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A Comparative Study of the Transport of Pyruvate in Liver Mitochondria from Normal and Diabetic Rats

A. Kielducka,[1,2] G. Paradies,[1] and S. Papa[1]

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Abstract

A comparative study of the transport of pyruvate in liver mitochondria from normal and diabetic rats has been carried out. The K_m for net pyruvate uptake in diabetic, ketotic mitochondria is practically equal to that measured in normal mitochondria, while the V_{max} is significantly lower. The lower activity of the pyruvate translocator in diabetic mitochondria compared to normal mitochondria is also shown by swelling experiments as well as by following the rate of pyruvate-supported respiration. Pre-exposure of mitochondria from normal rats to the ketone body acetoacetate and to 2-oxobutyrate results in a decrease of the K_m for pyruvate uptake. This effect is impaired in mitochondria from diabetic animals. The results indicate that the activity and the properties of the mitochondrial pyruvate translocator are modified in the diabetic, ketotic condition.

Key Words: Pyruvate; mitochondria; diabetes.

Introduction

The transport of pyruvate across the mitochondrial membrane is mediated by a specific system (Papa *et al.*, 1971; Papa and Paradies, 1974; Paradies and Papa, 1975–1978; Halestrap, 1975, 1978a). The kinetic properties, substrate specificity, and sensitivity to thiol reagents and to α-cyanocinnamate of this system have been investigated in detail (Papa and Paradies, 1974; Halestrap, 1975; Paradies and Papa, 1977, 1978). Recently it has been shown that the transport of pyruvate is stimulated in liver mitochondria from glucagon-treated rats (Adam and Hayne, 1969; Titheradge and Coore, 1976; Hale-

[1]Institutes of Biological Chemistry, Faculty of Medicine and Faculty of Science, Center for the Study of Mitochondria and Energy Metabolism, C.N.R., University of Bari, Bari, Italy.

[2]Supported by a joint grant from Consiglio Nazionale delle Ricerche, Rome, Italy, and the Polish Academy of Sciences, Warsaw, Poland. Permanent address: Nencki Institute of Experimental Biology, Warsaw, Poland.

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strap, 1978b). It has also been shown that pyruvate transport in tumor cell mitochondria exhibits different kinetic parameters as compared with normal mitochondria (Eboli *et al.*, 1977). Thus the pyruvate translocator may exhibit changes in its activity and kinetics properties in different metabolic situations.

The pyruvate carrier mediates also the transport of several substituted monocarboxylates involved in ketogenesis and fatty acid synthesis (Paradies and Papa, 1978). Among these, ketone bodies play an important role (Papa and Paradies, 1974; Halestrap, 1978a; Paradies and Papa, 1977, 1978). In addition it has been found that substituted monocarboxylates, including ketone bodies, regulate the activity of the pyruvate translocator (Papa and Paradies, 1974; Paradies and Papa, 1977, 1978). The level of ketone bodies rises severalfold in some metabolic states, like starvation, high fat diet, and particularly in the diabetic (Williamson *et al.*, 1967; Harano *et al.*, 1972).

The metabolism of pyruvate is decreased in mitochondria from diabetic animals (Koeppe *et al.*, 1959; Haft, 1964), the inhibition being attributed to impairment of pyruvate dehydrogenase (Garland *et al.*, 1964; Kerbey *et al.*, 1977). The inhibition of pyruvate utilization in diabetic mitochondria might also involve impairment of pyruvate transport across the mitochondrial membrane (Kerbey *et al.*, 1976).

These considerations prompted us to undertake a comparative study of the transport of pyruvate in liver mitochondria from normal and diabetic rats.

It is shown, in the present paper, that the pyruvate translocator exhibits in mitochondria from diabetic rats a decreased activity and different kinetic response to ketone bodies as compared to mitochondria from normal animals.

Material and Methods

Chemicals

Enzymes, coenzymes, and substrates were purchased from Sigma Chemical Co. or Boehringer Mannheim Corp. All radiochemicals were obtained from the Radiochemical Centre Amersham, England. 5,5-dimethyl[^{14}C]oxazolidine-2,4-dione was obtained from New England Nuclear. [U-^{14}C]Pyruvic acid, sodium salt, was treated as follows: the solid was dissolved in water, divided into 5-μCi samples, freeze-dried, and stored in sealed tubes at -20°C.

