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Decreased rate of ketone-body oxidation and decreased activity of D-3-hydroxybutyrate dehydrogenase and succinyl-CoA:3-oxo-acid CoA-transferase in heart mitochondria of diabetic rats

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Heart mitochondria from chronically diabetic rats ('diabetic mitochondria'), in metabolic State 3, oxidized 3-hydroxybutyrate and acetoacetate at a relatively slow rate, as compared with mitochondria from normal rats ('normal mitochondria'). No significant differences were observed, however, with pyruvate or L-glutamate plus L-malate as substrates. Diabetic mitochondria also showed decreased 3-hydroxybutyrate dehydrogenase and succinyl-CoA: 3-oxoacid CoA-transferase activities, but cytochrome content and NADH-dehydrogenase, succinate dehydrogenase, cytochrome oxidase and acetoacetyl-CoA thiolase activities proved normal. The decrease of 3-hydroxybutyrate dehydrogenase activity was observed in diabetic mitochondria subjected to different disruption procedures, namely freeze–thawing, sonication or hypo-osmotic treatment, between pH 7.5 and 8.5, at temperatures in the range 6–36 °C, and in the presence of L-cysteine. Determination of the kinetic parameters of the enzyme reaction in diabetic mitochondria revealed diminution of maximal velocity ($V_{max.}$) as its outstanding feature. The decrease in 3-hydroxybutyrate dehydrogenase in diabetic mitochondria was a slow-developing effect, which reached full expression 2–3 months after the onset of diabetes; 1 week after onset, no significant difference between enzyme activity in diabetic and normal mitochondria could be established. Insulin administration to chronically diabetic rats for 2 weeks resulted in limited recovery of enzyme activity. G.l.c. analysis of fatty acid composition and measurement of diphenylhexatriene fluorescence anisotropy failed to reveal significant differences between diabetic and normal mitochondria. The Arrhenius-plot characteristics for 3-hydroxybutyrate dehydrogenase in membranes of diabetic and normal mitochondria were similar. It is assumed that the variation of the assayed enzymes in diabetic mitochondria results from a slow adaptation to the metabolic conditions resulting from diabetes, rather than to insulin deficiency itself.

INTRODUCTION

Investigation of liver mitchondria of diabetic dogs (Roldan *et al.*, 1971) and diabetic rats (Boveris *et al.*, 1969; Vidal *et al.*, 1977, 1983; Brignone *et al.*, 1982) showed a depressed rate of 3-hydroxybutyrate oxidation and decreased activity of 3-hydroxybutyrate dehydrogenase (D-3-hydroxybutyrate:NAD^+ oxidoreductase, EC 1.1.1.30) in the mitochondrial inner membrane. The enzyme decrease, whose magnitude was related to the severity of the diabetes, was reversed by insulin treatment.

3-Hydroxybutyrate and acetoacetate, the main ketone bodies, are important sources of metabolic energy for the myocardium especially in diabetes (Robinson & Williamson, 1980). Oxidation of 3-hydroxybutyrate to acetoacetate is the first step in the process of ketone-body utilization in the peripheral tissues, the oxidation being catalysed by 3-hydroxybutyrate dehydrogenase. Acetoacetate, the reaction product, is converted into acetoacetyl-CoA in a reaction catalysed by 3-oxoacid CoA-transferase (succinyl-CoA:3-oxo-acid CoA-transferase, EC 2.8.3.5), and then acetoacetyl-CoA is cleaved by acetoacetyl-CoA thiolase (acetyl-CoA:acetyl-CoA *C*-acetyltransferase, EC 2.3.1.9) to form acetyl-CoA. The latter is finally condensed to form citrate and enters the tricarboxylic acid cycle. Both 3-hydroxybutyrate and acetoacetate are available in circulating blood for metabolic utilization by peripheral tissues, especially the myocardium (Robinson & Williamson, 1980).

Taking the foregoing into account, we decided to establish whether diabetes affects ketone-body oxidation by heart mitochondria. The results now reported confirm this hypothesis and allow one to correlate the depressed rates of 3-hydroxybutyrate and acetoacetate oxidation with the selective decrease in activity of 3-hydroxybutyrate dehydrogenase and 3-oxoacid CoA-transferase in mitochondria from diabetic rats ('diabetic mitochondria').

