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VARIATION IN ENDOGENOUS SUBSTRATES AND PYRUVATE METABOLISM IN ISOLATED HEART MITOCHONDRIA OF SEVERAL SPECIES*

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SUMMARY

1. The endogenous substrates of pigeon-, rat-, and rabbit-heart mitochondria isolated in the presence of [3-^{14}C]pyruvate have been compared. Freshly isolated rabbit-heart mitochondria contained citrate, glutamate, and alanine as the major endogenous substrates, while rat-heart mitochondria contained small amounts of glutamate and malate. Radioactive lactate was also present but probably does not constitute an endogenous substrate. Pigeon-heart mitochondria contained little radioactivity other than that present in aspartate.

2. Magnesium ions increased the State 3 rates and respiratory control ratios of rabbit-heart mitochondria, increased the State 4 rates and decreased the respiratory control ratios of pigeon-heart mitochondria, but had little effect on the respiratory rates or respiratory control ratios of rat-heart mitochondria.

3. The products of [3-^{14}C]pyruvate oxidation by pigeon-, rat-, and rabbit-heart mitochondria have been compared. α-Ketoglutarate accumulated in the case of pigeon- and rat-heart mitochondria. Addition of magnesium ions or of bovine serum albumin reduced the amount of α-ketoglutarate and increased the amounts of malate and succinate which accumulated.

4. The significance of these differences in heart mitochondria isolated from various species with respect to studies on mitochondrial metabolism is discussed.

INTRODUCTION

A number of investigators have noted the oxidation of endogenous substrates by mitochondria. This endogenous oxidation was reported to influence P/O ratios[1–3], reduction of NAD by energy-linked reactions[4–8], and the State 3 or activated state of respiration as well as the respiratory control ratio[9,10]. The nature of these substrates was first investigated by SCHNEIDER, STRIEBICH AND HOGEBOOM[11] who ob-

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served that citrate accumulated in liver mitochondria isolated from fluoroacetate-treated rats. BELLAMY[12] analyzed mitochondria isolated from a wide variety of organs and species, and found that citrate was the most widely distributed Krebs-cycle intermediate, and that smaller quantities of fumarate and α-ketoglutarate occurred frequently. However, the amino acids glutamate, glutamine, and aspartate were found more extensively and in greater amounts than Krebs-cycle intermediates. Other workers[1–8] speculated on the nature of the endogenous substrates of mitochondrial preparations but did not identify them. Previous work in this laboratory[13] resulted in the identification of ^{14}C-labeled substrates accumulating during the isolation of mitochondria from rabbit heart homogenized in medium containing [2-^{14}C]- or [3-^{14}C]-pyruvate. The data presented here show a distinct variation in the endogenous substrates of pigeon-, rat-, and rabbit-heart mitochondria isolated under similar conditions. Differences in the metabolism of added [3-^{14}C]pyruvate by the isolated heart mitochondria of these species is reflected by differences in the accumulation of radioactive substrates.

MATERIALS AND METHODS

Isolation of mitochondria

A modification of the procedure of R. W. ESTABROOK (personal communication) was used for isolation of pigeon-heart mitochondria. A domesticated pigeon was decapitated and its heart excised. A syringe containing cold medium (0.225 M mannitol, 0.075 M sucrose, 0.5 mM EDTA) was inserted into the aorta to force medium through the coronary vessels and cavities. The rinsed heart was minced in medium and transferred to a large Dounce homogenizer (Blaessig Glass Specialties, 645 Atlantic Ave., Rochester, N.Y.). The tissue was homogenized with several strokes of a loosely fitting pestle using 25 ml of isolation medium containing 6.3 mg Nagarse proteinase (Enzyme Development Corp., New York, N.Y.), 4 mM Tris buffer (pH 7.6) and [3-^{14}C]pyruvate at the concentrations specified for the individual experiments. The radioactive pyruvate was added in order to label the endogenous substrates of the isolated mitochondria. The homogenate was centrifuged for 5 min at 450 × *g* (average). The supernatant solution containing the mitochondrial fraction was then centrifuged for 10 min at 7000 × *g* (average). The mitochondrial pellet contained a lightly colored upper layer which was removed by swirling and discarded. The lower layer was rehomogenized in 15 ml of medium (without radioactive pyruvate) and the washed mitochondria resedimented at 7000 × *g* (average) for 5 min. The mitochondria were finally suspended in pyruvate-free medium to give a concn. of 18–25 mg mitochondrial protein per ml.