Treatment of Rats

Male Wistar rats (140–180 g) were made diabetic with an injection of alloxan (100 mg/kg body weight, subcutaneously). The animals were killed

30 min or 48 h after alloxan injection. Livers from normal and alloxan-treated rats were excised and the homogenates prepared at the same time. For diabetic rats only animals having blood sugar values higher than 300 mg/100 ml 48 h after alloxan injection were used. Mitochondria were isolated from liver homogenates in 0.25 M sucrose as described by Papa and Paradies (1974).

Pyruvate Transport

The initial rate of pyruvate uptake by rat liver mitochondria was measured by the centrifugation filtration technique as follows (see also Papa and Paradies, 1974; Paradies and Papa, 1977). Mitochondria were preincubated at 20°C in a reaction medium containing 150 mM sucrose, 30 mM Tris HCl, 1 mM $MgCl_2$, 0.5 mM EDTA, 1 mM arsenite, 10 μg/ml oligomycin, 1.4 μg/ml rotenone, and 0.34 μg/ml antimycin. After 3 min preincubation, mitochondria were layered on the top of a second incubation layer, at 4°C, and then spun down through this layer by rapid centrifugation. $HC10_4$ was immediately added to the mitochondrial pellet. The second incubation layer was of the same composition as the preincubation mixture and, in addition, contained [^{14}C]pyruvate at the concentrations shown in the tables and figures. A discontinuous density gradient increasing toward the bottom of the centrifuge tube was made by addition of dextran to the second layer. The exposure time of mitochondria to the second incubation layer was estimated to be about 15 sec by measuring the oxidation of β-hydroxybutyrate to acetoacetate (Papa and Paradies, 1974).

Measurements of the Rate of Oxygen Uptake

The rate of oxygen uptake was measured polarographically at 20°C with a Clark electrode.

Measurements of Mitochondrial Swelling

Mitochondrial swelling was monitored by following changes in absorbance at 520 nm in a Cary 15 spectrophotometer at room temperature.

pH Measurements

The external pH was determined potentiometrically on the supernatant obtained after rapid centrifugation of the mitochondrial suspension. The intramitochondrial pH (matrix space) was calculated on the basis of the distribution of 5,5-dimethyl[^{14}C]oxazoline-2,4-dione between the matrix space and medium by the equation of Addanki *et al.* (1968) with the only difference that the mitochondrial volume was estimated with [^{14}C]sucrose instead of [^{14}C]dextran (see also Papa and Paradies, 1974).

Assays

Mitochondrial protein was determined by the usual biuret method. Glucose and β-hydroxybutyrate concentration in plasma were determined by the method of Slein (1965) and Williamson and Mellambry (1974) respectively on blood samples collected 30 min or 48 h after alloxan injection.

Results

Figure 1 illustrates the kinetics of pyruvate uptake in mitochondria isolated from controls and from animals sacrificed 30 min or 48 h after alloxan injection.

Determinations on the blood of the rats just before killing showed that 30 min after alloxan injection the plasma glucose level, 200 ± 25 mg/100 ml, was about three times higher than in the controls (80 ± 6 mg/100 ml), while the plasma β-hydroxybutyrate level was practically unchanged (1.0 mM ± 0.1 plasma β-hydroxybutyrate in alloxan-treated rats, 0.75 mM ± 0.1 in the controls—values from eight animals). Forty-eight hours after alloxan injec-

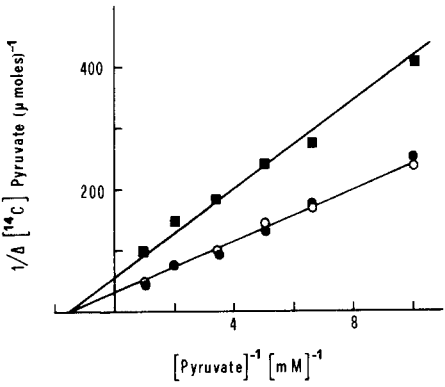

Fig. 1. Double reciprocal plots of pyruvate uptake by mitochondria from control and alloxan-treated rats. Mitochondria (8 mg protein/ml) were preincubated in the sucrose medium described under Methods. Final pH 7.0; Temperature 20°C. Three minutes later mitochondria were centrifuged through a second layer at 4°C containing the same components as the preincubation medium and in addition labeled pyruvate at the concentrations indicated. ○, control mitochondria; ●, mitochondria from rats sacrificed 30 min after alloxan injection; ■, mitochondria from rats sacrificed 48 h after alloxan injection.

tion there occurred a clear diabetic state characterized by more than five to six times enhancement of glycemia and more than ten times enhancement of plasma β-hydroxybutyrate concentration.