MATERIALS AND METHODS

Chemicals

The following were obtained from the supplier indicated: NAD^+ (grade III), sodium DL-3-hydroxybutyrate, L-cysteine, Trizma base, EDTA, bovine serum albumin, cytochrome *c* (type IV), sucrose, mannitol, ADP (grade I), L-malic acid, L-glutamic acid, malonic acid, succinic acid, rotenone, phenazine methosulphate, dichlorophenol-indophenol, succinyl-CoA, CoA, acetoacetate (lithium salt), 3-hydroxybutyrate dehydrogenase (type IV, from *Pseudomonas lemoignei*), acetoacetyl-CoA, iodoacetamide and 2,6-di-t-butyl-*p*-cresol from Sigma

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Chemical Co., St. Louis, MO, U.S.A.; streptozotocin from Upjohn International, Kalamazoo, MI, U.S.A.; insulin NPH from E. Lilly and Co., Buenos Aires, Argentina; Nagarse from Nagarse and Co., Osaka, Japan (1.5×10^6 proteolytic units/g); diphenylhexatriene from Serva, Heidelberg, Federal Republic of Germany; and BF_3 (50%, w/w, in methanol) from Aldrich Chemical Co., Milwaukee, WI, U.S.A. Other reagents were of analytical grade.

Animals and treatment

Male Williams rats from the Instituto de Química Biológica, weighing 180–220 g, were maintained on a Forramez or Purina diet and tap water *ad libitum*. Rats were rendered diabetic by intravenous injection of streptozotocin (65 mg/kg body wt.). Streptozotocin was dissolved in 0.1 M-citrate buffer, pH 4.5, 2–3 min before use. Control rats received the corresponding volume of citrate buffer. At 3 days after streptozotocin injection, average glycaemia values (mM) were 18 ± 3.7 (38) and 20 days after, 24 ± 4 (15). Rats showed polyphagia, polydipsia and polyuria, and also loss of weight. Investigation of ketone bodies in urine was positive in a few individuals during the first week of diabetes, but afterwards it was always negative. Unless stated otherwise, rats were used 2 months after streptozotocin treatment. The blood total ketone-body concentration (mM) of these rats was 0.15 ± 0.06 (8) [normal value 0.05 ± 0.02 (15)] and the 3-hydroxybutyrate/acetoacetate molar ratio was 2.0 (diabetic) and 0.9 (normal).

Assay methods

Glucose concentration in plasma was measured by the method of Nikkila & Hyvarinen (1962). Acetoacetate and 3-hydroxybutyrate concentrations in blood were determined by the methods of Mellanby & Williamson (1974) and Williamson & Mellanby (1974) respectively. Protein was determined by the method of Jacobs *et al.* (1956), with bovine serum albumin as a standard. Ketone bodies in urine were controlled by the Ketodiastix test (Ames).

Preparation of mitochondria

Unless otherwise stated, the Favelukes *et al.* (1975) procedure for skeletal-muscle mitochondria was used as follows. Rats were killed by decapitation and the hearts were rapidly excised and immersed in cold 0.15 M-KCl, the connective and auricular tissues were dissected, and the muscle tissue was thoroughly minced with scissors. The mince was suspended in 10 vol. of Chappell & Perry (1956) medium [100 mM-KCl, 50 mM-Tris/HCl, pH 7.2, 1 mM-ATP(Na), 5 mM-$MgSO_4$ and 1 mM-EGTA (instead of EDTA)] containing Nagarse (2 mg/g of tissue). After 15 min incubation at 0 °C, the suspension was homogenized with a Teflon/glass Potter–Elvehjem homogenizer, with three or four strokes of the pestle at moderate speed. Then 50 ml of medium was added to the homogenate and the suspension was centrifuged twice for 5 min at 500 ***g*** and 4 °C, to discard myofibrils and unbroken tissue. The supernatant was centrifuged at 7500 ***g*** for 20 min at 4 °C; the pellet was washed two to four times by centrifugation, at 4 °C, with medium containing serum albumin (1 mg/ml), and finally suspended in 0.25 M-sucrose at a concentration of 10–15 mg of protein/ml. When required, mitochondria were prepared by the method of Malmström & Carafoli (1976). Groups of at least two rats subjected to the same treatment were killed simultaneously, and the pooled hearts were used for the mitochondrial preparation.

Mitochondria were disrupted as follows. (1) Freeze–thawing. The mitochondrial suspension in 0.25 M-sucrose was frozen at −15 °C and then thawed at 4 °C. The number of freeze–thawing cycles is indicated in each case (see the Results section). (2) Sonic treatment (Beyer, 1967). The mitochondrial suspension in 0.25 M-sucrose was sonicated in a MSE disintegrator (500 W, 20 kHz), at an output of 0.4–0.5 mA, for 1 min, in periods of 10 s. During sonication the samples were kept in an ice–water bath (at 4 °C). 3. Osmotic shock (Parsons & Williams, 1967). The mitochondrial suspension in 0.25 M-sucrose was centrifuged and the pellet was resuspended in 20 mM-phosphate buffer, pH 7.5, containing bovine serum albumin (20 mg/100 ml). After standing for 20 min at 4 °C, the membranes (inner and outer) were centrifuged at 35000 ***g*** for 20 min at 4 °C, and the pellet was resuspended in 0.1 M-phosphate buffer, pH 7.5.

Measurement of enzyme activities

Unless otherwise stated, enzyme activities were measured at 30 °C.

