Experimental Diabetic Ketoacidosis

SEQUENTIAL CHANGES OF METABOLIC INTERMEDIATES IN BLOOD, LIVER, CEREBROSPINAL FLUID AND BRAIN AFTER ACUTE INSULIN DEPRIVATION IN THE STREPTOZOTOCIN-DIABETIC RAT

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Male rats rendered diabetic by the intravenous injection of streptozotocin (150mg/kg) were treated with a long-acting insulin for 1 week, then allowed to develop ketoacidosis. By using sampling techniques designed to avoid the use of anaesthesia and extended anoxic periods, sequential measurements of metabolic intermediates were made in blood, liver, cerebrospinal fluid and brain at 24h intervals after the last insulin injection. Measurements in blood and liver suggested a rapid increase in hepatic glycogenolysis and gluconeogenesis and peripheral-depot lipolysis between 24 and 48h after the last insulin injection, whereas blood and liver ketone-body and triglyceride concentrations rose more slowly. The changing metabolic patterns occurring with increasing time of insulin deprivation stress the importance of sequential compared with static measurements in experimental diabetes. Data are presented for brain metabolic intermediates in diabetic ketoacidosis, and support recent evidence that glucose plays a less important role in brain oxidative metabolism in ketotic states.

There is much fragmentary and contradictory evidence concerning measurements of metabolites in the tissues of animals with experimental diabetes. Much of the confusion is due to the lack of standardization of the duration, severity and type of diabetes, as well as the use of different forms of anaesthesia and blood and tissue sampling. To overcome this we have established a biochemically well-defined and reproducible type of experimental diabetic ketoacidosis. We describe below sequential measurements of several metabolic parameters in blood, liver, cerebrospinal fluid and brain after insulin withdrawal in the severely streptozotocin-diabetic rat. The procedure should prove to be of use in the assessment of different treatment regimes.

Experimental

Animals

Male Ash-Wistar rats weighing 130–170g were used. They were allowed free access to water and a standard laboratory rat diet (Diet 41B; Oxoid Ltd., London SE1 9HF, U.K.) at all times except where stated in the text.

Experimental design

Streptozotocin (150mg/kg body wt.) in approx. 0.5ml of 0.01M-disodium citrate buffer, pH4.5,

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was injected into a tail vein while the animal was under light ether anaesthesia. Control rats received buffer alone. All animals were given 5% (w/v) glucose to drink for the next 24h. At that time tail-vein blood glucose was monitored and 3 International Units of protamine-zinc insulin were administered subcutaneously. Animals with blood glucose of less than 23.3mm (420mg/100ml) were discarded (6% of streptozotocin-injected animals). At 48h, 4 units of insulin were administered; at 72h, blood glucose was again monitored and the insulin dose either increased to 5 units or maintained at 4 units. This dose was then continued for 5 more days unless an animal failed to gain weight in a given 24h period, in which case the insulin dose was raised or lowered by 1 unit from the preceding day's dose, depending on the blood glucose. Insulin was withdrawn after 7 days of treatment, and groups of 6–16 animals were killed at 24h intervals for 120h. Control rats received subcutaneously administered 0.9% NaCl instead of insulin. In certain animals the right kidney, liver, right epididymal fat-pad, brain, and right tibialis anterior were removed immediately *post mortem*, blotted to remove excess of blood, weighed, dried for 48h at 150°C and reweighed.

Results are expressed as the mean $\pm$ S.E.M.; significant differences were determined by using Student's *t* test (N.S., not significant).

Blood sampling

Heparinized blood was obtained after decapitation, and a 0.4ml sample deproteinized in 4.0ml of ice-

cold 3% (v/v) HClO_4 ; the remaining blood was centrifuged and the plasma stored at -20°C for later analyses. Cerebrospinal fluid was obtained immediately *post mortem* by cisternal puncture, and a sample (25–100 μl) was added to 1.0 ml of cold 3% HClO_4 . The acid extracts of cerebrospinal fluid and blood were processed as described previously (Schein *et al.*, 1971).

Tissue sampling

For assay of liver metabolites, rats were killed by cervical dislocation and the livers removed within 10 s and freeze-clamped (Wollenberger *et al.*, 1960). After grinding in liquid N_2 , the frozen powder was weighed rapidly and homogenized with a Teflon pestle in 4 vol. of ice-cold 6% (v/v) HClO_4 . The procedure followed was that of Williamson *et al.* (1967b). A portion of the freeze-dried powder was shaken with 16 vol. of chloroform-methanol (2:1, v/v) for triglyceride determination. A smaller portion of the acid homogenate was refrigerated for 12 h at 4°C and used for the determination of glycogen (Keppler & Decker, 1970).