For the experiments on this last group of rats, considered as diabetic, only animals having plasma glucose level higher than 300 mg/100 ml and plasma β-hydroxybutyrate higher than 10 mM were used. Double reciprocal plots of net pyruvate uptake followed saturation kinetics both in controls and alloxan-treated rats (30 min or 48 h). It can, however, be seen that while rats treated for 30 min with alloxan did not exhibit any change in the kinetic properties of pyruvate uptake by mitochondria, diabetic rats (48 h treatment) exhibited impaired pyruvate uptake.

The mean values (± S.E.M.) for the kinetic parameters obtained from 10 different experiments showed that while the K_m of pyruvate uptake remained practically the same, 683 ± 21 μM in normal and 665 ± 27 μM in diabetic mitochondria, there was a marked decrease in the maximum velocity in diabetic mitochondria, V_{max} = 9.88 ± 1.4 nmol/min per mg of protein, compared to normal mitochondria, V_{max} = 18.9 ± 1.6 nmol/min per mg protein.

The difference in the activity of the pyruvate translocator in liver mitochondria from diabetic rats with respect to normal animals is further documented by swelling experiments. A typical experiment reported in Fig. 2 shows that normal and diabetic mitochondria underwent large-amplitude swelling when suspended in isoosmotic solution of NH_4-pyruvate or NH_4-acetate. However, the following facts can be noted: (1) the rate of swelling in

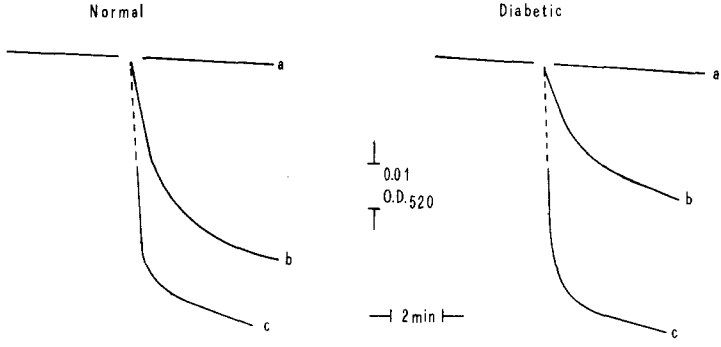

Fig. 2. Swelling of mitochondria from normal and diabetic rats suspended in isoosmotic ammonium pyruvate or ammonium acetate. Mitochondria swelling was monitored as described under Methods. Mitochondria (0.25 mg protein/ml) were suspended in a medium containing 20 mM Tris-HCl (pH 7.0), 0.5 mM EDTA, 1 μg/ml rotenone, 1.5 μg/ml antimycin; 150 mM sucrose (trace a), 125 mM NH_4-pyruvate (trace b), and 125 mM NH_4-acetate (trace c). The reaction was initiated by addition of mitochondria to the medium.

the NH_4 salt of acetate, which is known to freely permeate the mitochondrial membrane as undissociated acid (Papa and Paradies, 1974), was much higher than the rate of swelling in NH_4-pyruvate; (2) the rate of swelling in NH_4-pyruvate was markedly decreased in mitochondria from diabetic rats as compared to that from control animals; (3) no change in the rate of swelling in NH_4-acetate was, on the other hand, observed. These results confirm that there is a decreased activity of the mitochondrial pyruvate translocator in diabetic rats. It may be possible that at high pyruvate concentrations there occurs some free diffusion of pyruvic acid across the mitochondrial membrane. However the rate of free permeation is clearly minor compared to that of carrier-mediated pyruvate transport.

The metabolism of pyruvate has been reported to be inhibited in diabetic animals (Koeppe *et al.*, 1979; Haft, 1964; Garland *et al.*, 1964; Kerbey *et al.*, 1977). The experiment presented in Fig. 3 confirms this point. It is in fact shown here that the rate of pyruvate-supported oxygen consumption was significantly lower in diabetic than in normal mitochondria, being respectively 12.1 ± 0.41 and 20.4 ± 0.88 natoms O_2/min/mg of protein (values from eight experiments).