3-Hydroxybutyrate dehydrogenase activity. This was determined by measuring the reduction of NAD^+ in a Gilford model 2000 spectrophotometric unit, or a Beckman DB-G spectrophotometer attached to a recorder. Unless stated otherwise, the standard reaction mixture (1.0 ml) contained 50 mM-Tris/HCl buffer (pH 8.0), 2.0 mM-NAD^+, 0.3 μg of rotenone, 0.4 mg of albumin and 0.15–0.40 mg of mitochondrial protein. Samples were preincubated for 10 min at 30 °C and the reaction was then started by addition of 50 μl of 0.2 M-DL-3-hydroxybutyrate (sodium salt). The rate of NAD^+ reduction was calculated from the increase in A_{340}, measured for at least 2 min. The time of preincubation was essential in order to obtain maximal activity. Under the given experimental conditions, the initial velocity was linearly proportional to the concentration of mitochondrial protein throughout a range of 0–0.8 mg of protein/ml. Enzyme activity is expressed as nmol of NAD^+ reduced/min per mg of protein.

Kinetic parameters were determined by using double-reciprocal plots, the nomenclature proposed by Cleland (1963) and according to eqn. (1):

$$V_0 = \frac{V_{max.}[A][B]}{K_{ia}K_b + K_a[B] + K_b[A] + [A][B]} \quad [1]$$

V_0 is the initial velocity at given concentrations of A (NAD^+) and B (3-hydroxybutyrate), $V_{max.}$ is the maximal velocity at infinite concentration of NAD^+ and 3-hydroxybutyrate, K_{ia} is the dissociation constant for NAD^+, and K_a and K_b are the Michaelis constants for NAD^+ and 3-hydroxybutyrate respectively. The values of kinetic constants were calculated by the computation method determined by Cleland (1979).

3-Oxoacid CoA-transferase activity. The acetoacetyl-CoA formation from succinyl-CoA and acetoacetate was measured spectrophotometrically, at 313 nm and 25 °C, as described by Williamson *et al.* (1971), with sonicated mitochondria from three pooled rat hearts. The reaction mixture (1.0 ml) contained 50 mM-Tris/HCl buffer,

pH 7.4, 5.0 mM-iodoacetamide, 0.2 mM-succinyl-CoA, 5.0 mM-$MgSO_4$ and 50 mM-acetoacetate (lithium salt). The reaction was started by adding 5–25 μg (10 μl) of sonicated mitochondrial membranes (in 0.25 M-sucrose/ 10 mM-Tris/HCl buffer (pH 7.4)/1.0 mM-mercapto-ethanol] and the kinetics were followed for 2–3 min. Enzyme activity is expressed as nmol of acetoacetyl-CoA formed/min per mg of protein.

Acetoacetyl-CoA thiolase activity. Substrate cleavage was measured spectrophotometrically at 303 nm and 25 °C, as described by Williamson *et al.* (1971). The reaction mixture contained 50 mM-Tris/HCl buffer, pH 7.4, 5.0 mM-$MgSO_4$, 0.12 mM-CoA and 0.07 mM-acetoacetyl-CoA. The reaction was started by adding 5–10 μg of mitochondrial membranes, prepared as described for the transferase assay, and the reaction kinetics were followed for 2–3 min. Enzyme activity is expressed as nmol of acetoacetyl-CoA disappeared/min per mg of protein.

Other enzyme activities. Cytochrome oxidase was measured spectrophotometrically at 25 °C, as described by Smith (1954), with cytochrome *c* as substrate (14–17 μM), reduced at least 70% by the ascorbate method (Margoliash & Walasek, 1967). Activity is expressed as μmol of cytochrome *c* oxidized/min per mg of protein. NADH dehydrogenase was measured as described by Galante & Hatefi (1978), with ferricyanide concentrations in the range 0.4–2.0 mM. Activity is expressed as $V_{max.}$, in μmol of NADH oxidized/min per mg of protein. Succinate dehydrogenase was measured by the phenazine methosulphate/dichlorophenol-indophenol method as described by Ackrell *et al.* (1978). Activity is expressed as μmol of succinate oxidized/min per mg of protein.

Cytochrome composition of mitochondria

Difference spectra for cytochrome components of intact or frozen mitochondria were obtained with a split-beam spectrophotometer (Johnson Foundation, Philadelphia, PA, U.S.A.), by the method of Williams (1964). The mitochondrial fraction (3–4 mg) was suspended in 3 ml of 0.25 M-sucrose, in a 3 ml cell. One sample was oxidized by adding ferricyanide (final concn. 5 mM) and the other reduced with dithionite (2–3 mg).

