Phosphorylation, Oxidation, and Ubiquinone Content in Diabetic Mitochondria[1] (34174)

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Several investigators have put forward the view that diabetes affects mitochondrial function and structure. Thus (a) Hall *et al.* (1) and Vester and Stadie (2) reported depression of respiration and uncoupling of phosphorylation in diabetic liver mitochondria; (b) Haugaard and Haugaard (3) reported that oxidative phosphorylation is depressed in heart homogenates from diabetic rats; (c) diabetic mitochondria appear to be larger and more fragile than their normal counterpart (1); (d) the ubiquinone content of diabetic mitochondria is decreased [Shigeta *et al.* (4)]. However, these observations have not remained unchallenged. For example, Parks *et al.* (5) and Dow (6) did not observe a depression of the respiratory capacity and uncoupling of the liver (5) and muscle (6) diabetic mitochondria.

Most of the studies cited (1, 3–6) were performed with mitochondria from alloxan-diabetic rats. Alloxan is capable of inhibiting mitochondrial respiration and of uncoupling phosphorylation (7) and therefore, the effects attributed to the diabetic condition might reflect a direct action of alloxan on the respiratory systems. In order to avoid that difficulty, respiration and phosphorylation were investigated in liver mitochondria from rats made diabetic by pancreatectomy (8). Ubiquinone and cytochrome *c* content of mitochondria from diabetic and control rats were also determined.

Materials and Methods. White male rats, from the Institute of Physiology strain, were subjected to subtotal (95%) pancreatectomy according to Foglia (8). Laparotomized rats were used as controls. After 6–18 months, the depancreatized rats and the respective controls were killed by decapitation (without anesthesia) and the liver was immediately removed for mitochondrial preparation. The diabetic state was checked by determination of blood sugar (9).

Mitochondria were prepared by the method of Schneider (10). The liver cells were disrupted with a Potter-Elvehjem homogenizer consisting of a motor driven Teflon pestle fitted to a glass tube. The homogenization medium was 0.25 *M* sucrose, 1 m*M* EDTA, 5 m*M* Tris–HCl (pH 7.4). The homogenates were fractionated according to Myers and Slater (11). The mitochondria were suspended in the homogenization medium and the respiratory activity was measured not later than 3 hr after the end of the preparation. Mitochondrial respiration was determined polarographically at 30° with a vibrating platinum electrode (model K Oxigraph, Gilson Med. Elec.). The basic reaction medium contained 0.24 *M* sucrose, 34 m*M* KCl, 5 m*M* $MgCl_2$, 1 m*M* EDTA, 9 m*M* Tris–HCl (pH 7.4), 5 m*M* K_2HPO_4–NaH_2PO_4 (pH 7.4) and substrate as stated in Table I. When malate–glutamate was used as substrate, 2.5 m*M* malonate was added. The mitochondrial protein (1–2 mg/ml) and ADP (0.2–0.3 m*M*) were added successively. The final volume of the reaction mixture was 2 ml. The

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TABLE I. Phosphorylation and Substrate Oxidation in Mitochondria from Normal and Diabetic Rats.[a]

