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CONTROL OF NICOTINAMIDE NUCLEOTIDE-LINKED OXIDOREDUCTIONS IN RAT-LIVER MITOCHONDRIA

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SUMMARY

1. With α-oxoglutarate as the hydrogen donor and the substrate-linked phosphorylation step as the source of energy, hydrogen transfer to α-oxoglutarate (*plus* ammonia) in rat-liver mitochondria was inhibited by dicoumarol or oligomycin, and concomitantly, intramitochondrial NAD became more reduced. Hydrogen transfer to α-oxoglutarate (*plus* CO_2) was also inhibited by the uncoupler, but not that to acetoacetate or oxaloacetate.

2. Hydrogen transfer from β-hydroxybutyrate to α-oxoglutarate (*plus* ammonia) was stimulated by ATP. The stimulation was oligomycin-sensitive.

3. Hydrogen transfer from glutamate to acetoacetate or oxaloacetate was inhibited by ATP. This inhibition was oligomycin-sensitive.

4. With isocitrate as hydrogen donor, the reduction of acetoacetate was slightly stimulated by ATP. Reduction to α-oxoglutarate (*plus* ammonia) was not affected by ATP or by dicoumarol.

5. The reduction of acetoacetate or oxaloacetate with glutamate as hydrogen donor was inhibited by ATP, and concomitantly, NADP became more reduced. The effect of ATP was oligomycin-sensitive.

6. The effect of the mitochondrial energy level on the reductive amination of α-oxoglutarate in rat-liver mitochondria with α-oxoglutarate as the hydrogen donor was studied in detail. Following a preincubation in order largely to oxidize the intramitochondrial nicotinamide nucleotides, the overall reaction was separated into two steps: the reduction of intramitochondrial $NAD(P)^+$ by α-oxoglutarate, and the oxidation of the NAD(P)H formed by α-oxoglutarate (*plus* ammonia).

7. When dicoumarol *plus* oligomycin was present during the preincubation, the subsequent oxidation of intramitochondrial NAD(P)H by α-oxoglutarate (*plus* ammonia) was strongly inhibited. The inhibition could be prevented by removal of the dicoumarol after the preincubation and allowing α-oxoglutarate to be oxidized *via* the respiratory chain.

8. Preincubation with dicoumarol *plus* oligomycin led to inhibition of the

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accumulation of α-oxoglutarate by the mitochondria. However, a kinetic analysis showed that diminished substrate penetration was not the cause of the inhibition in the uncoupled mitochondria of the oxidation of intramitochondrial NAD(P)H by α-oxoglutarate (*plus* ammonia).

9. The results are discussed in relation to the NADP specificity of glutamate dehydrogenase in intact rat-liver mitochondria, the role of the energy-linked transhydrogenase, and a possible energy requirement for the oxidation of intramitochondrial nicotinamide nucleotides (previously reduced by NAD-linked substrates) by α-oxoglutarate (*plus* ammonia).

INTRODUCTION

Recent studies[1–18] on the transfer of hydrogens from one substrate to another in rat-liver mitochondria have shown that this may be influenced by the mitochondrial energy state. When the hydrogen donor is succinate, and the acceptor a nicotinamide nucleotide-linked substrate, there is a stoicheiometric requirement for energy[6,11,17,19], since the oxidoreduction involves an energy-linked reversal of the respiratory chain[20]. The energy state of the mitochondria would not be expected on thermodynamic grounds to influence hydrogen transfer from one nicotinamide nucleotide-linked substrate to another. Thus, the discovery[7,8,10,18] that the reduction of α-oxoglutarate (*plus* ammonia) with malate or α-oxoglutarate as the hydrogen donor is inhibited under conditions where the energy state of the mitochondria is low, was unexpected. Subsequent studies have suggested that at least three factors may influence these intramitochondrial oxidoreductions. Firstly, when malate is the hydrogen donor, energy appears to be necessary to prevent inhibition of malate dehydrogenase by oxaloacetate[21,22]. Secondly, the uptake of α-oxoglutarate is mediated by a specific "permease" (ref. 23), analogous to that for the tricarboxylic acids[24]. Furthermore, uncouplers of oxidative phosphorylation competitively inhibit the uptake of anionic substrates by mitochondria (see refs. 25–27) and increase the rate of efflux of α-oxoglutarate from mitochondria[28]. The third and most important factor is that glutamate dehydrogenase (EC 1.4.1.3) in rat-liver mitochondria appears to be NADP specific[29–32], in contrast to the isolated enzyme, which reacts with both NAD and NADP (ref. 33). Thus, when the hydrogen donor is NAD-linked, the formation of the NADPH required for the reduction of α-oxoglutarate (*plus* ammonia) would be promoted under conditions where the energy-linked transhydrogenase reaction can occur[34–36].