Mitochondrial respiration

Oxygen consumption and respiratory control ratio were measured polarographically with an oxygraph (Gilson Medical Electronics) at 30 °C, with the vibrating platinum electrode. The standard reaction mixture (1.9 ml) contained 0.25 M-mannitol, 10 mM-KCl, 2 mM-EDTA, 10 mM-Tris/HCl, 5.0 mM-K_2HPO_4/ KH_2PO_4, pH 7.4, and 2.0–3.0 mg of bovine serum albumin/ml. The mitochondrial suspension (about 1 mg/ml), the substrates and ADP (0.4 mM) were added in the given order. When using L-glutamate and L-malate, malonate (final concn. 2.5 mM) was added with the substrates. Other experimental conditions are given in Table 1.

Fatty acid composition of mitochondrial lipids

Total lipids of heart mitochondria were extracted with chloroform/methanol (2:1, v/v) by the method of Folch *et al.* (1957). Fatty acids were transesterified with 10% (w/v) BF_3 in methanol, for 2 min, in extraction tubes (Metcalfe & Schmitz, 1961). The antioxidant 2,6-di-t-butyl-*p*-cresol was used at a concentration of 50 mg/l throughout the procedure, to prevent peroxidative damage to polyunsaturated fatty acids. After extraction with diethyl ether, the methyl esters were analysed by g.l.c. A Perkin–Elmer model Sigma 300 FID with flame-ionization detector was used. Analyses were made on glass columns packed with Silar 10C on Gas Chrom QII, 100–120 mesh. The temperature of the oven was programmed in the 160–200 °C range and the carrier gas (N_2) flow rate was 40 ml/min. The peaks were identified by comparison with standards. Results are expressed as weight % for each fatty acid. The double-bond index

Table 1. Substrate oxidation by heart mitochondria of normal and diabetic rats

Mitochondria (0.9 mg of protein/ml) were prepared as described by Malmström & Carafoli (1976); respiration in State 3 was measured in the presence of 0.4 mM-ADP, and respiration in State 4 was measured after ADP was used up. Other experimental conditions were as described in the Materials and methods section. Values are means ± S.D., with the numbers of rats indicated in parentheses. Values that are statistically significantly different from normal values are indicated by *($P < 0.001$) or †($P < 0.05$).

Substrate	State of rats (number)	Rate of respiration (ng-atoms of O/min per mg of protein)		Respiratory control (State 3/ State 4)	P/O ratio
		State 3	State 4		
10 mM-DL-3-Hydroxybutyrate + 0.25 mM-L-malate	Control (9)	85.5 ± 11.2	37.9 ± 6.7	2.3 ± 0.4	2.74 ± 0.33
	Diabetic (11)	44.7 ± 6.8*	23.6 ± 3.4*	1.9 ± 0.5	2.77 ± 0.25
5.0 mM-Acetoacetate + 0.25 mM-L-malate	Control (7)	40.3 ± 4.7	18.2 ± 4.1	2.4 ± 0.6	2.79 ± 0.50
	Diabetic (11)	23.0 ± 2.5*	14.8 ± 3.1	1.6 ± 0.3†	2.61 ± 0.16
5.0 mM-Pyruvate + 0.25 mM-L-malate	Control (9)	242 ± 41.4	40.7 ± 7.7	6.2 ± 1.8	2.66 ± 0.14
	Diabetic (10)	217 ± 45.1	35.8 ± 7.5	6.2 ± 1.3	2.81 ± 0.18
5.0 mM-L-Glutamate + 5.0 mM-L-malate + 2.5 mM-malonate	Control (4)	251 ± 9.2	51.2 ± 7.4	4.9 ± 0.8	2.73 ± 0.22
	Diabetic (5)	236 ± 21.8	59.4 ± 11.6	4.2 ± 0.5	2.65 ± 0.35

Table 2. Activities of 3-hydroxybutyrate dehydrogenase, 3-oxoacid CoA-transferase and acetoacetyl-CoA thiolase in heart mitochondria of normal and diabetic rats

Diabetic rates were used 3 months after the streptozotozin treatment. Mitochondria disrupted by four freeze–thawing cycles. Other experiment conditions were as described in the Materials and methods section. Enzyme activities are expressed as defined with the corresponding method; numbers of measurements are given in parentheses. *$P < 0.001$ for significant difference from normal.

Enzyme	State of rats	
	Normal	Diabetic
3-Hydroxybutyrate dehydrogenase	55.0±5.4 (5)	15.1±5.2 (5)*
3-Oxoacid CoA-transferase	133±16.0 (4)	66.9±7.8 (4)*
Acetoacetyl-CoA thiolase	446±56.3 (4)	475±56.1 (4)

(DBI) was calculated by summing the (number of double bonds in fatty acid a)×(weight % occurrence of a) for each fatty acid a in the group of n fatty acids.

