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UTILIZATION OF RESPIRATORY SUBSTRATES IN CALF-RETINA MITOCHONDRIA

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SUMMARY

1. The utilization of respiratory substrates in calf-retina mitochondria has been studied. Calf-retina mitochondria oxidize pyruvate (+malate), α-oxoglutarate, succinate and glutamate at rates comparable to those reported for mitochondria from other mammalian tissues and with good P/O ratios and respiratory control. Added isocitrate is not oxidized. Glutamate is oxidized through the transaminase pathway.

2. In the absence of inorganic phosphate, succinate oxidation shows an initial inhibited phase followed by a progressive activation. Evidence is given that oxaloacetate is responsible for the inhibition of succinate oxidation and that the activation requires ATP formation. It is suggested that ATP removes inhibitory oxaloacetate.

3. Retina mitochondria do not oxidize added NADH, NADPH or β-hydroxybutyrate. α-Glycerophosphate is very slowly oxidized and only in the presence of added Mg^{2+}. These mitochondria are thus unable to oxidize extramitochondrial NADH directly, or through the β-hydroxybutyrate or the α-glycerophosphate cycles.

INTRODUCTION

It has been known for many years that retina has the most active energy metabolism of all animal tissues[1-3]. A Q_{O_2} of 31 and a $Q_{CO_2}^{N_2}$ of 88 have been reported for slices of rat retina with glucose as substrate[1]. This high respiratory activity raises the question of the properties of retina mitochondria, about which only limited data are available[4,5].

We have shown that by using an improved method of isolation, mitochondria with good oxidative and phosphorylative activity can be obtained[6]. The utilization of respiratory substrates by calf-retina mitochondria is examined in more detail in this paper. These mitochondria exhibit the phenomenon of "inhibition" and "activation" of succinate oxidation. Evidence is presented that the inhibition is due to oxaloacetate and that the activation requires internally generated or added ATP. Some of the experiments on succinate oxidation have been presented in a preliminary form[7].

Attention has also been paid to the problem of the mechanism by which extramitochondrial NADH can be oxidized by the mitochondria in this tissue.

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METHODS

Calf-retina mitochondria

Calf retinae were prepared as described previously[6]. Mitochondria were isolated by method B of ref. 6. Electron-microscope studies showed that the mitochondrial fraction obtained consisted of a large proportion of mitochondria with a double membrane and well preserved cristae[8].

Oxygen uptake and oxidative phosphorylation

Respiration was assayed either manometrically or polarographically with a Clark vibrating oxygen electrode. The standard reaction mixture used for the manometric measurements contained: 20 mM potassium phosphate buffer (pH 7.5), 30 mM KCl, 5 mM $MgCl_2$, 20 mM Tris–HCl buffer (pH 7.4), 30 mM glucose, 60 mM sucrose, 2 mM ATP, hexokinase (150 KUNITZ–MCDONALD units) and 15 mM substrate; the final volume was 1 ml, the pH 7.4, and the reaction temperature 30°. The polarographic assays were carried out in a reaction mixture containing 0.13 M KCl and 20 mM Tris–HCl buffer (pH 7.2); the final volume was 1.5 ml and the reaction temperature 25°. Oxidative phosphorylation was measured by determining the disappearance of inorganic phosphate.

Analytical methods

Inorganic phosphate was determined according to LOWRY AND LOPEZ[9].

Protein was determined by the method of CLELAND AND SLATER[10] and by a micro-Kjeldahl procedure.

Ammonia was determined by the method of KIRSTEN, GEREZ AND KIRSTEN[11].

Aspartate was determined by the method of PFLEIDERER, GRÜBER AND WIELAND[12].

Oxaloacetate, pyruvate and phosphoenolpyruvate were determined as described previously[13].

Special chemicals and enzymes

ATP, ADP, AMP, NADH, NADPH, sodium pyruvate, sodium succinate, α-oxoglutarate, malate dehydrogenase (EC 1.1.1.37), lactate dehydrogenase (EC 1.1.1.27), glutamate dehydrogenase (EC 1.4.1.3), aspartate transaminase (EC 2.6.1.1), pyruvate kinase (EC 2.7.1.40) from Boehringer und Soehne; yeast hexokinase (EC 2.7.1.1) (type III), DL-α-glycerophosphate DL-β-hydroxybutyrate, oligomycin and 2-methyl-1,4-naphthoquinone from Sigma Chemical Co.; 2,4-dinitrophenol and L-glutamate from British Drug Houses; DL-isocitrate from Light and Co.; dicoumarol from Calbiochem. Rotenone was a gift of Dr. J. M. TAGER and atractyloside a gift of Professor T. AJELLO and Professor A. QUILICO.

