7 days P	(6) 270 $\pm$ 14 274 $\pm$ 5	6.95 $\pm$ 0.42 10.90 $\pm$ 0.58 <0.01	2.60 $\pm$ 0.17 4.05 $\pm$ 0.32 <0.01	13.62 $\pm$ 0.35 17.41 $\pm$ 1.72 <0.05	4.86 $\pm$ 0.35 6.54 $\pm$ 0.68	11.71 $\pm$ 0.53 15.45 $\pm$ 0.92 <0.01	4.11 $\pm$ 0.20 5.75 $\pm$ 0.37 <0.01	24.75 $\pm$ 0.98 18.75 $\pm$ 1.00 <0.01	9.20 $\pm$ 0.53 7.22 $\pm$ 1.15 8.90 $\pm$ 0.52
Streptozotocin- diabetic 2 days P	(6) 248 $\pm$ 4	7.28 $\pm$ 0.59	2.94 $\pm$ 0.22	10.25 $\pm$ 0.37	4.09 $\pm$ 0.15	15.10 $\pm$ 0.20	6.10 $\pm$ 0.22	22.05 $\pm$ 1.17	<0.001

content in the alloxan-treated group. The fall in glucose 6-phosphatase concentration noted in the starved chronic alloxan-diabetic animal has also been recorded by Ashmore, Hastings & Nesbitt (1954).

In conjunction with enzyme measurements, hepatic glycogen, RNA, free amino acid and protein measurements were carried out. Total free amino acid contents per g of tissue in general were little changed by any of the treatments, including starvation, though somewhat diminished per unit of DNA. Protein content per g of tissue was increased by starvation and diabetes. The most striking difference between alloxan- and streptozotocin-diabetes was the much greater glycogen content of the streptozotocin-diabetic animals. The reason for this is not known.

DISCUSSION

The activities of the enzymes recorded here are in the same range as generally found by other workers. Böttger, Wieland, Brdiczka & Pette (1969) claimed an activity of pyruvate carboxylase of $10 \mu\text{mol/min per g}$, but most other estimates are in the range $2.6\text{--}4.5 \mu\text{mol/min per g}$ (Henning & Seubert, 1964; Prinz & Seubert, 1964; Wagle, 1964). Surprisingly there are no published values for the activity of phosphoenolpyruvate carboxykinase per g of tissue when activated by Mn^{2+} as well as Mg^{2+} . Our assays in the presence of these ions give activities about twice those published for Mg^{2+} alone (Shrago *et al.* 1963). All the activities observed are considerably in excess of the likely rate of gluconeogenesis either *in vivo* or in the perfused liver (Hornbrook, Burch & Lowry, 1965; Ross, Hems & Krebs, 1967; Exton & Park, 1968; Williamson, Browning & Olsen, 1968), but of course are measured under conditions of optimum activation which are not necessarily operative *in vivo*. Thus the low content of oxaloacetate in liver, even in the cytoplasm, is likely to limit activity of phosphoenolpyruvate carboxykinase if the K_m is as high as

$120 \mu\text{M}$ (Foster *et al.* 1967) and the activity of fructose 1,6-diphosphatase may be regulated by the concentration of AMP. It is perhaps notable that the two enzymes not at present known to be subject to allosteric regulation show the largest proportional rises in activity, namely glucose 6-phosphatase and phosphoenolpyruvate carboxykinase, and any diminution in substrate concentrations in starvation or diabetes, e.g. for glucose 6-phosphate or oxaloacetate (Start & Newsholme, 1968; Wieland, 1968), will affect the increased potential activity arising from enhanced enzyme concentration. The location of the enzymes will also be important; a cytoplasmic location for phosphoenolpyruvate carboxykinase benefits from higher oxaloacetate concentrations than present in the mitochondrion. It does appear, however, that rat liver mitochondria contain appreciable phosphoenolpyruvate-synthesizing capacity (E. J. Harris, personal communication) and the kinetics of the enzyme under these conditions remain to be explored.

The location of pyruvate carboxylase is also of consequence since it is activated by acetyl-CoA whose highest concentration is found in the mitochondrion. The kinetics of rat liver pyruvate carboxylase and its control by acetyl-CoA and other metabolites in relation to its intracellular location is discussed in the accompanying paper (Wimhurst & Manchester, 1970a).

The aetiology of diabetes induced by streptozotocin is different in several respects from that with alloxan. Streptozotocin displays less general toxicity and produces much less rise in blood ketone bodies and fatty acids although the plasma insulin concentration is similar in both conditions (Gonet & Reynold, 1966; Dulin, Lund & Gerritsen, 1967; Junod *et al.* 1967; Mansford & Opie, 1968). In the present study, however, relatively little difference was seen in the response of the enzymes to diabetes induced by the two agents. In extracts from starved animals activity of only fructose 1,6-diphosphatase was consistently enhanced by

Table 5. *Correlation between the changes in activity of the gluconeogenic enzymes*

The linear regression coefficient, r , was calculated for the contents of the four enzymes, a pair at a time, for all the different experiments (13) reported. The values of P are those given by Fisher & Yates (1963) for the appropriate number of degrees of freedom.

		Glucose 6-phosphatase	Fructose diphosphatase	Phosphoenol- pyruvate carboxykinase	Pyruvate carboxylase
Glucose 6-phosphatase	r	—	0.55	0.81	0.82
P		—	—	<0.001	<0.001
Fructose diphosphatase	r	—	—	0.21	0.71
P		—	—	—	<0.01
Phosphoenolpyruvate carboxykinase	r	—	—	—	0.69
P		—	—	—	<0.01

streptozotocin, whereas the effects of alloxan under these conditions were more widespread.

It has been suggested (Weber, Singhal, Stamm, Lea & Fisher, 1966) that the enzymes of a single pathway such as glycolysis might be controlled by a single gene unit on the basis of the similarities of the response of the enzymes concerned both to metabolites and to changes in the condition of the rat, e.g. prolonged starvation. The results presented here question the existence of an equally simple control for all four gluconeogenic enzymes. Table 5 compares the changing hepatic content of each of the four enzymes with the others and shows a lack of correlation between fructose 1,6-diphosphatase and either glucose 6-phosphatase or phosphoenolpyruvate carboxykinase. These results point to a possible single control for only three of the four enzymes. It is to be noted that pyruvate carboxylase, alone of the four, can respond in the perfused organ to changing glucose concentration (Wimhurst & Manchester, 1970b).

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