Measurement of fluorescence of diphenylhexatriene in mitochondrial membranes

Fluorescence anisotropy values (Shinitzky & Barenholz, 1974) were obtained by measuring the fluorescence intensities polarized parallel and perpendicular to the direction of polarization of the excitation beam respectively. Measurements were made with an Aminco–Bowman spectrofluorimeter equipped with Glan prisms. The assay medium contained 250 mM-sucrose, 0.2 mM-EDTA, 50 mM-Tris/HCl buffer, pH 7.4, 2.0 μM-diphenylhexatriene and 50 μg of mitochondrial protein. Other experimental conditions were as described by Vidal *et al.* (1983).

Expression of results

The values are given as means±S.D. of the mean and, in parentheses, the number of samples. Statistical analysis of results was performed by Student's t test.

RESULTS

Substrate oxidation by normal and diabetic mitochondria

Oxidative-phosphorylation studies were carried out using the substrates listed in Table 1. The respiration rate of diabetic mitochondria in metabolic State 3 (Chance & Williams, 1956) was significantly depressed with 3-hydroxybutyrate and acetoacetate (48 and 42% respectively), but not with pyruvate or L-glutamate. On the other hand, the phosphorylating capacity (expressed as the P/O ratios) appeared to be normal with all the substrates assayed, thus indicating that diabetic mitochondria were not deficient in the ability to transfer electrons from NADH to oxygen or to couple oxidation to phosphorylation. Addition of L-malate was essential for a rapid oxidation of ketone bodies, as otherwise the oxidation rate decreased to about 30% and no significant differences between diabetic and normal mitochondria could then be detected (Grinblat *et al.*, 1981).

Effect of diabetes on mitochondrial enzyme activities

Specific activities of mitochondrial enzymes involved in ketone-body utilization are shown in Table 2.

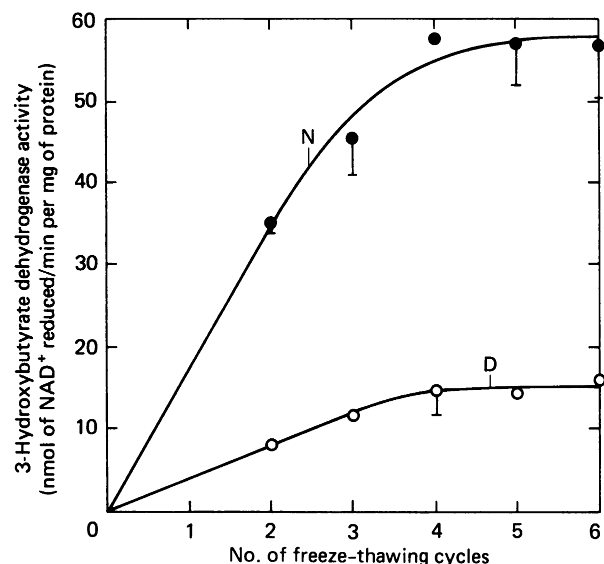

Fig. 1. Effect of freeze–thawing on 3-hydroxybutyrate dehydrogenase activity of mitochondrial membranes from normal (N) and diabetic (D) rats

Standard experimental conditions were used, except that mitochondria were subjected to freeze–thawing for the number of cycles indicated on the abscissa; 0.27 (N) and 0.34 (D) mg of mitochondrial protein were used. The points represent the averages of two independent experiments; the bars indicate the deviation of the experimental values from the mean.

3-Oxoacid CoA-transferase was measured in the direction of acetoacetyl-CoA synthesis, a condition in which the enzyme activity appears to be about 20% as active as in the reverse direction (Williamson *et al.*, 1971). Accordingly, the actual ratio of 3-oxoacid CoA-transferase to acetoacetyl-CoA thiolase activities should be about 1.5, a value approaching that calculated from the data of Williamson *et al.* (1971) for sonicated heart extracts. Comparison of enzyme activities in normal and diabetic mitochondria revealed in the latter a significant diminution of the dehydrogenase and transferase activities (72 and 50% respectively). However, the thiolase activity did not vary. Remarkably enough, the decrease

Table 3. 3-Hydroxybutyrate dehydrogenase, NADH dehydrogenase, succinate dehydrogenase, cytochrome oxidase activity and cytochrome composition of mitochondria from normal and diabetic rats

Diabetic rats were used 1 month after the streptozotocin treatment. Experimental conditions were as described in the Materials and methods section. Enzyme activity is expressed as defined with the corresponding method; numbers of measurements are given in parentheses. The cytochrome composition was determined with mitochondria (1.0–1.3 mg of protein/ml) from three pooled hearts. $*P < 0.001$ for significant difference from normal.