Rat brain was sampled by using the apparatus and technique of Veech *et al.* (1973) in which up to 1 g of supratentorial brain is displaced and frozen within 1 s between aluminium plates precooled in liquid N_2 . Brains from three to five rats were pooled. Tissue preparation was the same as for liver.

Assays

Enzymic assays were performed for glucose (Slein, 1963), acetoacetate and 3-hydroxybutyrate (Williamson *et al.*, 1962), glycerol (Eggstein & Kreutz, 1966), lactate (Hohorst *et al.*, 1959), pyruvate (Bücher *et al.*, 1963), glucose 6-phosphate and ATP (Lamprecht & Trautschold, 1963), ADP and AMP (Adam, 1963), glycerolphosphate (Hohorst, 1963a), phosphoenolpyruvate, 2-phosphoglycerate, 3-phosphoglycerate (Czok & Eckert, 1963), citrate (Gruber & Moellering, 1966), 2-oxoglutarate (Bergmeyer & Bernt, 1963), malate (Hohorst, 1963b), L-alanine (Williamson *et al.*, 1967b), L-glutamine (Lund, 1970), L-glutamate (Bernt & Bergmeyer, 1963) and L-aspartate (Pfleiderer, 1963). When metabolites in frozen-tissue extracts were assayed, the adenosine nucleotides, α -oxo acids and glycolytic intermediates were determined immediately; other metabolites were measured within 48 h. Florisil-treated tissue extracts were used for the assay of the α -oxo acids and glucose (Frisell & Mackenzie, 1959). Plasma non-esterified fatty acids were determined colorimetrically (Itaya & Ui, 1965); plasma and liver triglyceride was determined by the method of Eggstein & Kreutz (1966), serum non-esterified cholesterol by the method of MacIntyre & Ralston

(1954), and plasma immunoreactive insulin was measured by micro-modification of the method of Soeldner & Stone (1965). Tail-vein blood glucose concentrations were monitored 24 and 72 h after the injection of streptozotocin by using Dextrostix and an Ames reflectance meter (Jarrett *et al.*, 1970).

Special chemicals

The streptozotocin was a gift from Dr. W. E. Dulin, Upjohn Co., Kalamazoo, Mich., U.S.A. Protamine-zinc insulin (40 units/ml) was obtained from Burroughs Wellcome and Co., London HP4 2DY, U.K. Enzymes and coenzymes were supplied by Boehringer Corp. (London) Ltd., London W5 2T, U.K., with the exception of glutaminase, which was supplied by Sigma (London) Chemical Co. Ltd., London KT2 7BH, U.K. Florisil was obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K. Dextrostix were supplied by Ames Inc., Slough, Bucks., U.K. Rat insulin standard was a gift from Novo Industries, Copenhagen, Denmark; ^{125}I -labelled insulin was purchased from The Radiochemical Centre, Amersham, Bucks., U.K., and antisera were from Wellcome Laboratories, Beckenham, Kent, U.K.

Results

Effects of streptozotocin and insulin treatment

(a) *Blood glucose.* At 24 h after streptozotocin injection, the mean blood glucose was $33.3 \pm 0.9 \text{ mm}$ ($n = 34$) compared with $5.4 \pm 0.1 \text{ mm}$ in 14 controls. At 48 h after the first insulin injection, the tail-vein glucose values obtained with Dextrostix were $8.2 \pm 1.6 \text{ mm}$ in the diabetic animals, compared with $5.7 \pm 0.2 \text{ mm}$ in 0.9% NaCl-injected controls (N.S.).

(b) *Weight change; food and water consumption.* The diabetic rats lost 3% total body weight in the first 24 h, whereas control rats gained 5%; after 7 days of insulin treatment, however, both the diabetic and the control groups had increased body weight by 26% (Fig. 1). Food and water consumption were not significantly greater in the diabetic rats.

Effects of insulin withdrawal

(a) *Body weight; food and water consumption.* On cessation of insulin treatment, the diabetic rats showed a progressive weight loss of 27% in 4 days; this was accompanied by a corresponding decline in food consumption, which decreased by 50% between 24 and 48 h after the last insulin injection. Water consumption remained constant for 72 h, after which it decreased rapidly. Only eight of 34 (24%) animals survived 120 h after insulin withdrawal, and all were stuporous. Metabolite measurements

Fig. 1. Rat weights during induction of diabetes, during insulin maintenance and after insulin withdrawal

Values are expressed as % of weight at day 0 $\pm$ S.E.M. ○, Diabetic rats ($n = 10-18$); ●, controls ($n = 11-18$). Arrow indicates streptozotocin injection (150mg/kg, intravenously). N.S., differences not significant. Other details are described in the Experimental section.

Fig. 2. Whole blood glucose, plasma immunoreactive insulin and cerebrospinal fluid|blood glucose ratio after the last injection of insulin

Diabetics, ○; controls, ●. Values are means $\pm$ S.E.M. Details are described in the Experimental section.

at 120h are therefore biased in that only survivors are included.