RESULTS AND DISCUSSION

Oxidation of tricarboxylic-acid cycle substrates and of glutamate

In Table I the rates of oxygen uptake and the P/O ratios found when retina mitochondria were supplied with various respiratory substrates, are listed. With pyruvate + malate, succinate, α-oxoglutarate and glutamate, respiratory rates comparable to those reported for mitochondria from other animal tissues were observed.

When isocitrate, β-hydroxybutyrate or α-glycerophosphate were added the oxygen consumption was not significantly higher than in the absence of added substrate. In the case of isocitrate at least, this may be due to a permeability barrier in the mitochondria[14,15]. Glutamate oxidation was accompanied by aspartate formation.

TABLE I

RESPIRATORY RATES AND P/O RATIOS IN CALF-RETINA MITOCHONDRIA

The standard components of the reaction mixture and the experimental procedure are given in METHODS. 4–6 mg mitochondrial protein. Arsenite, where present, 1 mM. Oxygen uptake was measured manometrically. Incubation time, 10–15 min.

Additions	*O uptake (natoms O/min per mg protein)*	*P/O*
None	10.8	—
Succinate	100.0	1.74
α-Oxoglutarate	70.0	2.45
Isocitrate	11.5	—
Glutamate	95.0	2.40
Glutamate + arsenite	14.2	—
Pyruvate + malate	110.3	2.50
α-Glycerophosphate	14.1	—
β-Hydroxybutyrate	11.0	—

The mean ratio of oxygen consumption:aspartate formation was about 3. Arsenite completely inhibited glutamate oxidation and aspartate formation. In the presence of glutamate no ammonia formation was found either in the absence or in the presence of arsenite. Thus retina mitochondria, like those of muscle[16,17] and mammary gland[18], oxidize glutamate exclusively through the transaminase pathway[19]. Polarographic measurements showed that when ADP was added to a reaction mixture containing mitochondria, inorganic phosphate and respiratory substrate, the respiratory rate was enhanced 3–4 fold in the case of glutamate and 2–3 fold in the case of succinate. With both substrates the stimulation persisted until the ADP added was phosphorylated, after which the respiratory rate returned to that observed before adding ADP.

AMP, added in the inhibited phase, stimulated oxygen uptake almost as much as ADP, showing that calf-retina mitochondria contain an active adenylate kinase (EC 2.7.4.3).

Succinate oxidation

With succinate as substrate, the order of additions has a marked effect on the respiratory rate. When succinate was added before phosphate (Fig. 1a), oxygen uptake showed a lag phase followed by a striking activation. The inhibited phase was considerably lengthened if mitochondria were preincubated for a few minutes in the absence of succinate and phosphate.

If ATP was present during the preincubation, on addition of succinate the active phase of respiration started directly (Fig. 1b). When mitochondria were preincubated in the presence of arsenate, succinate oxidation showed only a partial activation, which was completely abolished by the addition of dinitrophenol (Fig. 1c). No activation was observed if mitochondria were preincubated with dinitrophenol and arsenate