Male rats (400 g) of the Holtzman strain were killed with 100 % CO_2 and mitochondria isolated from the hearts using the procedure described for pigeons.

Albino rabbits (4 pounds) were killed with 100 % CO_2 and the hearts rinsed as described for pigeon heart. The minced heart was forced through a stainless-steel tissue press to facilitate homogenization. The subsequent procedure was identical to that used for isolation of pigeon- and rat-heart mitochondria. In all cases, protein was determined by a biuret method[14] after solubilization of the mitochondria with 0.1 % sodium deoxycholate.

Other procedures

Sodium [3-^{14}C]pyruvate, obtained from New England Nuclear Corporation, was dissolved in a stoichiometric amount of HCl and stored at −20° prior to use[15]. A sample was assayed for radiopurity[15] by chromatography on a 1 cm × 7 cm Dowex 1-X8 (Cl^-) column and for pyruvate concentration using lactate dehydrogenase[16] (EC 1.1.1.27).

Oxygen consumption was measured with a vibrating platinum electrode polarograph (Gilson Medical Electronics, Middleton, Wisc., U.S.A.) in a 2-ml open-type cell. The usual reaction medium contained: 0.225 M mannitol; 0.075 M KCl; 5.5 mM potassium phosphate (pH 7.2); mitochondria, approx. 2 mg protein/ml; and either 100 μM EDTA, or 50 μM EDTA *plus* 4 mM $MgCl_2$, as specified. Non-radioactive pyruvic acid (Eastman Kodak Co.) was twice distilled *in vacuo* and stored at −20°. Pyruvic, malic (Calbiochem), glutamic (Eastman Kokak Co.) and α-ketoglutaric (Calbiochem) acids were diluted from stock solutions and neutralized just prior to a series of experiments. State 3 rates of oxidation were induced by addition of ADP. After utilization of the amount of oxygen specified for each experiment, a 1-ml sample was withdrawn and deproteinized with 0.05 ml of 70% $HClO_4$. The supernatant solutions were stored at −20° until chromatographed using methods previously described[17]. Using these methods, radioactive compounds are initially separated on Dowex 1-X8 (Cl^-) columns and peaks containing more than one component are further resolved with chromatography on silicic acid. The citrate *plus* pyruvate peak was treated with potassium borohydride to reduce the pyruvate to lactate which can easily be separated from citrate by rechromatography on Dowex 1. Amino acids were identified using the procedure of MOORE AND STEIN[18].

RESULTS

Fig. 1 illustrates the ^{14}C-labeled endogenous substrates of pigeon-, rat- and rabbit-heart mitochondria isolated in the presence of [3-^{14}C]pyruvate. Aspartate was the only radioactive substrate found in appreciable amounts in pigeon-heart mitochondria. [^{14}C]Glutamate was the major oxidizable substrate of rat-heart mitochondria, although a larger amount of [^{14}C]lactate was present. The total radioactivity present in isolated rabbit-heart mitochondria far exceeded that present in either pigeon- or rat-heart mitochondria. Rabbit-heart mitochondria contained substantial amounts of radioactive alanine, glutamate, and citrate and small amounts of malate and lactate. The respective percentages correlated well with those of rabbit-heart mitochondria isolated by a different procedure[13].

The total amounts of aspartate, glutamate, and alanine in freshly isolated pigeon-heart mitochondria were 2.5, 0.5 and 0.0 μmoles/g mitochondrial protein, respectively while the comparable amounts in rabbit-heart mitochondria were 1.1, 4.2 and 2.7 μmoles/g mitochondrial protein*.

Table I shows a comparison of the labeled substrates in the supernatant solutions above mitochondrial pellets obtained during isolation of mitochondria from pigeon, rat and rabbit heart. Reduction of pyruvate by the soluble lactate dehydrogenase during the isolation procedure accounts for the large amounts of [^{14}C]lactate in these

* The authors are indebted to M. S. OLSON for the amino acid determinations. A Beckman 120 B autoanalyzer was used.

solutions. While only rabbit-heart mitochondria contained a substantial amount of radioactive glutamate, the supernatant solutions of all 3 species contained comparable amounts. Negligible quantities of radioactivity were found in the solutions used for washing the mitochondria.