(b) *Organ weights and water content.* In the diabetic animals, liver dry weight showed a decrease of 25% between 24 and 48h, presumably owing to loss of glycogen, and then remained constant; tibialis anterior lost 35% in dry weight by 48h and ended with a total decrease of 45% at 120h. Epididymal fat-pad lost 11% of dry weight by 48h, and the 120h dry weight was only 50% of the original dry weight. Neither brain nor kidney displayed any significant change in weight or water content. Liver water content fell from $72.5 \pm 0.2\%$ at 24h to $69.8 \pm 1.0\%$ at 120h ($P < 0.05$) and fat-pads were severely dehydrated 120h after insulin injection ($11.1 \pm 2.0\%$ compared with $23.4 \pm 1.4\%$ at 24h; $P < 0.01$).

(c) *Blood and cerebrospinal-fluid metabolites 24h after the last insulin injection.* Blood and cerebrospinal-fluid metabolite values 24h after the last injection of insulin did not differ significantly from control values. Plasma insulin was still detectable in the diabetic animals.

(d) *Blood and cerebrospinal-fluid metabolites 48-120h after the last insulin injection.* (1) Blood glucose and plasma insulin. At 48h after the last insulin injection, blood glucose had increased nearly 3-fold, continuing to rise to a plateau of about 20mm at 72h (Fig. 2). No insulin was detectable in the plasma of the diabetic animals at 96h (Fig. 2).

(2) Blood lactate and pyruvate. Blood lactate and pyruvate both decreased significantly from control values (Fig. 3). In the diabetic animals, lactate

Fig. 3. Whole blood lactate and pyruvate concentrations after insulin withdrawal

Experimental details are as in Fig. 2. Lactate: diabetics, ○; controls, ●. Pyruvate: diabetics, △; controls, ▲.

decreased from a 24h mean of 2.35 ± 0.31 mm to remain at approx. 1.5 mm until 120h, when a slight rise was observed; pyruvate decreased from 0.14 ± 0.02 mm at 24h to remain at less than half that value for the next 72h. Blood [lactate]/[pyruvate] ratios first decreased from 18 at 24h (fed control ratio = 21 ± 2), then rose to 66, 44 and 34 at 72, 96 and 120h respectively.

(3) Fat metabolites. Blood glycerol and plasma free fatty acids increased abruptly by 3-fold and 5-fold respectively, reaching plateaux at 48h (Fig. 4). In contrast, plasma triglycerides increased from a low value of 36.5 ± 9.8 mg/100 ml at a relatively constant rate of approx. 180 mg/100 ml per 24h period, reaching a mean value of 918 ± 257 mg/100 ml at 120h (Fig. 4). Serum non-esterified cholesterol was unchanged at 48h, but by 96h had risen sixfold.

Blood ketone bodies showed a pattern similar to triglycerides (Fig. 5), with 3-hydroxybutyrate increasing more rapidly than acetoacetate. The 3-hydroxybutyrate and acetoacetate concentrations

Fig. 4. Plasma glycerol, free fatty acids, and triglyceride and serum non-esterified cholesterol concentrations after insulin withdrawal

(a) Glycerol: diabetics, ○; controls, ●. Free fatty acids: diabetics, □; controls, ■. (b) Triglyceride: diabetics, ○; controls, ●. Cholesterol: diabetics, □; controls, ■. Values are means $\pm$ S.E.M. Experimental details are as in Fig. 2.

Fig. 5. Whole blood 3-hydroxybutyrate and acetoacetate concentrations and cerebrospinal fluid/blood ratios for total ketone bodies after insulin withdrawal

(a) 3-Hydroxybutyrate: diabetics, $\circ$; controls, $\bullet$. Acetoacetate: diabetics, Δ ; controls, $\blacktriangle$. (b) Cerebrospinal fluid/blood ratio: diabetics, $\circ$; controls, $\bullet$. Values are means $\pm$ S.E.M. Experimental details are as described in Fig. 2.

reached maxima of 7.00 ± 0.73 mm and 2.90 ± 0.46 mm respectively, with the greatest rate of increase occurring after 48h, in marked contrast to glycerol and free fatty acids. Blood [3-hydroxybutyrate]/[acetoacetate] ratios declined slightly initially, then remained virtually unchanged at approx. 2.5.

(4) Cerebrospinal-fluid metabolites. The cerebrospinal-fluid glucose concentration was 66% of the blood glucose at 24h, and ultimately reached 80% of blood glucose concentration at 96h (Fig. 2). On the other hand, the cerebrospinal fluid concentrations of ketone bodies, which were 96% of those in blood at 24h, decreased to 30% at 48h, eventually returning to 92% at 96h (Fig. 5).