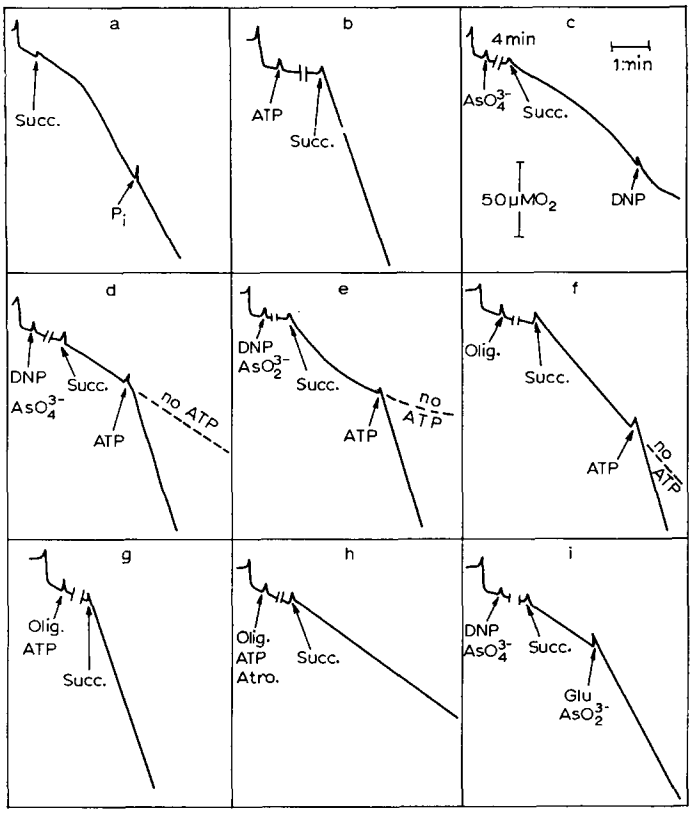

Fig. 1. Oxygen-electrode recordings of respiration during the course of succinate oxidation in calf-retina mitochondria. The standard components of the reaction mixture and the experimental procedure are given in METHODS. Additions: 4 mM succinate (Succ.), 5 mM inorganic phosphate (P_i), 2 mM ATP, 0.1 mM dinitrophenol (DNP), 3 mM arsenate, 1 mM arsenite, 15 µg oligomycin (Olig.), 0.3 mM atractyloside (Atro.), 4 mM glutamate (Glu). Mitochondrial protein, 2.2 mg (Expts. a–h) or 1.5 mg (Expt. i).

(Fig. 1d) or dinitrophenol and arsenite (Fig. 1e). Preincubation of mitochondria in presence of oligomycin partly suppressed the activation (Fig. 1f). However, the addition of ATP brought about, in all cases, an activation of respiration (Fig. 1b, d, e, f, g). The activation by ATP was abolished by atractyloside (Fig. 1h) but not by oligomycin. These findings suggest that the activation of succinate oxidation is due to the formation of ATP either by the respiratory chain or by the substrate level phosphorylation system.

In other experiments (not shown) it was found that preincubation of retina mitochondria in the presence of rotenone or of glutamate + arsenite eliminated the initial lag phase of succinate oxidation. Furthermore, the addition of glutamate + arsenite (Fig. 1i) relieved the inhibition caused by dinitrophenol + arsenate.

These results suggest that the inhibition of succinate oxidation is due to an accumulation of oxaloacetate (*cf.* refs. 20, 21). We have recently found[13] that the inhibition of succinate oxidation in rabbit-kidney mitochondria, treated with uncouplers, is due to oxaloacetate accumulation and that the reactivation of succinate

oxidation by ATP is probably due to the conversion of oxaloacetate to phosphoenolpyruvate. In the experiments reported in Table II, the levels of oxaloacetate (measured as aspartate), phosphoenolpyruvate and pyruvate were determined at different times during succinate oxidation in retina mitochondria. As shown above preincubation with dinitrophenol and arsenate caused inhibition of succinate oxidation. After the preincubation 4.2 nmoles oxaloacetate had accumulated. When ATP, oligomycin and arsenite were added together with succinate, the inhibition was largely prevented. This was accompanied by a lowering in the level of oxaloacetate and by a significant formation of pyruvate. A few nmoles of phosphoenolpyruvate were also formed.

TABLE II

OXYGEN UPTAKE AND LEVELS OF OXALOACETATE (MEASURED AS ASPARTATE), PYRUVATE AND PHOSPHOENOLPYRUVATE DURING SUCCINATE OXIDATION IN CALF-RETINA MITOCHONDRIA

Mitochondria (4–6 mg protein) were preincubated for 6 min in 1 ml of a reaction mixture containing: 0.13 M KCl, 20 mM Tris–HCl buffer (pH 7.2), 0.1 mM dinitrophenol and 2 mM arsenate. At zero time 10 mM succinate (Expt. A) or 10 mM succinate, 2 mM ATP, 1 mM arsenite and 15 μg oligomycin (Expt.B) were added. Respiration was measured polarographically. At the times indicated, the oxidation of succinate was stopped by the addition of 2 μg antimycin and oxaloacetate was converted to aspartate by adding 10 mM glutamate and 2 μg rotenone as described previously[13].

	Time (min)	*Respiration (natoms O/min per mg protein)*	*Aspartate (nmoles)*	*Pyruvate (nmoles)*	*Phosphoenol-pyruvate (nmoles)*
	0	0	4.2	6	0
Expt. A	4	25	6.1	4	0
Expt. B	1	75	0	9	2
	2	75	0.2	10	3
	4	75	3.0	16	3

These data further substantiate the hypothesis that the inhibition of succinate oxidation is due to oxaloacetate, and that ATP activates succinate oxidation by preventing oxaloacetate accumulation in the vicinity of succinate dehydrogenase.