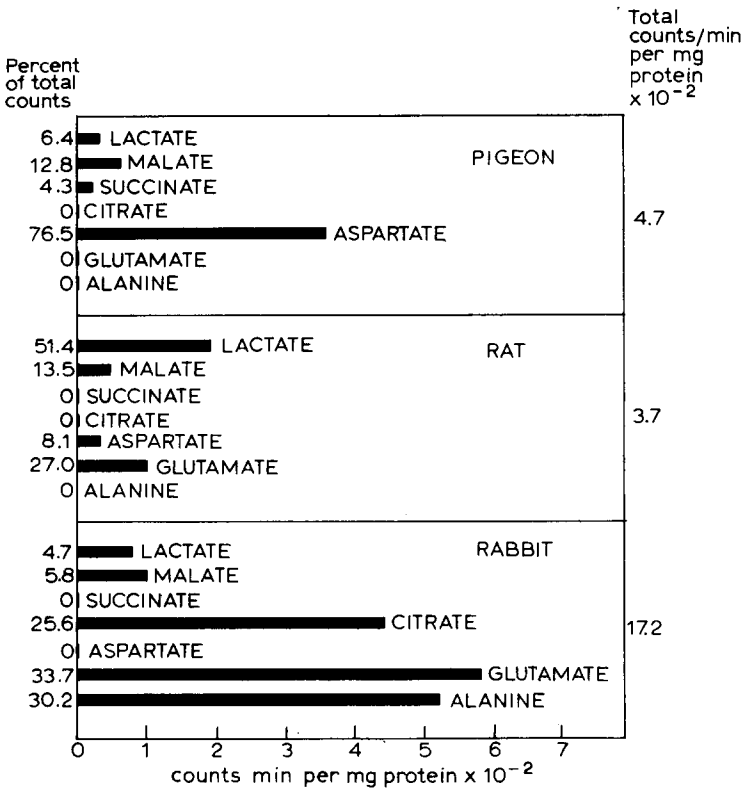

Fig. 1. Endogenous substrates of pigeon-, rat-, and rabbit-heart mitochondria labeled by isolation in the presence of [^{14}C]pyruvate as described in the text. The concentrations of [^{14}C]pyruvate used were: pigeon, 150 μM; rat, 113 μM; rabbit, 150 μM.

TABLE I

^{14}C-LABELED PRODUCT ACCUMULATION IN THE SUPERNATANT SOLUTIONS FROM PIGEON-, RAT-, AND RABBIT-HEART MITOCHONDRIA ISOLATED IN THE PRESENCE OF [3-^{14}C]PYRUVATE

Species	*Pyruvate added (μM)*	*Total counts/min per ml of supernatant $\times 10^{-3}$*	*Product (% of total counts/min)*								
			*Lac**	*Mal*	*Suc*	*Cit*	*Asp*	*Glu*	*Ala*	*Ace*	*Pyr*
Pigeon	120	150	89	0.2	0.7	—	3.1	4.2	0.4	1.2	1.6
Rat	113	138	90	1.3	1.6	—	1.2	3.9	0.6	0.9	0.3
Rabbit	150	176	80	2.6	3.9	0.6	2.0	4.4	3.7	0.8	1.9

* Abbreviations used: Lac, lactate; Mal, malate; Suc, succinate; Cit, citrate; Asp, Aspartate; Glu, glutamate; Ala, alanine; Ace, acetate; Pyr, pyruvate.

While isolated pigeon- and rat-heart mitochondria oxidized pyruvate at maximal rates only after addition of a dicarboxylic acid, rabbit-heart mitochondria oxidized pyruvate at comparable rates with or without added dicarboxylic acid.

TABLE II

RATES OF OXIDATION AND RESPIRATORY CONTROL RATIOS OF PIGEON-, RAT-, AND RABBIT-HEART MITOCHONDRIA IN THE PRESENCE AND ABSENCE OF ADDED MAGNESIUM IONS

Mitochondria were incubated at 28° in the reaction medium noted in the text. Substrates added for pigeon- and rat-heart mitochondria; pyruvate (250 μM) *plus* malate (100 μM); for rabbit-heart mitochondria, pyruvate (250 μM). State 3 was induced by addition of ADP (200 μM). Numbers in parentheses indicate the number of determinations.