It has previously been demonstrated that the uptake of pyruvate by mitochondria is driven by transmembrane ΔpH (Papa and Paradies, 1974). It has also been shown that acetoacetate and other substituted oxomonocarboxylates can strongly influence the transport of pyruvate (Paradies and Papa, 1977, 1978). It should be recalled that the level of acetoacetate is known to largely increase in diabetic rats (Williamson *et al.*, 1967; Harano *et al.*, 1972).

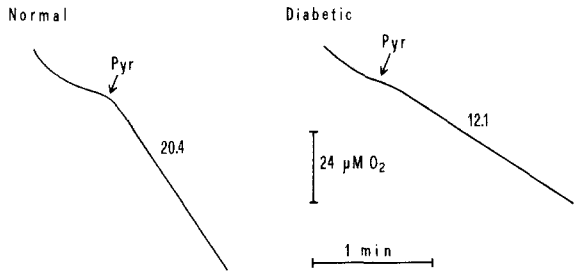

Fig. 3. Pyruvate-supported respiration in mitochondria isolated from normal and diabetic rats. Mitochondria (5 mg protein/ml) were incubated in a reaction medium containing 75 mM sucrose, 30 mM Tris-HCl, 50 mM KCl, 1 mM $MgCl_2$, 0.5 mM EDTA, and 0.5 μM FCCP (carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone). Final pH 7.0. Temperature 20°C. Where indicated 0.2 mM pyruvate was added. The numbers on the trace refer to the respiratory rates in ngatoms O_2/min/mg protein.

Table I. Transmembrane ΔpH in Mitochondria from Normal and Diabetic Rats[a]

	Number of observations	ΔpH ($pH_{in} - pH_{out}$)
Normal mitochondria	4	0.78 ± 0.09
Diabetic mitochondria	4	0.56 ± 0.06

[a] Mitochondria were preincubated in the sucrose medium described under Methods. External pH 7.0. Temperature 20°C. After 3 min preincubation mitochondria were separated from the medium by rapid centrifugation. The intramitochondrial pH was determined by the distribution of [^{14}C]DMO as described under Methods. The external pH was determined potentiometrically on the supernatant obtained after centrifugation of mitochondria.

The data reported in Table I show that in liver mitochondria from diabetic rats the transmembrane ΔpH was somewhat lower than that found in normal mitochondria.

The results presented in Fig. 4 and Table II show, in agreement with previous observations (Paradies and Papa, 1976–1978), that preexposure of normal mitochondria to acetoacetate or 2-oxobutyrate led to an increase in the affinity of pyruvate for its carrier with no change in V_{max}. In diabetic mitochondria, on the other hand, acetoacetate was unable, and 2-oxobutyrate much less effective, in enhancing the affinity of pyruvate for the carrier when

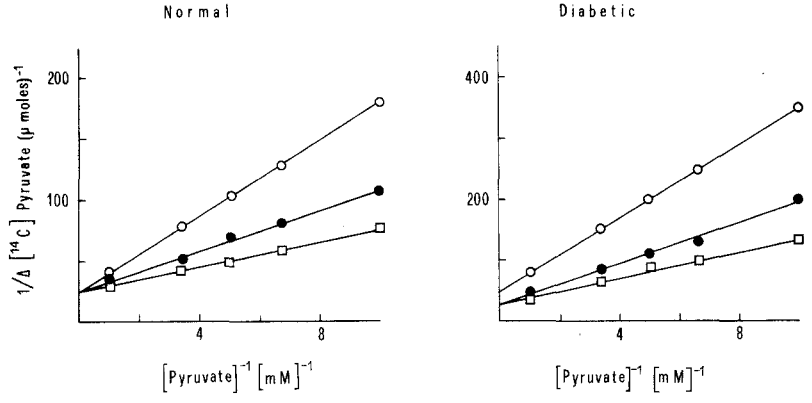

Fig. 4. Double reciprocal plots of the effect of preincubation of mitochondria with 2-oxobutyrate and acetoacetate on the initial rate of pyruvate uptake. Mitochondria (8 mg protein/ml) were preincubated in the sucrose medium described under Methods. After 3 min of preincubation normal or diabetic mitochondria were centrifuged through a second incubation layer containing the same components as the preincubation medium and in addition labeled pyruvate at the concentrations indicated in the figure. Symbols: ○, control; ●, acetoacetate (2 mM); □, 2-oxobutyrate (2 mM) added in the preincubation phase.