Enzyme or cytochrome measured	Disruption of mitochondria	State of rats		Decrease of activity (%)
		Normal	Diabetic	
3-Hydroxybutyrate dehydrogenase	Freeze–thawing	50.5±2.7 (11)	24.9±2.0 (12)*	51
	Same; activity measured in the presence of 50 mM-cysteine	50.1±3.3 (8)	25.9±1.9 (8)*	50
	Sonication	65.2±15.5 (3)	32.0±13.1 (3)*	51
	Osmotic shock	17.5±0.05†	6.7±0.3†	61
NADH dehydrogenase	Osmotic shock	1.36±0.17 (4)	1.80±0.38 (4)	—
Succinate dehydrogenase	Osmotic shock	0.14±0.01 (4)	0.15±0.01 (7)	—
Cytochrome oxidase	Osmotic shock	1.85±0.19 (4)	1.81±0.18 (5)	—
Cytochrome composition (nmol/mg of protein)				
Cytochrome aa_3	None	1.20±0.05 (8)	1.10±0.14 (5)	—
Cytochrome b		0.88±0.05 (8)	0.81±0.06 (5)	—
Cytochrome c_1		0.26±0.01 (8)	0.26±0.02 (5)	—
Cytochrome c		0.68±0.04 (3)	0.60±0.04 (5)	—

†Mean±deviation from two samples.

Table 4. Kinetic parameters of 3-hydroxybutyrate dehydrogenase reaction in heart mitochondria

The values represent best fits (±S.D.) and were obtained with mitochondria from pooled hearts of normal (8) or diabetic (12) rats. Diabetic rats were used 1 month after the streptozotocin treatment. Mitochondria were disrupted by five freeze–thawing cycles. Other conditions were as described in the Materials and methods section. $*P < 0.01$ for significant difference from normal.

Kinetic parameter	State of rats	
	Normal	Diabetic
$V_{max.}$ (nmol/min per mg of protein)	62.1±3.84	42.9±2.21*
K_a (mM)	0.086±0.073	0.284±0.04
K_b (mM)	0.629±0.158	0.481±0.100
K_{ia} (mM)	0.833±0.355	0.809±0.250

of the dehydrogenase activity in diabetic mitochondria was a tardy phenomenon. Thus, 1 week after the onset of diabetes the average enzyme-activity values were not significantly different from the normal. Maximal decay of 3-hydroxybutyrate dehydrogenase activity was reached at 2–3 months after streptozotocin injection, with activity values as in Table 2.

The decay of 3-hydroxybutyrate dehydrogenase activity was confirmed by using mitochondria subjected to different disruption procedures. Fig. 1 shows that freeze–thawing released enzyme activity as a function of the number of freezing cycles, but the relative depression of the dehydrogenase activity in diabetic mitochondria was roughly the same with all the samples assayed. Similar results were obtained with frozen, sonicated or osmotically disrupted mitochondria (Table 3). Cysteine, an enzyme activator (Gotterer, 1967; Latruffe & Gaudemer, 1974), failed to re-establish 3-hydroxybutyrate dehydrogenase activity (Table 3). The maximal difference between normal and diabetic mitochondria was obtained between pH 7.5 and 8.5 (results not shown).

In contrast with 3-oxoacid CoA-transferase and acetoacetyl-CoA thiolase, 3-hydroxybutyrate dehydrogenase is an inner-membrane-bound enzyme. This special linkage prompted comparison with other inner-membrane enzymes, in order to ascertain the selectivity of its variation in diabetic mitochondria. The results in

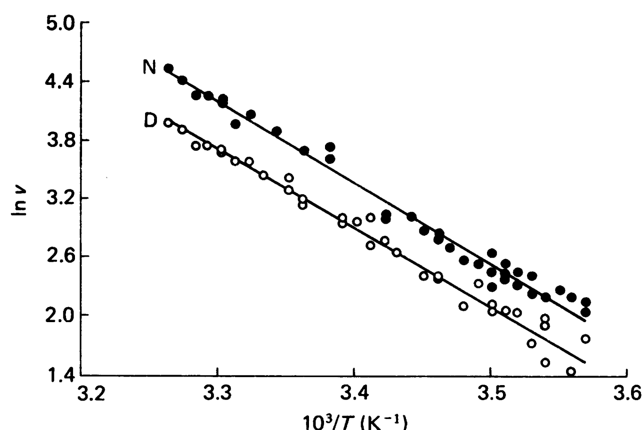

Fig. 2. Arrhenius plots of 3-hydroxybutyrate dehydrogenase activity of heart mitochondrial membranes of normal (N) and diabetic (D) rats

The temperature-dependence of the initial reaction velocities was determined with 0.09–0.17 mg of mitochondrial protein/ml. The values correspond to mitochondria from 10 (N) and 8 (D) rats. Other conditions were as described in the Materials and methods section, except for the temperature, which is indicated on the abscissa. Enzyme activity (v) is expressed as nmol/min per mg of mitochondrial protein. The correlation coefficients were 0.98 ± 0.01 (N) and 0.97 ± 0.03 (D).