Effect of starvation on development of ketoacidosis

To assess the possible role of decreased food intake on the observed metabolic changes, a

group of rats was starved for 48h from 24h after the last insulin injection and compared with fed diabetic rats and 48h-starved control rats (Table 1). Glucose increased less in the starved animals (15.3 ± 2.2 versus 19.8 ± 1.5 mm) and total ketone-body concentration more (9.64 versus 6.43 mm). In starved control animals total blood ketone-body concentration reached only 2.66 mm. The concentrations of the gluconeogenic substrates lactate and glycerol were lower in starved diabetic animals, whereas plasma free fatty acids were 5.57 ± 0.54 mequiv./litre in the starved animals compared with 1.56 ± 0.19 mequiv./litre in the fed diabetics.

Effect of insulin withdrawal on liver metabolite content (Table 2)

(a) Carbohydrate intermediates. Glucose concentrations in liver paralleled those in blood, rising to 25.8 ± 2.3 μ mol/g at 72h. [Glycogen] decreased rapidly by 5-fold in the diabetics from 24 to 48h, then more slowly to reach values similar to those found in 48h-starved controls by 96h. Since the glycogen loss between 24 and 48h after insulin was accompanied by a 25% decrease in liver dry weight, and since our results are expressed in μ mol/g wet wt., decreasing hepatic metabolite concentrations reported here will be underestimates of the true decline in intermediate concentrations per whole liver, and reported increases will be correspondingly higher when compared with fed controls or fed 24h diabetics. [Lactate] was significantly lower in animals 24h after the last insulin injection than in fed controls, but then changed little. Concentrations were greater than in starved control animals. [Pyruvate] showed more dramatic changes, with an 8-fold decline. The resulting ratios of mean [lactate]/mean [pyruvate] increased from 5.7 to 53 in the diabetics, and from 5.1 to 11 in the fed and starved controls. [3-Phosphoglycerate], [2-phosphoglycerate] and [phosphoenolpyruvate] were still unchanged at 48h, but were significantly decreased by 96h in the diabetics compared with both fed and starved controls. Of the tricarboxylic acid-cycle intermediates, [malate] had increased and [citrate] and [2-oxoglutarate] decreased by 48h in the diabetics. By 96h [malate] had increased 4-fold, [2-oxoglutarate] was decreased by 50%, whereas [citrate] had returned to the 24h values. Citrate and 2-oxoglutarate concentrations were similar to those found in starved controls, but [malate] was more than 2-fold higher.

(b) Fat metabolites. Both [3-hydroxybutyrate] and [acetoacetate] rose steadily with the progression of ketoacidosis in the diabetic animals, reaching 15.4 ± 2.4 μ mol/g by 96h. As with blood the greatest increase occurred between 48h and 72h after insulin

Table 1. *Effect of 48 h starvation on blood metabolites in control and 72 h-diabetic rats*

For experimental details see the text. Values are means $\pm$ S.E.M.; the relevant units are to the right of each compound. Numbers in parentheses refer to numbers of animals in each group. Fed versus starved controls: * $P < 0.05$; ** $P < 0.01$. Fed versus starved 72h-diabetic animals: † $P < 0.05$; †† $P < 0.01$. Starved 72h-diabetic animals versus starved controls: ‡ $P < 0.05$; ‡‡ $P < 0.01$.

Metabolite	Fed controls (14)	48h-Starved controls (8)	72h-Diabetics, fed (16)	72h-Diabetics, 48h-starved (6)
Glucose (mM)	5.89 $\pm$ 0.31	3.95 $\pm$ 0.17**	19.8 $\pm$ 1.5	15.3 $\pm$ 2.2††
Lactate (mM)	2.57 $\pm$ 0.24	2.33 $\pm$ 0.41	1.46 $\pm$ 0.20 (10)	0.94 $\pm$ 0.10† (5)
Pyruvate (mM)	0.132 $\pm$ 0.010	0.168 $\pm$ 0.030	0.037 $\pm$ 0.010 (10)	0.044 $\pm$ 0.005† (5)
[Lactate]/[pyruvate]	21 $\pm$ 2	15 $\pm$ 2	66 $\pm$ 15 (10)	24 $\pm$ 5 (5)
3-Hydroxybutyrate (mM)	0.172 $\pm$ 0.041 (13)	2.01 $\pm$ 0.25**	4.57 $\pm$ 0.47	7.06 $\pm$ 0.73†††
Acetoacetate (mM)	0.048 $\pm$ 0.006 (13)	0.55 $\pm$ 0.04**	1.86 $\pm$ 0.23	2.58 $\pm$ 0.21††
[3-Hydroxybutyrate] [acetoacetate]	4.0 $\pm$ 1.1 (13)	3.7 $\pm$ 0.4	2.4 $\pm$ 0.2	2.8 $\pm$ 0.2
Glycerol (mM)	0.145 $\pm$ 0.01 (18)	0.210 $\pm$ 0.14**	0.38 $\pm$ 0.02 (10)	0.231 $\pm$ 0.02††
Plasma free fatty acids (mequiv./l)	0.45 $\pm$ 0.06 (6)	1.14 $\pm$ 0.09** (7)	1.56 $\pm$ 0.19 (9)	5.57 $\pm$ 0.54††††
Plasma triglyceride (mg/100ml)	70.3 $\pm$ 8.9 (7)	73.0 $\pm$ 2.6	360 $\pm$ 56 (10)	404 $\pm$ 44††
Serum non-esterified cholesterol (mg/100ml)	39.1 $\pm$ 5.5 (6)	43.4 $\pm$ 3.1 (5)	251 $\pm$ 87 (6)	58.8 $\pm$ 8.6 (5)