Species	*Additions*	*Average State-3 rate (μmoles O_2/sec per g protein per l)*	*Average State-4 rate (μmole O_2/sec per g protein per l)*	*Average respiratory control ratio*
Pigeon	EDTA, 100 μM	0.29 (3)	0.07 (3)	4.5
	EDTA, 50 μM; $MgCl_2$, 4 mM	0.36 (3)	0.20 (3)	1.8
Rat	EDTA, 100 μM	1.0 (4)	0.26 (4)	3.8
	EDTA, 50 μM; $MgCl_2$, 4 mM	1.2 (3)	0.32 (3)	3.9
Rabbit	EDTA, 100 μM	0.23 (2)	0.17 (2)	1.4
	EDTA, 50 μM; $MgCl_2$, 4 mM	0.85 (2)	0.18 (2)	4.7

Table II shows the effects of added magnesium ions on the respiratory rates and respiratory control ratios of heart mitochondria isolated from the 3 species. Magnesium ions notably enhanced the State-3 rates and respiratory control ratios of rabbit-heart mitochondria; increased the State-4 rates and decreased the respiratory control ratios of pigeon-heart mitochondria but had little effect on the respiratory rates or respiratory control ratios of rat-heart mitochondria.

Table III lists the ^{14}C-labeled substrates formed during metabolism of [3-^{14}C]-pyruvate by pigeon-heart mitochondria. The α-ketoglutarate accumulation shown for Expt. 1 was typical of pigeon-heart mitochondria. The presence of magnesium ions reduced the accumulation of α-ketoglutarate (Expt. 2) while decreasing the respiratory control ratio (Table II). The addition of 1 % bovine serum albumin in the absence of magnesium ions also reduced the accumulation of α-ketoglutarate and increased that of succinate and malate (Expt. 3). The respiratory control ratio was increased by serum albumin.

Substrates accumulating during [3-^{14}C]pyruvate metabolism by rat- and rabbit-heart mitochondria are shown in Table IV. With rat-, as with pigeon-heart mitochondria, α-ketoglutarate was the major product in the absence of magnesium ions. In the presence of magnesium ions, the amount of α-ketoglutarate was decreased while that of succinate and malate was increased. Under all conditions with rabbit-heart mitochondria, radioactive α-ketoglutarate failed to accumulate to the extent observed with pigeon- or rat-heart mitochondria. In quickly frozen pigeon and rabbit hearts, however, comparable concentrations of α-ketoglutarate (3.9 and 6.0 μM, respectively) were found*.

* The authors are indebted to Dr. N. D. GOLDBERG for performing these analyses.

TABLE III

THE EFFECT OF MAGNESIUM ION AND BOVINE SERUM ALBUMIN ON THE PRODUCTS OF [3-^{14}C]PYRUVATE OXIDATION BY PIGEON-HEART MITOCHONDRIA

Mitochondria were incubated at 28° in the reaction medium described in the text with the addition of [3-^{14}C]pyruvate (225 μM) and malate (100 μM). ADP added: Expt. No. 1, 200 μM; Expt. No. 2, 200 μM; Expt. No. 3, 500 μM *plus* bovine serum albumin, 1 % final concn. Samples were taken after one State 3 to State 4 transition.

Expt. No.	*Mitochondrial protein (mg/ml)*	*Mg^{2+} (mM)*	*EDTA (μM)*	*Reaction time (min)*	*Total ΔO_2 (μM)*	*Product (counts/min per ml × 10^3)*											
						Cit	*α-KG*	*Suc*	*Fum*	*Mal*	*Glu*	*Asp*	*Ala*	*Ace*	*βOH*	*Lac*	*Pyr*
1	2.3	0	100	12.0	156	0.5	71.0	4.1	5.8	11.8	2.5	0.0	0.8	9.0	0.5	1.1	144
2	1.9	4	50	8.7	166	2.4	34.6	8.0	5.6	37.8	2.1	0.3	0.7	11.9	0.8	0.7	103
3	2.0	0	100	18.5	182	8.9	2.6	16.3	8.3	62.5	0.9	1.5	0.8	8.1	3.0	1.4	75

Abbreviations used: Cit, citrate α-KG, α-ketoglutarate; Suc, succinate; Fum, fumarate; Mal, malate; Glu, glutamate; Asp, aspartate; Ala, alanine; Ace, acetate; βOH, β-hydroxybutyrate; Lac, lactate; Pyr, pyruvate.