Table 5. Fatty acid composition of total lipids from heart mitochondria of normal and diabetic rats

Experimental conditions were as described in the Materials and methods section. Diabetic rats were used 3 months after the streptozotocin treatment. The data given for the fatty acid analysis does not include minor components [18:3(9,12,15), 20:1(11), 20:3(5,8,11), 20:3(8,11,14) and 20:5(5,8,11,14,17)], which together constituted 3% of the total fatty acids and did not vary significantly between the normal and diabetic samples.

	State of rats (6)	
Fatty acid	Normal	Diabetic
16:0	13.5 ± 0.6	13.7 ± 2.3
16:1(9)	1.5 ± 0.6	1.0 ± 0.4
17:0	1.2 ± 0.3	1.2 ± 0.4
18:0	20.3 ± 1.5	20.4 ± 2.0
18:1(9)	8.0 ± 1.0	6.5 ± 0.5
18:2(9,12)	24.0 ± 1.2	20.0 ± 1.3
20:4(5,8,11,14)	15.0 ± 1.0	15.8 ± 1.5
22:5(7,10,13,16,19)	1.5 ± 0.3	1.3 ± 0.3
22:6(4,7,10,13,16,19)	10.9 ± 1.1	17.0 ± 1.6
DBI	203 ± 8.9	230 ± 12.7

Table 3 indicate that no significant differences between normal and diabetic mitochondria could be established as regards NADH dehydrogenase, succinate dehydrogenase or cytochrome oxidase, or the cytochrome content.

The kinetic parameters for the 3-hydroxybutyrate dehydrogenase reaction were determined by using normal and diabetic mitochondrial membranes. The double-reciprocal plots of initial velocity were a family of intersecting lines, and the replots of their slopes and intercepts were a linear function of the second substrate (results not shown). The data from initial-velocity measurements were fitted to eqn. (1) to obtain values for K_a, K_{ia} and K_b, and Table 4 summarizes the values obtained. K_a and $V_{max.}$ values were in close agreement with those obtained by Tucker & Dawson (1979), whereas K_b and K_{ia} approached the values reported by Nielsen *et al.*(1973). The ratio of $V_{max.}$ for normal mitochondria to that for diabetic mitochondria was about 1.5, in good agreement with results by Vidal *et al.* (1983) for the liver enzyme. The K_a for diabetic mitochondria was apparently higher than for the normal enzyme, but variation was within the limit of significance.

Effect of temperature

The activity of 3-hydroxybutyrate dehydrogenase in diabetic and normal mitochondrial membranes was measured at different temperatures, and the Arrhenius plots are presented in Fig. 2. No break in these plots is apparent, as reported by Menzel & Hammes (1973), Gazzotti *et al.* (1975) and Vidal *et al.* (1978) with similar enzyme preparations. The values for the apparent activation energy were, in kJ/mol (kcal/mol), 39.1 (9.3) and 36.6 (8.7) for the control and diabetic enzyme preparations respectively. These values approached those reported by Latruffe & Gaudemer (1974) and Vidal *et al.* (1978) for the liver enzyme.

Fatty acid composition of mitochondrial membranes

3-Hydroxybutyrate dehydrogenase forms lipoprotein complexes with unsaturated phosphatidylcholines, which are essential for the enzyme activity (Gazzotti *et al.*, 1975; McIntyre *et al.*, 1978; Churchill *et al.*, 1983). Since unsaturated fatty acid synthesis is decreased in diabetes (Mercuri *et al.*, 1966; Mercuri & DeTomas, 1977), the observed variation of 3-hydroxybutyrate dehydrogenase activity in diabetic mitochondria might then depend on changes in the mitochondrial lipid structure. However, no significant differences between diabetic and normal mitochondrial lipids were observed (Table 5), as reported by Rösen *et al.* (1983), despite the fact that changes in dietary fatty acids may affect the composition of heart lipids (Tahin *et al.*, 1981; McMurchie *et al.*, 1984; Royce & Holmes, 1984). Furthermore, measurement of fluorescence anisotropy of diphenylhexatriene, which is an index of membrane fluidity (Shinitzky & Barenholz, 1974), yielded similar values for normal and diabetic mitochondria [0.18 ± 0.012 (10) and 0.18 ± 0.015 (10) respectively].

Effect of insulin

Treatment of diabetic rats with insulin for periods of 3–15 days decreased glycaemia to normal or even lower values and increased 3-hydroxybutyrate activity to a relatively minor extent. Nevertheless, when rats received insulin for 3 weeks, starting 7 days after the onset of diabetes, the decay of 3-hydroxybutyrate dehydrogenase activity was prevented to a highly significant degree (Table 6).