withdrawal. The ratio of mean [3-hydroxybutyrate]/mean [acetoacetate] rose from 1.2 to 1.6 in the diabetics, being similar to the 1.9 found in starved controls. Hepatic [triglyceride], unchanged at 48h, rose 2½-fold to 65.5 $\pm$ 40.3 μ mol/g by 96h in the diabetics, increasing only slightly in the starved controls.

(c) *Adenine nucleotides*. [ATP] had decreased significantly by 96h in the diabetic animals compared with the concentration in diabetic animals at 24h, although not when compared with controls, whereas [ADP] increased slightly and [AMP] increased almost 2-fold. The mass-action ratio of the myokinase system, [ATP][AMP]/[ADP]², decreased from 0.44 at 24h to 0.34 and 0.29 at 48h and 72h respectively in the diabetics, then increased to 0.48 at 96h.

(d) *Amino acids*. [Glutamate] and [glutamine] showed an initial increase, but then decreased 2-fold in the diabetics by 96h, whereas starvation had no significant effect in control animals. Both [aspartate] and [alanine] fell to nadirs at 72h and increased again at 96h in the diabetic animals. [Alanine] fell to similar concentrations in starved normal animals. The mass-action ratio of the alanine aminotransferase system [alanine][2-oxoglutarate]/[pyruvate][glutamate], decreased from 0.72 to 0.34 at 48h in the diabetics, then rose again.

Effect of insulin withdrawal on brain metabolite content (Table 3)

Brain metabolites remained relatively stable during the development of severe ketoacidosis. When the metabolite concentrations in brains from 96h-

diabetic animals were compared with those from the brains of 24h-diabetic animals, only [glucose], [3-hydroxybutyrate], [pyruvate] and [citrate] showed highly significant increases ($P < 0.01$), whereas [ADP] and [total adenine nucleotides] decreased at a borderline level of significance ($P < 0.05$). However, as in liver, by 24h the brains from the diabetic animals already showed some differences from controls: [glucose] and [3-hydroxybutyrate] were higher and [malate], [2-oxoglutarate] and [glutamine] were lower. Adenine nucleotides were unchanged in concentration.

The elevation of brain [glucose] cannot be accounted for by blood contamination, assuming 2.6% of blood by weight in the frozen samples (Veech *et al.*, 1973). However, by the same assumption, blood contamination could evidently account for a significant portion of the ketone-body concentrations in the brains of ketotic rats.

Discussion

Diabetic ketoacidosis has been described in the rat after near-total pancreatectomy (Chernick & Scow, 1959) and after the injection of either alloxan (Steiner *et al.*, 1961; Prahl & Steenrod, 1965) or streptozotocin (Förster & Rudas, 1969; Schein *et al.*, 1971). Pancreatectomy has the obvious disadvantage of removing the glucagon-producing pancreatic α -cells, whereas both acute and chronic nephrotoxic as well as hepatotoxic effects have been attributed to alloxan (Vargas *et al.*, 1970; Steen Olsen *et al.*, 1966; Lazarow & Palay, 1946). Streptozotocin is less toxic than

Table 2. *Effects of insulin withdrawal on the concentrations of metabolic intermediates in rat livers*

See the text for other experimental details. Mean values are expressed as $\mu\text{mol/g}$ liver wet wt. $\pm$ S.E.M. Numbers in parentheses refer to number of animals in each group. * $P < 0.05$; ** $P < 0.01$.