In this paper the effects of uncoupler, oligomycin and ATP on nicotinamide nucleotide-linked oxidoreductions in rat-liver mitochondria are reported. In particular, the control by the mitochondrial energy level of the reductive amination of α-oxoglutarate is examined, attention being focussed on the reaction of the intramitochondrial reduced nicotinamide nucleotides with α-oxoglutarate (*plus* ammonia), and on the penetration of α-oxoglutarate into the mitochondria. In the accompanying paper[37], the role of the energy state of the mitochondria in reactions involving glutamate and isocitrate dehydrogenases is analysed further. A preliminary report of this investigation has been presented[38].

METHODS

Rat-liver mitochondria were prepared by the method of HOGEBOOM[39], exactly as described by MYERS AND SLATER[40].

Experimental conditions. The standard reaction mixture contained 15 mM KCl, 5 mM $MgCl_2$, 2 mM EDTA, 50 mM Tris–HCl buffer, 5, 10 or 20 mM potassium phosphate buffer and 25 mM sucrose (derived from the mitochondrial suspension). Other additions, the reaction temperature and the incubation procedure are indicated in the legends to the tables and figures. The final pH was 7.4 or 7.5. The reaction was stopped with 0.1 ml 55 % $KClO_4$ or (for the determination of NAD(P)H) with 0.5 ml 1 M KOH in ethanol per ml reaction mixture. The acid and alkaline extracts were neutralized as described in ref. 41.

Assays. α-Oxoglutarate[42], ammonia[43], nicotinamide nucleotides[44,45] and acetoacetate[46] were determined enzymically (see ref. 41 for details). Protein was determined by the biuret method as described by CLELAND AND SLATER[47].

The oxidation of intramitochondrial nicotinamide nucleotides by α-oxoglutarate (*plus* ammonia) was directly monitored in the mitochondrial suspension by using the Aminco-Chance dual-wavelength spectrophotometer at the wavelength pair 360–374 mμ.

The penetration of α-oxoglutarate into the mitochondria was studied by using the centrifugation-filtration technique of WERKHEISER AND BARTLEY[48] as modified by PFAFF[49]. α-Oxoglutarate was determined enzymically in the mitochondrial extract and in the supernatant, using the Aminco-Chance dual-wavelength spectrophotometer (see ref. 32). Mitochondrial water was determined gravimetrically, correction being made for adherent supernatant in the pellet. The intramitochondrial concentration of α-oxoglutarate was calculated by correcting the amount found in the mitochondrial extract for that present in the adherent supernatant.

For the determination of adherent water, [*carboxy*-^{14}C]dextran (mol. wt. 60000–90000) was included in the reaction mixture. The [*carboxy*-^{14}C]dextran was obtained from New England Nuclear Corporation, Boston.

Special chemicals and enzymes. ADP, NAD^-, NADH, α-oxoglutarate, glucose 6-phosphate, the enzymes used in the assays, and hexokinase (EC 2.7.1.1) were obtained from Boehringer und Soehne, and dicoumarol, L-malate, β-hydroxybutyrate, glutamate, malonate and rotenone, from British Drug Houses. The hexokinase was diluted with serum albumin and dialysed against 1 % glucose before use to remove ammonia. Acetoacetate was prepared by the method of HALL[50], and *threo*-D_S-isocitrate was isolated from *Bryophyllum* leaves[51].