Table II. Statistical Analysis of the Effect of Preexposure of Mitochondria from Normal and Diabetic Rats to 2-Oxobutyrate and Acetoacetate on the Kinetic Parameters of Pyruvate Uptake[a]

Addition in the preincubation phase	Normal		Diabetic	
	K_m (μM)	V_{max} (nmol/min/mg protein)	K_m (μM)	V_{max} (nmol/min/mg protein)
None	683 ± 21 (8)	18.9 ± 1.6	665 ± 27 (8)	9.88 ± 1.4
2-Oxobutyrate	195 ± 35 (3)	19.4 ± 2.7	321 ± 34 (3)	17.4 ± 2.4
Acetoacetate	345 ± 28 (4)	19.2 ± 2.3	642 ± 39 (4)	16.8 ± 2.3

[a]The values for K_m and V_{max} are the means ± S.E.M. for the number of different experiments indicated in brackets. The experimental conditions for pyruvate uptake by normal and diabetic mitochondria are those described in the legends to Figs. 1 and 4. For other details see Methods.

added in the preincubation phase. In addition these monocarboxylates almost completely restored the V_{max} of pyruvate transport to the value found in the normal state.

Discussion

The kinetic analysis with radioisotopic tracer shows that the capacity for pyruvate uptake is significantly decreased in liver mitochondria from diabetic ketotic rats as compared to normal animals. The driving force for pyruvate uptake by mitochondria, i.e., transmembrane ΔpH, is somewhat reduced in mitochondria from diabetic rats. This may represent one of the factors responsible for impairment of pyruvate uptake in mitochondria from diabetic rats. However, the swelling experiments and the kinetic analysis of the effect of added oxomonocarboxylates on [^{14}C]pyruvate uptake by mitochondria document an *intrinsic* decrease of the activity of the pyruvate translocator and change in its properties. Mitochondria isolated from diabetic, ketotic rats exhibit the same affinity for pyruvate uptake as normal mitochondria. Preexposure of mitochondria from normal rats to added oxomonocarboxylates results in a significant decrease of the K_m for pyruvate uptake (cf. Paradies and Papa, 1977). In mitochondria from diabetic rats this positive effect on the affinity of pyruvate for its translocator is impaired in the case of 2-oxobutyrate and completely lost in the case of acetoacetate. The consequence is that in the presence of added ketone bodies the K_m for pyruvate is higher in mitochondria from diabetic rats than in normal animals. This change in the response of the pyruvate translocator to added oxomonocarboxylic acids might represent another factor for functional deficiency of this system in diabetic conditions.

The change in the regulation of pyruvate translocation by added

oxomonocarboxylic acids indicates that the molecular properties of the transport system are modified in diabetic conditions. The possibility that other factors, such as substances formed during the diabetic state, may affect directly the activity of the system cannot be excluded. In this respect it is worth noting that the citrate carrier is also depressed in mitochondria isolated from diabetic or fasted rats (Cheema-Dhali and Halperin, 1973). The inhibition is attributed to long-chain fatty acyl-CoA (Cheema-Dhali and Halperin, 1973) which are known to increase in this state.

The transport of pyruvate from cytosol to the mitochondrial matrix may represent a rate-limiting step for the utilization of this substrate in mitochondria (Papa and Paradies, 1974; Paradies and Papa, 1978; Halestrap, 1978c). Thus depression of the activity of the mitochondrial pyruvate translocator and its altered properties can account, in addition to other factors like inhibition of pyruvate dehydrogenase, etc., for the impaired metabolism of this substrate in diabetes and can be involved, as well, in the particular pattern of ketone body metabolism, characteristic of this pathological condition. It should be mentioned in this respect that the pyruvate translocator, in addition to mediating the transport of cytosolic pyruvate into mitochondria—a step which is essential for aerobic breakdown of glucose to CO_2 and H_2O—plays also a role, through pyruvate-acetoacetate exchange-diffusion, in the net transfer of acetyl-CoA from the mitochondrial matrix to the cytosol (Paradies and Papa, 1978; Stern, 1971; Rous, 1976). Further investigations are, however, necessary to define the cause–effect relationship between alterations of the properties of the oxomonocarboxylate transport system and the metabolic disorders, like ketosis, characteristic of diabetes.

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