DISCUSSION

The observed variations of 3-hydroxybutyrate dehydrogenase and 3-oxoacid CoA-transferase in Fig. 1 and

Table 6. 3-Hydroxybutyrate dehydrogenase activity in mitochondrial membranes of diabetic rats after insulin treatment

Values are means ± S.D.; numbers of measurements are given in parentheses. Mitochondrial samples were made from at least three pooled hearts each. Mitochondria were disrupted by three freeze–thawing cycles. Insulin treatment was 5.0 (Expt. A) or 2.0 units (Expt. B) per 100 g body wt., injected daily, subcutaneously, for the number of days indicated below. Other experimental conditions were as described in the Materials and methods section. *$P < 0.05$, as compared with normal rats; †$P < 0.05$, as compared with non-treated diabetic rats.

Expt.	Time after the onset of diabetes at which the insulin treatment was started (days)	Duration of insulin treatment (days)	3-Hydroxybutyrate dehydrogenase activity (nmol/min per mg of protein)
A	30	No treatment	21.4 ± 3.5 (3)*
		3	26.7 ± 2.9 (3)*†
		7	25.5 ± 0.5 (3)*
		15	27.9 ± 3.1 (3)*†
B	7	23	38.8 ± 8.8 (4)
Enzyme activity in mitochondria of normal rats ...			39.2 ± 0.7 (3)

Tables 2 and 3 may explain the depressed rate of ketone-body oxidation by diabetic heart mitochondria (Table 1). In this regard, our results fit in well with the overall decrease of ketone-body utilization by peripheral tissues in diabetic organisms (Hall *et al.*, 1984; Nosadini *et al.*, 1985).

The information available on biochemical alterations in diabetic mitochondria is extensive but contradictory. Concerning muscle mitochondria, impairment of oxidative phosphorylation and decrease of succinate dehydrogenase activity was reported by Goranson & Erulkar (1949), Haugaard & Haugaard (1964) and Armstrong & Ianuzzo (1976). However, Dow (1967) and Favelukes *et al.* (1973) observed normal respiration and phosphorylation. Our results with pyruvate, or L-glutamate plus L-malate, as substrates (Table 1) are in good agreement with the latter observations. Concerning liver mitochondria, the following effects have been described: decrease in P/O ratio and respiration rate (Vester & Stadie, 1957; Hall *et al.*, 1960; Matsubara & Tochino, 1969); increase in respiration but normal P/O ratio (Beyer & Shamoian, 1961; Mackerer *et al.*, 1971); increase in palmitate oxidation and decrease in oxidation of 2-oxoglutarate and succinate (Harano *et al.*, 1969, 1972); normal P/O ratio with several substrates (Parks *et al.*, 1955); decrease in cytochrome oxidase and cytochrome aa_3 (Lerner *et al.*, 1972); and, finally, decrease in 3-hydroxybutyrate oxidation with normal P/O ratio (Boveris *et al.*, 1969; Brignone *et al.*, 1982). Our results do not agree with some of these reports.

The slow decrease of 3-hydroxybutyrate dehydrogenase activity in diabetic heart mitochondria and its limited recovery after insulin treatment (Table 6) are at variance with the rapid evolution of other expressions of insulin deficiency (Weber *et al.*, 1965). Concerning 3-oxoacid CoA-transferase, a slow decay during diabetes may also be inferred, since Bässler *et al.* (1973) failed to observe a significant variation of this enzyme activity in heart mitochondria 3 weeks after onset of diabetes. Therefore the selective variations of 3-hydroxybutyrate dehydrogenase and 3-oxoacid CoA-transferase activities in Table 2 may be envisaged as the result of a slow metabolic adaptation to hormonal imbalance and/or modified substrate conditions, rather than as a direct mitochondrial response to insulin deficiency. Studies on skeletal muscle (Chen & Ianuzzo, 1982) or isolated perfused hearts from acute or chronically diabetic rats lend support to this assumption (Chen *et al.*, 1984). On the other hand, liver 3-hydroxybutyrate dehydrogenase seems to be more responsive to hormonal control, as shown by its rapid restoration to normal values after insulin treatment (Roldan *et al.*, 1971; Vidal *et al.*, 1977, 1983; Brignone *et al.*, 1982).

3-Hydroxybutyrate dehydrogenase has long been recognized as a sensor of the physical properties of the mitochondrial inner membrane (Sandermann, 1978) and, accordingly, the decrease in the enzyme activity after chronic intense diabetes suggests a membrane alteration not detected by the measurement of other mitochondrial properties or functions. The activity decay might then reflect abnormal phospholipid constitution of the mitochondiral inner membrane, owing to deficient synthesis of unsaturated fatty acids in diabetes. Earlier experiments with liver mitochondria by Vidal *et al.* (1977) lent support to this hypothesis, but more recent observations (Vidal *et al.*, 1983) have demonstrated that the lowered dehydrogenase activity in liver mitochondria of diabetic rats is due to decreased enzyme in the mitochondrial inner membrane, not to the modification of the lipid environment. The fatty acid composition of the heart mitochondrial lipids (Table 5), the measurement of diphenylhexatriene fluorescence and the Arrhenius plots (Fig. 2) fit in well with this latter possibility.

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