RESULTS

General considerations

The nicotinamide nucleotide-linked oxidoreductions studied in this paper are shown in Table I. The hydrogen donors α-oxoglutarate and β-hydroxybutyrate, and the hydrogen acceptors acetoacetate and oxaloacetate, are NAD-linked substrates. Isocitrate can donate hydrogens either to NAD^+ or to $NADP^+$. The reduction of α-oxoglutarate (*plus* CO_2) requires NADPH (see DISCUSSION).

The presence of rotenone *plus* malonate in the experiments of Tables II–VII

TABLE I

EFFECT OF DICOUMAROL, OLIGOMYCIN AND ATP ON NICOTINAMIDE NUCLEOTIDE-LINKED OXIDOREDUCTIONS IN RAT-LIVER MITOCHONDRIA

Hydrogen donor	*Nucleotide specificity*	*Hydrogen acceptor*	*Nucleotide specificity*	*Energy supplied by*	*Effect of*		
					Dicoumarol	*Oligomycin*	*ATP*
α-Oxoglutarate	NAD^+	Acetoacetate	NADH	Substrate linked	None	None	—
		Oxaloacetate	NADH	phosphorylation	None	None	—
		α-Oxoglutarate (+ CO_2)	NADPH		Inhibits	—	—
		α-Oxoglutarate (+ NH_3)	(NADPH)		Inhibits	Inhibits	—
β-Hydroxybutyrate	NAD^+	α-Oxoglutarate (+ NH_3)	(NADPH)	—	—	None	Stimulates*
Glutamate	($NADP^+$)	Acetoacetate	NADH	—	—	—	Inhibits*
		Oxaloacetate	NADH		—	—	Inhibits*
Isocitrate	NAD^+ or $NADP^+$	Acetoacetate	NADH	—	Stimulates	—	—
		α-Oxoglutarate (+ NH_3)	(NADPH)		None	—	None

* Effect oligomycin-sensitive.

TABLE II

EFFECT OF DICOUMAROL ON HYDROGEN TRANSFER FROM α-OXOGLUTARATE TO ACETOACETATE, OXALOACETATE, α-OXOGLUTARATE (+ CO_2) AND α-OXOGLUTARATE (+ AMMONIA) IN RAT-LIVER MITOCHONDRIA

Reaction mixture (1 ml) contained the basic components *plus* 0.1 mM ADP, 20 mM glucose, hexokinase, 10–20 mM α-oxoglutarate, 1 μg rotenone, and 3.1–4.0 mg mitochondrial protein. Reaction time, 10–20 min. Further additions were, 10 mM acetoacetate (A), 10 mM oxaloacetate (B), 16.6 mM $KHCO_3$ (C), 20 mM NH_4Cl (D), 8–20 mM malonate (A, B and D) and 4–6 mM L-malate (C). The gas phase was air (A, B and D) or 95% O_2–5% CO_2 (C). The concentration of dicoumarol used was 20 μM. The purpose of the L-malate added in Expt. C was to facilitate the efflux of tricarboxylic acids[24] and, at the same time, the entry of α-oxoglutarate[23].

Expt.	*Hydrogen acceptor*	*Additions*	*α-Oxoglutarate (nmoles/min per mg protein)*		*No. of measurements*
			Mean	*Range*	
A	Acetoacetate	None	37	32, 42	2
		Dicoumarol	38	36, 41	2
B	Oxaloacetate	None	14	—	1
		Dicoumarol	14	—	1
C	α-Oxoglutarate (+ CO_2)	None	16	14–20	3
		Dicoumarol	9	6–14	3
D	α-Oxoglutarate (+ NH_3)	None	84	74–90	6
		Dicoumarol	47	44–56	6

eliminated respiratory-chain phosphorylation. However, when α-oxoglutarate was the hydrogen donor, the substrate-linked phosphorylation step was a source of energy.