The amounts of phosphoenolpyruvate formed in retina mitochondria in the presence of ATP are much smaller than those found, under the same conditions, in rabbit-kidney mitochondria[13], which have a high phosphoenolpyruvate carboxylase activity[22]. In retina mitochondria one possible mechanism by which ATP lowers the level of oxaloacetate at the site of inhibition could be by decarboxylation.

NADH oxidation

Fig. 2a shows that the addition of NADH and NADPH, in the presence of phosphate and ADP, had no effect on the slow rate of respiration supported by endogenous substrates. Also α-glycerophosphate gave no extra oxygen consumption (Fig. 2b). Mg^{2+} slightly stimulated oxygen uptake in the presence of α-glycerophosphate (Fig. 2b; *cf.* ref. 23).

Whilst the addition of catalytic amounts of β-hydroxybutyrate did not affect the oxygen uptake in the presence of NADH + NADPH, 10^{-5} M 2-methyl-1,4-

naphthoquinone greatly stimulated their oxidation. The 2-methyl-1,4-naphthoquinone-mediated respiration was insensitive to rotenone but was strongly inhibited by dicoumarol, indicating that a NAD(P)H dehydrogenase (EC 1.6.99.2), similar to that described in rat liver[24] and other tissues[25], was operative. As reported above, slices of rat retina have a Q_{O_2} and a $Q^{N_2}_{CO_2}$, with glucose as substrate, which are by far the highest found in normal tissues under the above reported conditions. However, our data show that the rates of oxidation of respiratory substrates by calf-retina mitochondria are not higher than those of other tissues of the same or other mammals.

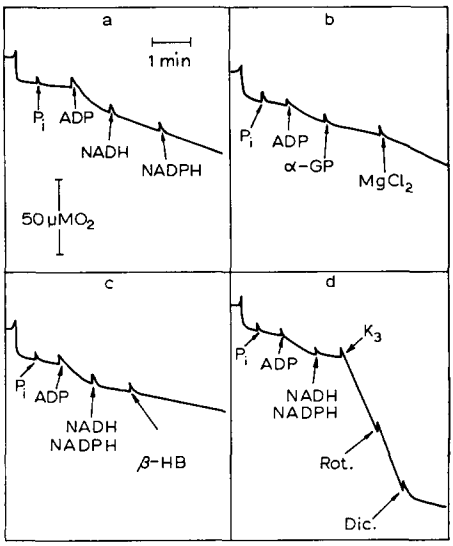

Fig. 2. Oxygen-electrode recordings of oxygen uptake in calf-retina mitochondria in the presence of NAD(P)H, β-hydroxybutyrate and α-glycerophosphate. The standard components of the reaction mixture and the experimental procedure are given in METHODS. Additions: 5 mM P_i, 270 µM ADP, 1 mM NADH, 1 mM NADPH, 4 mM α-glycerophosphate (α-GP), 5 mM $MgCl_2$, 0.1 mM β-hydroxybutyrate (β-HB), 10^{-5} M 2-methyl-1,4-naphthoquinone (K_3), 2 µg rotenone (Rot.), 60 µM dicoumarol (Dic.). Mitochondrial protein, 2.2 mg.

The question arises of the mechanism of reoxidation of extramitochondrial NAD(P)H in retina. The very high rate of glycolysis (KINI AND COOPER[5] reported a $Q^{N_2}_{La}$ of 24 for slices of ox-retina) requires an efficient system for the reoxidation of the cell sap NADH.

The finding that retina mitochondria do not oxidize β-hydroxybutyrate at all, and α-glycerophosphate only sluggishly shows that neither the β-hydroxybutyrate cycle[26] nor the α-glycerophosphate cycle[27] can be of any physiological significance in the oxidation of glycolytic NADH in this tissue.

Trace d of Fig. 2 shows that calf-retina mitochondria contain a NAD(P)H dehydrogenase which in the presence of added 2-methyl-1,4-naphthoquinone mediates a very efficient oxidation of NAD(P)H. CONOVER[28] and BORST[25] have pointed out why this enzyme is unlikely to play a role in the reoxidation of extramitochondrial NADH in liver and ascites cells. More detailed studies of the kinetics of this NAD(P)H dehydrogenase, as well as of the concentration of NAD(P)H in retina, could give information about its role in the oxidation of reduced nicotinamide nucleotides in this

tissue. In this regard it is interesting to mention the suggestion of FUTTERMAN[29] that a light-induced oxidoreduction cycle of vitamin A may play a role in the oxidation of NADH and NADPH in retina.