	Controls						Diabetics						A versus						C versus						B versus					
	Fed (8)		48h-Starved(7)		24h (5)		48h(11)		72h (5)		96h (5)		B			C			D			E			F					
	A	B	A	B	C	D	D	E	E	F	F	F	B	C	D	E	F	B	C	D	E	F	B	C	D	E	F			
Glycogen	259±5	17±3	236±23	46±6	28±8	14±8																								
Glucose	6.39±0.36	4.46±0.32	6.97±1.04	16.8±1.0	25.8±2.3	20.1±1.0																								
Glucose 6-phosphate	0.096±0.010	0.070±0.005	0.133±0.024	0.153±0.010	0.130±0.037	0.082±0.008																								
3-Phosphoglycerate	0.292±0.020	0.201±0.028	0.236±0.024	0.267±0.031	0.125±0.022	0.077±0.026																								
2-Phosphoglycerate	0.045±0.005	0.026±0.004	0.041±0.005	0.040±0.014	0.016±0.004	0.014±0.002																								
Phosphoenolpyruvate	0.101±0.007	0.076±0.010	0.097±0.009	0.110±0.014	0.050±0.020	0.040±0.009																								
Pyruvate	0.162±0.014	0.027±0.004	0.080±0.030	0.049±0.009	0.049±0.007	0.010±0.001																								
Lactate	0.82±0.10	0.30±0.04	0.46±0.08	0.40±0.07	0.52±0.18	0.53±0.08																								
Citrate	0.33±0.02	0.36±0.05	0.35±0.04	0.27±0.04	0.26±0.02	0.39±0.04																								
2-Oxoglutarate	0.185±0.030	0.070±0.005	0.115±0.022	0.061±0.005	0.073±0.007	0.059±0.008																								
Malate	0.26±0.02	0.30±0.06	0.17±0.02	0.30±0.02	0.26±0.03	0.68±0.04																								
3-Hydroxybutyrate	0.072±0.014	1.66±0.16	0.17±0.07	1.43±0.27	5.67±3.10	9.58±1.68																								
Acetoacetate	0.092±0.005	0.85±0.06	0.14±0.02	0.87±0.13	3.28±1.05	5.87±0.76																								
Total ketone bodies	0.164±0.017	2.51±0.19	0.31±0.09	2.30±0.38	8.94±4.04	15.4±2.4																								
Triglyceride	15.0±1.3	21.6±1.5	27.2±4.4	24.7±3.5	41.7±19.5	65.5±40.3																								
ATP	2.28±0.37	2.61±0.20	3.21±0.20	2.71±0.13	2.13±0.22	2.38±0.16																								
ADP	1.11±0.07	1.48±0.12	1.37±0.09	1.84±0.06	1.90±0.13	1.52±0.22																								
AMP	0.28±0.06	0.35±0.03	0.26±0.03	0.42±0.02	0.49±0.06	0.47±0.09																								
Total adenine nucleotides	3.67±0.31	4.44±0.30	4.84±0.15	4.98±0.16	4.51±0.27	4.37±0.35																								
Glutamate	2.02±0.11	1.82±0.25	1.85±0.06	2.27±0.16	1.37±0.26	0.73±0.22																								
Glutamine	2.91±0.23	2.76±0.19	3.27±0.42	4.24±0.29	2.06±0.37	1.62±0.23																								
Aspartate	0.52±0.08	0.77±0.10	0.67±0.07	0.61±0.04	0.12±0.05	0.50±0.07																								
Alanine	1.34±0.08	0.52±0.04	0.93±0.21	0.62±0.05	0.56±0.06	0.66±0.17																								
[ATP]/[AMP] [†]	0.52	0.42	0.45	0.34	0.29	0.48																								
[ADP] [‡]																														
[Lactate] [†]	5.1	11	5.7	8.3	11	53																								
[pyruvate]																														
[3-Hydroxybutyrate] [†]	0.78	2.0	1.2	1.6	1.7	1.6																								
[acetoacetate]																														
[Alanine]/[2-oxoglutarate] [†]	0.76	0.75	0.72	0.34	0.61	1.0																								
[pyruvate]/[glutamate]																														

[†] Ratios of mean values

Table 3. *Effects of insulin withdrawal on the concentrations of metabolic intermediates in rat brain*

See the text for other experimental details. Mean values are expressed as $\mu\text{mol/g}$ brain wet wt. $\pm$ S.E.M. Numbers in parentheses refer to number of samples in each group; three to five brains were pooled for each sample. Versus fed controls: * $P < 0.05$; ** $P < 0.01$. Versus 24h-diabetics: † $P < 0.05$; †† $P < 0.01$.