Effect of dicoumarol on hydrogen transfer from α-oxoglutarate to different acceptors

DANIELSON AND ERNSTER[18] first showed that hydrogen transfer from α-oxoglutarate to α-oxoglutarate (*plus* ammonia) is inhibited by uncouplers or oligomycin. We have confirmed this (Tables II and VI). Table II shows that dicoumarol inhibited hydrogen transfer not only to α-oxoglutarate (*plus* ammonia), but also to α-oxoglutarate (*plus* CO_2). The uncoupler had no effect on hydrogen transfer to acetoacetate or oxaloacetate.

Hydrogen transfer from β-hydroxybutyrate to α-oxoglutarate (plus ammonia)

Table III shows that the transfer of hydrogens from β-hydroxybutyrate to α-oxoglutarate (*plus* ammonia), in the presence of arsenite to prevent the oxidation of α-oxoglutarate, was stimulated by ATP in an oligomycin-sensitive reaction.

Hydrogen transfer from glutamate to acetoacetate and oxaloacetate

These hydrogen transfers (measured as ammonia formation) were inhibited by ATP (Table IV; see also ref. 52), the effect being partly prevented by oligomycin.

TABLE III

EFFECT OF ATP AND OLIGOMYCIN ON HYDROGEN TRANSFER FROM β-HYDROXYBUTYRATE TO α-OXOGLUTARATE (+ AMMONIA) IN RAT-LIVER MITOCHONDRIA

Reaction mixture (1 ml) contained the standard components *plus* 0.1 mM ADP, 40 mM β-hydroxybutyrate, 1 mM arsenite, 5 mM α-oxoglutarate, 20 mM NH_4Cl, 4 mM malonate, 2 % ethanol, 2 μg rotenone and 5.0 mg mitochondrial protein. Reaction time, 10 min.

Additions	*Glutamate (μmoles)*
None	1.58
ATP (10 μmoles)	2.11
Oligomycin (5 μg)	1.44
ATP + oligomycin	1.70

TABLE IV

OLIGOMYCIN-SENSITIVE INHIBITION BY ATP OF HYDROGEN TRANSFER FROM GLUTAMATE TO ACETOACETATE AND OXALOACETATE IN RAT-LIVER MITOCHONDRIA

Reaction mixture contained the standard components *plus* 1 mM arsenite, 20 mM glutamate, 1 μg rotenone, 10–20 mM malonate, 4.1–7.8 mg mitochondrial protein, and (where present) 10 mM acetoacetate, 10 mM oxaloacetate, 10 mM ATP and 5 μg oligomycin. Reaction time, 20–30 min.

Hydrogen acceptor	*Additions*	*NH_3 (nmoles/min per mg protein) in Expt.*		
		1	*2*	*3*
Acetoacetate	None	10	5	8
	ATP	5	2	4
	ATP + oligomycin	8	4	6
Oxaloacetate	None	9	6	10
	ATP	0.8	0.7	0.6
	ATP + oligomycin	5	3	3

Hydrogen transfer from isocitrate to α-oxoglutarate (plus ammonia) and to acetoacetate

When isocitrate (*plus* arsenite) was the hydrogen donor, and α-oxoglutarate (*plus* ammonia) the hydrogen acceptor, neither dicoumarol nor ATP had any effect on glutamate synthesis (Table V). On the other hand, dicoumarol stimulated the transfer of hydrogens to acetoacetate.

Oxidoreduction state of intramitochondrial NAD(P) during hydrogen transfer from α-oxoglutarate to α-oxoglutarate (plus ammonia) and from glutamate to acetoacetate or oxaloacetate

During hydrogen transfer from α-oxoglutarate to α-oxoglutarate (*plus* ammonia) both NAD and NADP are largely oxidized (Table VI). When dicoumarol or oligomycin was present, glutamate synthesis was inhibited, and concomitantly, the degree of reduction of NAD was increased from 5 % to 14 or 12 %, respectively. No change was observed in the oxidoreduction state of NADP.