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REFERENCES

1 O. WARBURG, K. POSENER AND E. NEGELEIN, *Biochem. Z.*, 152 (1924) 205.
2 A. C. AISENBERG, *The Glycolysis and Respiration of Tumors*, Academic, New York, 1961, p. 1.
3 L. H. COHEN AND W. K. NOELL, in C. N. GRAYMORE, *Biochemistry of the Retina*, Academic, New York, 1965, p. 36.
4 A. PYRIE, *Biochemistry of the Eye*, Blackwell Scientific Publ., 1956, p. 205.
5 M. M. KINI AND J. R. COOPER, *Biochem. J.*, 82 (1962) 164.
6 S. PAPA, A. G. SECCHI, N. E. LOFRUMENTO, F. D'ERMO AND E. QUAGLIARIELLO, *Ital. J. Biochem.*, 14 (1965) 175.
7 S. PAPA, A. G. SECCHI, N. E. LOFRUMENTO, F. D'ERMO AND E. QUAGLIARIELLO, *Boll. Soc. Ital. Biol. Sperim.*, 41 (1965) 1199.
8 S. PAPA, A. G. SECCHI, N. E. LOFRUMENTO, F. D'ERMO AND E. QUAGLIARIELLO, *Atti XLVIII Congr. Soc. Oftalm. Ital., Siena, 1964.*
9 O. H. LOWRY AND Y. A. LOPEZ, *J. Biol. Chem.*, 162 (1946) 421.
10 K. W. CLELAND AND E. C. SLATER, *Biochem. J.*, 53 (1953) 547.
11 E. KIRSTEN, C. GEREZ AND R. KIRSTEN, *Biochem. Z.*, 337 (1963) 312.
12 G. PFLEIDERER, W. GRÜBER AND T. WIELAND, *Biochem. Z.*, 326 (1955) 446.
13 S. PAPA, N. E. LOFRUMENTO AND E. QUAGLIARIELLO, *Biochim. Biophys. Acta*, 110 (1965) 442.
14 S. G. VAN DEN BERGH AND E. C. SLATER, *Biochem. J.*, 82 (1962) 362.
15 J. B. CHAPPELL, *Proc. Intern. Congr. Biochem., 6th, New York, 1964*, Abstr. p. VIII-S12.
16 P. BORST AND E. C. SLATER, *Biochim. Biophys. Acta*, 41 (1960) 170.
17 P. BORST, *Biochim. Biophys. Acta*, 57 (1962) 256.
18 E. A. JONES AND H. GUTFREUND, *Biochem. J.*, 79 (1961) 608.
19 H. A. KREBS AND D. BELLAMY, *Biochem. J.*, 75 (1960) 523.
20 E. C. SLATER AND W. C. HÜLSMANN, *Proc. Natl. Acad. Sci. U.S.*, 47 (1961) 1109.
21 J. B. CHAPPELL, *Proc. 1th IUB/IUBS Intern. Symp. on Biological Structure and Function*, Vol. 2, Academic, New York, 1961, p. 71.
22 R. BANDURSKY AND F. LIPMANN, *J. Biol. Chem.*, 219 (1956) 741.
23 B. SACTOR, L. PACKER AND R. W. ESTABROOK, *Arch. Biochem. Biophys.*, 80 (1959) 68.
24 T. E. CONOVER AND L. ERNSTER, *Biochim. Biophys. Acta*, 58 (1962) 189.
25 P. BORST, *Funktionelle und Morphologische Organization der Zelle*, Springer Verlag, Berlin, 1963, p. 137.
26 T. M. DEVLIN AND B. M. BEDELL, *J. Biol. Chem.*, 235 (1960) 2134.
27 E. ZEBE, A. DELBRÜCK AND T. BÜCHER, *Ber. Ges. Physiol.*, 189 (1957) 115.
28 T. E. CONOVER, *Proc. 1th IUB/IUBS Intern. Symp. on Biological Structure and Function*, Vol. 2, Academic, New York, 1961, p. 169.
29 S. FUTTERMAN, *J. Biol. Chem.*, 238 (1963) 1145.