	Fed controls (5)	Diabetics	
		24h (6)	96h (6)
Glucose	1.71 ± 0.09	$2.67 \pm 0.21^{**}$	$6.64 \pm 0.16^{**\dagger\dagger}$
Glucose 6-phosphate	0.064 ± 0.009	0.088 ± 0.016	$0.128 \pm 0.017^{**}$
3-Phosphoglycerate	0.133 ± 0.014	0.118 ± 0.009	$0.118 \pm 0.002^*$
2-Phosphoglycerate	0.005 ± 0.001	0.036 ± 0.021	0.025 ± 0.008
Phosphoenolpyruvate	0.016 ± 0.014	0.052 ± 0.020	0.038 ± 0.019
Pyruvate	0.153 ± 0.002	0.139 ± 0.009	$0.174 \pm 0.005^{**\dagger\dagger}$
Lactate	1.69 ± 0.02	1.73 ± 0.10	1.75 ± 0.16
2-Oxoglutarate	0.17 ± 0.02	$0.11 \pm 0.01^*$	$0.13 \pm 0.00^*$
Citrate	0.34 ± 0.01	0.33 ± 0.01	$0.38 \pm 0.01^{*\dagger\dagger}$
Malate	0.26 ± 0.01	$0.22 \pm 0.01^*$	0.24 ± 0.01
3-Hydroxybutyrate	0.078 ± 0.007	$0.107 \pm 0.010^*$	$0.48 \pm 0.11^{**\dagger\dagger}$
Acetoacetate	—	0.067 ± 0.033	0.18 ± 0.05
Glutamate	12.1 ± 0.1	$11.2 \pm 0.2^{**}$	11.7 ± 0.1
Alanine	0.72 ± 0.01	0.68 ± 0.04	0.70 ± 0.01
ATP	1.80 ± 0.03	1.76 ± 0.05	1.69 ± 0.08
ADP	1.27 ± 0.07	1.34 ± 0.04	$1.19 \pm 0.03\dagger$
AMP	0.61 ± 0.02	0.55 ± 0.02	$0.54 \pm 0.02^*$
Total adenine nucleotides	3.68 ± 0.09	3.64 ± 0.05	$3.43 \pm 0.08\dagger$
$\frac{[\text{ATP}][\text{AMP}]\dagger}{[\text{ADP}]^2}$	0.68	0.54	0.65
$\frac{[\text{Lactate}]\dagger}{[\text{Pyruvate}]}$	11	12	10
$\frac{[\text{Alanine}][2\text{-oxoglutarate}]\dagger}{[\text{pyruvate}][\text{glutamate}]}$	0.065	0.049	0.045

† = Ratios of mean values

alloxan (Arison *et al.*, 1967; Hohenegger & Rudas, 1971), but results acutely in a variable ketoacidosis and in liver [NAD] and [NADH] depletion (Schein & Loftus, 1968). A method of overcoming these problems is the use of streptozotocin induction and 7 days of insulin maintenance followed by insulin withdrawal, by using the dose of streptozotocin that was previously found to give the most severe acute diabetes, 150mg/kg body wt. (Junod *et al.*, 1969; Schein *et al.*, 1971). After 1 week of insulin treatment under a regime similar to that used by Steiner *et al.* (1961), metabolic and physical parameters gave evidence of good control of the diabetes. A long-acting insulin (protamine-zinc insulin) gave the best diabetic control, but by 96h after the last dose of insulin there was no detectable immunoreactive insulin in the circulation, implying a complete absence of pancreatic β -cell function.

After insulin withdrawal, sequential measurements of blood and liver metabolites revealed a continually

changing pattern of metabolic events, leading eventually to severe ketoacidosis. We thus consider the elapsed time after insulin deprivation to be the most important of the many factors leading to discrepant data on metabolite concentrations found in the literature. At 24h after the last insulin injection, few metabolic changes could be demonstrated, owing to the use of a long-acting insulin preparation. During the next 24h, however, rapid hepatic glycogenolysis ensued, as shown by the simultaneous fall in liver glycogen and rise in liver and blood glucose concentrations. Gluconeogenesis was probably also enhanced during this early phase, as shown by the concentration decreases in both blood and liver lactate and pyruvate and liver alanine at 48h, although the decline in liver alanine concentration may be due in part to decreased peripheral glycolysis (Felig, 1973; Alleyne *et al.*, 1970). Other glycolytic intermediates in liver changed little by 48h. After 48h, when the liver glycogen stores were almost

completely exhausted, gluconeogenesis must have made the primary contribution to the constant, grossly elevated blood glucose concentrations. The changes in the hepatic gluconeogenic pathway are summarized in Fig. 6; the low concentrations of glycolytic intermediates, and the continued depression of gluconeogenic-precursor concentrations during the later stages of ketoacidosis, support the evidence of Singhal *et al.* (1971) that key gluconeogenic enzyme activities are increased in liver from streptozotocin-diabetic rats. The increasing liver [lactate]/[pyruvate] ratios may reflect a more reduced redox state in the hepatic cytosol, as may the elevated malate concentrations (Wieland & Löffler, 1963). Although intracellular acidosis or anoxia could result in an increased [lactate]/[pyruvate] ratio (Alberti *et al.*, 1972), the relatively constant [3-hydroxybutyrate]/[acetoacetate] ratios, indicating an unchanged mitochondrial redox state (Klingenberg & von Häfen,

1963; Williamson *et al.*, 1967a), suggest that neither is the case.