As shown in Table VII, the inhibition by ATP of hydrogen transfer from glutamate to acetoacetate and oxaloacetate was accompanied by a large increase in the percentage reduction of NADP; oligomycin partly abolished both effects. No significant change occurred in the oxidoreduction state of NAD.

TABLE V

EFFECT OF DICOUMAROL AND ATP ON HYDROGEN TRANSFER FROM ISOCITRATE TO α-OXOGLUTARATE (+ AMMONIA) AND TO ACETOACETATE

The reaction mixture contained the standard components *plus* 0.5 mM ADP, 20 mM *threo*-D_s-isocitrate, 4 mM L-malate (see Table II), 1 mM arsenite and 5.5 mg mitochondrial protein. Reaction time, 14 min.

Additions	*Acetoacetate (nmoles)*	*Glutamate (nmoles)*
α-Oxoglutarate (4 mM) + NH_4Cl (20 mM)	—	5.9
α-Oxoglutarate + NH_4Cl + dicoumarol (30 μM)	—	6.1
α-Oxoglutarate + NH_4Cl + ATP (10 mM)	—	6.0
Acetoacetate (10 mM)	2.1	—
Acetoacetate + dicoumarol	2.8	—

TABLE VI

EFFECT OF DICOUMAROL AND OLIGOMYCIN ON THE DEGREE OF REDUCTION OF NAD(P) DURING HYDROGEN TRANSFER FROM α-OXOGLUTARATE TO α-OXOGLUTARATE (+ AMMONIA) IN RAT-LIVER MITOCHONDRIA

Reaction mixture (1 ml) contained the basic components *plus* 0.1 mM ADP, 20 mM glucose, 20 mM α-oxoglutarate, 20 mM NH_4Cl, 10 mM malonate, 2 μg rotenone, 2 % ethanol, hexokinase, and 3.1 mg mitochondrial protein. After 10 min the reaction was stopped in parallel incubations with either $HClO_4$ (for the determination of $NAD(P)^+$ and glutamate) or alcoholic KOH (for the determination of NAD(P)H).

Additions	*Glutamate (μmoles)*	*% Reduction of*	
		NAD	*NADP*
None	1.13	5	12
Dicoumarol (20 μM)	0.61	14	13
Oligomycin (5 μg)	0.87	12	13

The curves of Fig. 1, obtained with the data from Table VII, show the striking correlation between the rate of ammonia formation during hydrogen transfer from glutamate to the two NAD-linked substrates, and the extent of oxidation of NADP.

Effect of the mitochondrial energy level on the rate of oxidation of intramitochondrial NAD(P)H by α-oxoglutarate (plus ammonia)

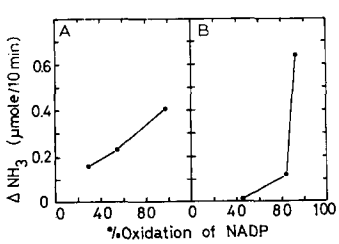
In the experiments described in this and the following sections, the synthesis of glutamate with α-oxoglutarate as hydrogen donor (the KREBS–COHEN dismutation[53]) was separated into two steps: the reduction of intramitochondrial $NAD(P)^+$ by α-oxoglutarate, and the oxidation of the NAD(P)H formed by α-oxoglutarate (*plus* ammonia). The first step was arrested by adding arsenite, and the second initiated with NH_4Cl.

TABLE VII

EFFECT OF OLIGOMYCIN ON HYDROGEN TRANSFER FROM GLUTAMATE TO ACETOACETATE AND TO OXALOACETATE IN RAT-LIVER MITOCHONDRIA

Reaction mixture (1 ml) contained the standard components *plus* 1 mM arsenite, 20 mM malonate, 20 mM glutamate, 2 μg rotenone, 2% ethanol, 6.3 mg mitochondrial protein and 10 mM acetoacetate or 10 mM oxaloacetate. After 10 min, the reaction was stopped in parallel incubations with either $HClO_4$ or alcoholic KOH.