TABLE IV

PRODUCTS OF THE OXIDATION OF [3-^{14}C]PYRUVATE BY RAT- AND RABBIT-HEART MITOCHONDRIA

Mitochondria were incubated at 28° in the presence of 225 μM [3-^{14}C]pyruvate and the medium described in the text. In addition, for rat-heart mitochondria the medium contained 100 μM malate. Samples were taken after three State 3 to State 4 transitions induced by successive additions of 200 μM ADP. Mitochondrial protein: rat, 1.8 mg/ml; rabbit, 2.0 mg/ml.

Species	*Mg^{2+} (mM)*	*EDTA (μM)*	*Reaction time (min)*	*Total ΔO_2 (μM)*	*Product (counts/min per ml × 10^3)*											
					Cit	*α-KG*	*Suc*	*Fum*	*Mal*	*Glu*	*Asp*	*Ala*	*Ace*	*βOH*	*Lac*	*Pyr*
Rat	0	100	8.1	220	0.0	120.0	6.0	2.3	9.3	6.5	0.0	1.9	2.9	3.6	0.2	74
Rat	4	50	7.3	220	7.3	20.9	22.4	9.4	71.2	3.8	1.2	2.3	11.6	12.1	1.4	54
Rabbit	4	50	11.2	230	5.0	3.6	5.3	3.1	13.0	6.1	5.8	8.0	3.7	10.8	0.9	83

DISCUSSION

Although the amounts of oxidizable substrates retained in mitochondria of intact heart during normal cellular activity are not known, mitochondria isolated from heart cells of various species show not only differences in their content of endogenous substrates, but also differences in the nature of exogenous substrate metabolism. The substrates present in freshly isolated rabbit-heart mitochondria are retained throughout several hours anoxic storage at 0° (see ref. 13) and are capable of promoting the oxidation of added pyruvate. Freshly isolated pigeon- and rat-heart mitochondria contained insufficient substrates for maximal rates of pyruvate oxidation, whereas rabbit heart contained adequate sources of dicarboxylic acids.

In experiments, not reported here, an added dicarboxylic acid served as a pool to trap a radioactive product. The accumulation of α-[^{14}C]ketoglutarate could not have been such an effect since α-ketoglutarate was not added. The data presented here suggest that the α-ketoglutarate accumulation in rat-heart mitochondria may reflect the requirement for Mg^{2+} in the succinate thiokinase (EC 6.2.1.4) step of α-ketoglutarate oxidation[19]. In pigeon-heart mitochondria, bovine serum albumin was more effective in preventing α-ketoglutarate accumulation than was magnesium ion. Albumin may preserve or reconstitute a structure necessary for the oxidation of α-ketoglutarate to free succinate, or may aid in retention of intramitochondrial magnesium. BJÖRNTORP[20] has noted increased rates of α-ketoglutarate oxidation by rat-liver mitochondria in the presence of bovine serum albumin.

Distinctly opposite effects of Mg^{2+} on the respiratory control ratios of pigeon- and rabbit heart-mitochondria were noted. In this connection, CHANCE[21] reported restitution of control on addition of Mg^{2+} during succinate oxidation by rat-liver mitochondria, while BALTSCHEFFSKY[22] found that the respiratory control of liver mitochondria oxidizing β-hydroxybutyrate in the absence of Mg^{2+} was lost after several minutes at 25°. PACKER[23] observed that the addition of Mg^{2+} to rat-heart mitochondria increased oxidation rates, ATPase activity and decreased respiratory control. KLINGENBERG AND SCHOLLMEYER[24] observed similar effects on the respiratory rates of rat skeletal muscle mitochondria with decreased respiratory control when Mg^{2+} was added. The observations reported in this paper show a beneficial effect of Mg^{2+} on the respiratory control of rabbit-heart mitochondria and an uncoupling effect on pigeon-heart mitochondria.

The insensitivity of rat-heart mitochondrial respiratory rates to the presence of Mg^{2+} observed in these studies, concomitant with a pronounced effect on α-ketoglutarate oxidation, demonstrates the fact that similar rates of oxygen consumption can mask considerable variations in substrate transformations and that little information as to the nature of substrate metabolism can be gained by measurement of oxidation rates alone.

The data presented in this paper indicate that differences in endogenous substrate content of isolated mitochondria of various species, in the metabolism of added substrates, and in mitochondrial response to added cofactors, necessitate that careful consideration be given to the conditions under which isolated mitochondria are studied. Conclusions based on experiments with mitochondria from one organ or species do not necessarily extend to mitochondria isolated from another organ or species, or to mitochondria from the same source when studied under slightly different conditions.

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