Condition of rats	Blood sugar (mg/100 ml ± SEM)	P:O	Maximal rate of mitochondrial respiration (*n* g-atoms of O/min /mg of protein)	Respiratory control	
				Before[b]	After[c]
	Substrate: β-hydroxybutyrate (10 m*M*)				
Control	93 ± 3	2.34 ± 0.10	92 ± 4	4.7 ± 0.2	5.9 ± 0.6
Depancreatized	140 ± 7	2.56 ± 0.12	76 ± 5[d]	3.9 ± 0.2	5.2 ± 0.6
	300 ± 14	2.39 ± 0.19	56 ± 4[e]	3.1 ± 0.5	2.9 ± 0.6
	Substrate: L-malate (5 m*M*)-L-glutamate (5 m*M*)				
Control	93 ± 3	2.38 ± 0.06	135 ± 7	6.0 ± 0.3	6.0 ± 0.5
Depancreatized	140 ± 7	2.43 ± 0.13	119 ± 7	4.9 ± 0.3	5.8 ± 0.8
	300 ± 14	2.40 ± 0.14	103 ± 6[f]	4.6 ± 0.4	4.4 ± 0.5
	Substrate: succinate (5 m*M*)				
Control	93 ± 3	1.64 ± 0.07	214 ± 9	4.5 ± 0.2	4.4 ± 0.3
Depancreatized	140 ± 7	1.74 ± 0.09	193 ±12	3.7 ± 0.2	4.9 ± 0.3
	300 ± 14	1.73 ± 0.12	181 ± 9[g]	3.9 ± 0.1	4.2 ± 0.3

[a] The values correspond to lots of 10 rats. Mean values are followed by the standard error of the mean.

[b] Ratio of respiratory rate in the presence of ADP vs. the respiratory rate before ADP addition.

[c] Ratio of respiratory rate in the presence of ADP vs. the respiratory rate after the expenditure of ADP.

[d] $p < 0.05$.

[e] $p < 0.001$.

[f] $0.001 < p < 0.01$.

[g] $p = 0.05$.

oxygen concentration in the reaction media was taken as 0.22 m*M*. The ADP:O ratio was determined according to Estabrook (12) (corrected for the small quantity of hydrolysed ADP, about 8% and is expressed as P:O ratio in Table I.

Total ubiquinone was measured by the Redfearn method (13). Spectra of oxidized and reduced quinone were recorded in a Beckman DK2 spectrophotometer. Cytochromes $c + c_1$ were determined with the Aminco-Chance spectrophotometer at 550–540 mμ and are expressed as cytochrome *c*. Antimycin A (1 μg/mg) was added to the mitochondria to assure a full oxidized state. After recording of absorbance in the oxidized state, 4 m*M* KCN and a few crystals of sodium dithionite were added and absorbance was measured again.

The protein concentration of mitochondrial preparations was determined with the biuret method (14) in the presence of 0.1% (w/v) sodium deoxycholate. Statistical analysis of the results was performed by applying the variance test. The values are given as mean values followed by the standard error of the mean (SEM). Other materials and methods were as described previously (15, 16).

Results. The metabolic parameters employed to test mitochondrial function were (a) the P:O ratio which measures the oxidative phosphorylating capacity of mitochondria; (b) the maximal rate of respiration [mitochondria in the presence of substrate and ADP, metabolic state "3" (17)]; and (c) the respiratory control ratio which is a function of the coupling of electron transfer and phosphorylation. Each substrate was assayed with mitochondria from (a) normal rats; (b) rats with incipient diabetes (blood

TABLE II. Ubiquinone and Cytochrome *c* Content in Mitochondria from Normal and Diabetic Rats.[a]

Condition of rats	Blood sugar (mg/100 ml ± SEM)	Ubiquinone (nmoles/mg of protein)	Cytochrome *c* (nmoles/mg of protein)	Mitochondria (mg of protein /g of wet liver)
Control	93 ± 3	2.81 ± 0.12	0.32 ± 0.02	13.3 ± 0.7
Depancreatized	140 ± 7	2.77 ± 0.23	0.32 ± 0.02	13.1 ± 0.9
	300 ±14	4.55 ± 0.35[b]	0.29 ± 0.02	11.3 ± 0.6[c]

[a] The values correspond to lots of 10 rats. Mean values are followed by the standard error of the mean.

[b] $p < 0.001$.

[c] $p > 0.05$.

sugar 140 ± 7 mg/100 ml); and (c) rats with manifest diabetes (blood sugar 300 ± 14 mg/100 ml).