Hydrogen acceptor	*Additions*	NH_3 (μmole)	% *Reduction of*	
			NAD	*NADP*
Acetoacetate	None	0.41	18	2
	ATP (10 mM)	0.16	14	71
	ATP + oligomycin (5 μg)	0.23	13	46
Oxaloacetate	None	0.64	4	7
	ATP	0.01	4	55
	ATP + oligomycin	0.12	3	16



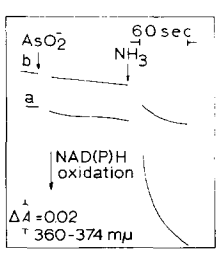


Fig. 1. Relationship between rate of hydrogen transfer from glutamate to acetoacetate (A) or oxaloacetate (B) and degree of oxidation of NAD(P). Data from Table VII.

Fig. 2. Dual-wavelength spectrophotometer recording of the oxidation of intramitochondrial NAD(P)H by α-oxoglutarate (*plus* ammonia). Mitochondria (10.2 mg protein) were preincubated in a medium (final volume, 3 ml) containing the standard components *plus* 2 mM ADP. In Expt. a, 20 μM dicoumarol and 15 μg oligomycin were present. This preincubation was carried out at 25°. After 2 min, 6 μmoles α-oxoglutarate were added, followed 2 min later by 3 μg rotenone. In Expt. b, α-oxoglutarate and rotenone were added together after the 2-min precinubation. In both cases, 5 min after the addition of α-oxoglutarate, 3 μmoles arsenite were added. The reaction was started 2 min later with 30 μmoles NH_4Cl. The oxidation of NAD(P)H was directly monitored at 360–374 mμ, the temperature being maintained at 5° at this stage.

Fig. 2 shows a spectrophotometric recording of the second step. Rat-liver mitochondria were preincubated with ADP and P_i in order largely to oxidize the intramitochondrial NAD(P)H. α-Oxoglutarate was then added to reduce $NAD(P)^+$, and rotenone to block the oxidation of NADH *via* the respiratory chain*. When the nicotinamide nucleotides had become maximally reduced, arsenite was added. On the addition of NH_4Cl, a very rapid oxidation of the nicotinamide nucleotides occurred (Trace a).

In Expt. b of Fig. 2 mitochondria were preincubated with dicoumarol (*plus* oligomycin). α-Oxoglutarate was then added together with rotenone. Although the same extensive reduction of the nicotinamide nucleotides was obtained as in Expt. a, the addition of NH_4Cl after this preincubation resulted in a very slow oxidation of NAD(P)H. With 10 mM α-oxoglutarate and 10 mM NH_4Cl, maximal inhibition (91 %) was found at 30–50 μM dicoumarol.

Lineweaver–Burk plots show that the inhibition by dicoumarol was almost completely of the non-competitive type with respect to α-oxoglutarate (Fig. 3).

Penetration of α-oxoglutarate into rat-liver mitochondria

In the experiment of Fig. 4, the penetration of α-oxoglutarate into rat-liver mitochondria was measured under exactly the same experimental conditions as those