The first effects of insulin lack on lipid metabolism are seen in the simultaneous rapid elevations in blood glycerol and free fatty acid concentrations between 24 and 48 h after insulin, indicating a rapid increase in depot triglyceride lipolysis without a matching increase in re-esterification. This increased flux of free fatty acid to the liver results secondarily in a steady increase in total ketone-body concentrations in blood and liver, although several authors have recently suggested that peripheral uptake of these substances may be impaired as well in states of insulin lack (Balasse & Havel, 1971; Bäessler *et al.*, 1972). The increase in plasma and liver triglyceride may stem from increased free fatty acid re-esterification in the liver (Meier *et al.*, 1972); however, the decreased lipoprotein lipase activity and resulting impaired peripheral clearance of plasma triglyceride seen in

Fig. 6. Patterns of glycolytic intermediates in liver and brain after insulin withdrawal

Values are expressed as percentages of those for 24h-diabetic or fed control animals as follows. Liver: (a) (fed controls/24h diabetics) $\times 100$; (b) (48h diabetics/24h diabetics) $\times 100$; (c) (72h diabetics/24h diabetics) $\times 100$; (d) (96h diabetics/24h diabetics) $\times 100$; (e) (starved controls/fed controls) $\times 100$; brain: (f) (96h diabetics/24h diabetics) $\times 100$. The absolute concentrations of metabolites are given in Tables 2 and 3. Abbreviations for metabolites: Lac, lactate; Pyr, pyruvate; Ala, alanine; Asp, aspartic acid; α -OGl, 2-oxoglutarate; Cit, citrate; Mal, malate; Pyr-P, phosphoenolpyruvate; 2P-Gri, 2-phosphoglycerate; 3P-Gri, 3-phosphoglycerate; Glc-6-P, glucose 6-phosphate; Glc, glucose. For other experimental details, see the text.

insulin-deficient states (Schnatz & Williams, 1963; Bierman *et al.*, 1970) could also account for the triglyceride accumulation. In contrast with our findings of elevated serum non-esterified cholesterol concentrations during the later stages of ketoacidosis, previous authors could find no significant cholesterol elevation after near-total pancreatectomy in rats (Chernick & Scow, 1959) or after insulin deprivation in streptozotocin-diabetic rats (Schnatz *et al.*, 1972). However, the normal cholesterol concentrations found in 48h-starved diabetic rats 72h after insulin withdrawal (Table 1) suggests that a considerable portion of the increase in cholesterol in the fed diabetic animals could be of exogenous origin.

In contrast to the data for liver, the data for brain presented here reflect a different pattern of metabolic changes. When compared with the 24h-diabetic animals, only the brain concentrations of glucose, pyruvate, 3-hydroxybutyrate and citrate showed highly significant changes in the 96h-diabetic animals. Since much of the elevation in ketone-body concentrations in brain can be attributed to blood contamination, the relatively small elevation of these substances compared with their increase in blood and cerebrospinal fluid point to either a low rate of uptake by the brain cells or, more likely, rapid utilization (Williamson *et al.*, 1971). This low concentration of ketone bodies in brain makes them unlikely as the source of the neurological symptoms of diabetic coma (Ohman *et al.*, 1971). The high glucose and low glycolytic-intermediate concentrations seen in the brains of ketotic animals (Fig. 6) support the recent data of Berger *et al.* (1973) suggesting that glucose accounts for a decreased proportion of the brain's oxidative metabolism in experimental ketoacidosis; our finding of significantly elevated brain citrate concentrations in ketotic rats supports their contention that a possible inhibition of phosphofructokinase by citrate occurs under these conditions (Passoneau & Lowry, 1964). Finally, although our data showing no change in brain water content eliminate cerebral oedema (Young & Bradley, 1967) or dehydration *per se* (Ohman *et al.*, 1971) as the cause of the neurological symptoms observed under these conditions, cerebrospinal-fluid hyperosmolality may still play a part, as it supposedly does in human non-ketotic hyperglycaemic coma (Danowski & Nabarro, 1965).

In conclusion, the experimental model described in the present paper should provide a useful tool both in the study of other metabolic aberrations occurring during the development of diabetic ketoacidosis and in the assessment of various forms of treatment for this condition. The use of severely streptozotocin-diabetic rats after a period of insulin treatment minimizes the side effects seen in both acute diabetes and those in which other diabetogenic agents or

animal preparations are used. The sampling procedures for liver and brain preclude the introduction of variables such as anaesthesia and different periods of anoxia (Dale, 1965; Veech *et al.*, 1973). Finally, since all other variables were kept constant, the time-courses of metabolite concentrations described in this study permit the examination at any stage during the development of ketoacidosis with an idea of the complex metabolic interactions occurring at that time.

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