Table I shows that pancreatectomy did not affect P:O quotients whatever the substrate employed. The obtained P:O values were in the normal range according to the substrates. On the other hand, the maximal rate of respiration decreased with all substrates used and the diminution was related to the severity of diabetic state. The largest variation was obtained with β-hydroxybutyrate while with succinate as substrate the difference between normal and diabetic values was in the limit of significance ($p = 0.05$). Diminution of maximal rate of respiration was the cause of the lower respiratory control values, as it can be calculated from the data presented in Table I.

Pancreatectomy did not affect the sensitivity of mitochondrial respiration towards steroids (15, 16) since with L-malate-L-glutamate as substrates (exptl. conditions as in Table I), nor-ethisterone acetate (5 μM), androsterone (25 μM) and testosterone (50 μM) inhibited to the same extent (about 45%) the ADP activated respiration of mitochondria from normal and diabetic rats.

Table II shows the effect of pancreatectomy on the ubiquinone and cytochrome *c* content of mitochondria. The ubiquinone values increased with manifest diabetes while cytochrome *c* levels remained unaffected by pancreatectomy. Furthermore, the quantity of mitochondria (mg of mitochondrial protein) per gram of liver showed a slight apparent diminution ($p > 0.05$) only with manifest diabetes.

Discussion. In agreement with Parks *et al.* (5), Vester and Stadie [expts. with rats (2)], and Dow (6), the phosphorylating activity (P:O ratio) of diabetic mitochondria did not significantly depart from normal values, whatever the substrate employed as electron donor. Therefore, a key element in mitochondrial function, such as the energy conserving mechanism was not primarily affected in diabetes. This stability is particularly significant considering that pancreatectomy did actually affect mitochondria as shown by the lower respiratory activity and the higher ubiquinone content of diabetic mitochondria.

Concerning previous observations by Hall *et al.* (1), and Haugaard and Haugaard (3) with alloxan-diabetic rats it is possible that direct damage of mitochondria may have been produced by high doses of alloxan (1, 3) since the latter agent is an uncoupler of oxidative phosphorylation and an inhibitor of mitochondrial respiration, at concentrations in the range of those used to cause diabetes (7, 18). Similarly, it cannot be ruled out that metabolic conditions accompanying the diabetic state, such as fatty infiltration of the liver, would cause the uncoupling of liver mitochondria from diabetic cats (1, 2).

Diabetic mitochondria showed a relatively lower respiratory activity which depended firstly on the substrate and secondly on the severity of the diabetic condition (Table I). It seems reasonable to postulate that surgical diabetes affected the level of substrate-

linked dehydrogenases in mitochondria but whether these changes were primary effects of the lack of insulin is an open question. In this connection, Schäfer and Nägel (19) reported a higher level of β-hydroxybutyrate, L-glutamate, L-malate, and succinate dehydrogenases in liver mitochondria after insulin treatment of rats. The depression of respiratory activity of diabetic mitochondria would fit in with the more striking effects observed by other workers either with tissue homogenates (3) or mitochondrial suspensions (1). Nevertheless, the relatively higher inhibitions reported by Hall *et al.* (1) and Haugaard and Haugaard (3) may be partially due to the already-mentioned effect of alloxan on respiratory enzymes (7).

Concerning the ubiquinone level in diabetic mitochondria the discrepancies between the present results and those reported by Shigeta *et al.* (4) could be accounted for by the different experimental conditions in which the diabetic state was developed.

Summary. Respiratory and phosphorylating activities were determined in liver mitochondria from mild and severely diabetic rats. Diabetes was caused by 95% pancreatectomy performed 6–18 months before the investigation of mitochondrial function. Diabetic mitochondria showed depressed respiratory rates with β-hydroxybutyrate, L-malate–L-glutamate, and succinate as substrates while the P:O ratios did not depart from the normal control values, with all the employed substrates. Ubiquinone levels were significantly increased in mitochondria from severely diabetic rats while cytochrome *c* and mitochondrial protein remained unchanged.

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