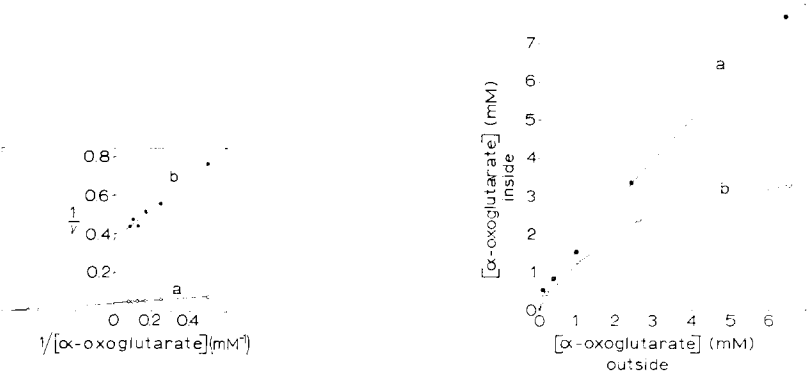


Fig. 3. Lineweaver–Burk plot of the rate of NAD(P)H oxidation by α-oxoglutarate (*plus* ammonia) *versus* the concentration of α-oxoglutarate in the absence (a) and in the presence of dicoumarol *plus* oligomycin (b). The experiment was carried out exactly as described in Fig. 1, except that the amount of mitochondrial protein was 9.9 mg and the α-oxoglutarate concentration was varied as indicated. The maximum velocity was 4.2 nmoles/sec in the absence and 0.45 nmole/sec in the presence of dicoumarol *plus* oligomycin. Similar results were obtained when the initial rate of NAD(P)H oxidation by α-oxoglutarate (*plus* ammonia) was measured by direct analyses of NAD^+ and $NADP^+$ in extracts of the reaction mixture (*cf.* Table VII and ref. 37).

Fig. 4. Penetration of α-oxoglutarate into rat-liver mitochondria. Rat-liver mitochondria (4.6 mg protein/ml) were incubated with the standard components, 2 mM ADP and 0.5 mg [*carboxy*-^{14}C]-dextran (440000 counts/min). In Expt. b, 20 μM dicoumarol and 5 μg/ml oligomycin were also present. Final volume, 5 ml. In Expt. a, after 2 min at 25°, α-oxoglutarate was added, followed 2 min later by rotenone (1 μg/ml). In Expt. b, α-oxoglutarate and rotenone were added together after the 2-min preincubation. In both cases, 4 min after the addition of α-oxoglutarate, the mitochondrial suspension was transferred to thermostatted beakers at 5°; 1 min later 1 mM arsenite was added. 2 Min after the addition of arsenite the mitochondria were separated from the incubation mixture by centrifugation filtration and the intra- and extramitochondrial concentrations of α-oxoglutarate was determined.

* In control experiments, it was found that no reduction of $NAD(P)^+$ occurred if α-oxoglutarate was omitted (see ref. 37).

used to study the oxidation of NAD(P)H by α-oxoglutarate (*plus* ammonia). The mitochondria preincubated with ADP and P_i accumulated α-oxoglutarate from the suspending medium (Curve a). Pretreatment with dicoumarol (*plus* oligomycin) led to a marked inhibition of α-oxoglutarate penetration (Curve b).

Relationship between α-oxoglutarate penetration and the rate of NAD(P)H oxidation by α-oxoglutarate (plus ammonia)

This was examined in a single experiment using the same mitochondrial preparation. In mitochondria pretreated with dicoumarol (*plus* oligomycin), both the penetration of α-oxoglutarate and the oxidation of NAD(P)H were inhibited with respect to those preincubated with ADP *plus* P_i. A Lineweaver–Burk plot of the rate of oxidation of NAD(P)H by α-oxoglutarate (*plus* ammonia) *versus* the intramitochondrial concentration of α-oxoglutarate shows that, even at equal concentrations of α-oxoglutarate, there was an inhibition by dicoumarol of NAD(P)H oxidation. Furthermore, this inhibition was not of the competitive type with respect to α-oxoglutarate (Fig. 5).

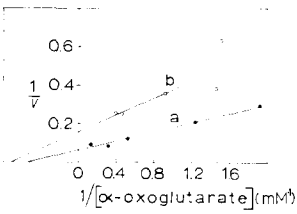


Fig. 5. Lineweaver–Burk plot of the rate of oxidation of intramitochondrial NAD(P)H by α-oxoglutarate (*plus* ammonia) as a function of the intramitochondrial concentration of α-oxoglutarate. Reaction mixture and additions as in Fig. 4, except that 4.2 mg mitochondrial protein/ml was present. The maximum velocity was 2.7 nmoles NAD(P)H/sec in the absence of dicoumarol and 1.0 in its presence. Experimental procedure as in Figs. 2 and 4.

TABLE VIII

EFFECT OF SERUM ALBUMIN ON THE INHIBITION OF NAD(P)H OXIDATION BY α-OXOGLUTARATE (+ AMMONIA), CAUSED BY PREINCUBATION OF THE MITOCHONDRIA WITH DICOUMAROL AND OLIGOMYCIN

Mitochondria (4.7 mg protein/ml) were incubated in the reaction mixture described in Fig. 1. Final volume, 5 ml. In Expts. 2–4, 20 μM dicoumarol and 5 μg/ml oligomycin were also present. After 2 min at 25° (phase I) 10 mM α-oxoglutarate, 5 μg rotenone, 40 mg dialysed bovine serum albumin and 1 mM Na_2S were added as indicated in the table. After 2 min (phase II) rotenone was added in Expts. 1 and 3 (phase III). 4 Min after adding α-oxoglutarate, the reaction mixture was transferred to beakers kept at 5°. After 1 min, 1 mM arsenite was added, followed 2 min later by 10 mM NH_4Cl. The reaction was stopped 10 sec later with $HClO_4$, and NAD^+ and $NADP^+$ were determined in the extracts. Parallel samples were stopped 2 min after the addition of arsenite. The values in the last column represent the amount of NAD(P)H oxidized in 10 sec.

Expt.	*Additions in phase*			*NAD(P)H oxidation (nmoles)*
	I	*II*	*III*	
1	None	α-Oxoglutarate	Rotenone	22.8
2	Dicoumarol, oligomycin	α-Oxoglutarate, rotenone	None	12.0
3	Dicoumarol, oligomycin	α-Oxoglutarate, albumin	Rotenone	22.3
4	Dicoumarol, oligomycin	α-Oxoglutarate, albumin, Na_2S, rotenone	None	14.0

Reversibility of the inhibition by dicoumarol

Table VIII shows that the inhibition of the oxidation of NAD(P)H by α-oxoglutarate (*plus* ammonia) brought about by pretreating the mitochondria with dicoumarol (*plus* oligomycin), could be completely reversed by adding serum albumin together with the α-oxoglutarate and delaying the addition of rotenone. Albumin served to bind dicoumarol and to restore the coupled state[45,51], and α-oxoglutarate oxidation *via* the respiratory chain provided the energy-rich bond. In Expt. 4, sulphide and rotenone were added at the same time as α-oxoglutarate and albumin, and in this case the inhibition of NAD(P)H oxidation was not reversed.

These results exclude the possibility that inhibition of glutamate synthesis was due to some irreversible change in the system, or to an effect of dicoumarol *per se*. Furthermore, they indicate that the energy-rich bond is required for maximal oxidation of intramitochondrial reduced nicotinamide nucleotides by α-oxoglutarate (*plus* ammonia).

DISCUSSION

The experimental results allow the oxidoreductions studied to be grouped as follows (see Table I):

1. *NAD → NAD type* (α-oxoglutarate → acetoacetate or oxaloacetate). Depleting the system of energy has no effect.

2. *NAD → NADP type* (α-oxoglutarate → α-oxoglutarate (*plus* CO_2)). Although two isocitrate dehydrogenases are present in mitochondria, one NAD-specific (EC 1.1.1.41) and the other NADP-specific (EC 1.1.1.42), the reduction of α-oxoglutarate (*plus* CO_2) has been demonstrated with the NADP-linked enzyme only[50,51] and not with the NAD-linked one (ref. 51; see also ref. 37). With α-oxoglutarate as hydrogen donor, energy would be expected to promote the transfer of hydrogens, by promoting the energy-linked transhydrogenase reaction (see also ref. 52). Indeed dicoumarol inhibits the transfer of hydrogens from α-oxoglutarate to α-oxoglutarate (*plus* CO_2).

3. *NAD or NADP → NAD type*. The transfer of hydrogens from isocitrate to acetoacetate could be of the type NAD → NAD or NADP → NAD. In the former case, low concentrations of uncoupler would not be expected to have any effect. However, the operation of the energy-linked transhydrogenase would tend to keep NADP reduced, and the oxidation of NADPH by NAD^+ would not occur readily. Uncoupler, by inhibiting the energy-linked transhydrogenase reaction, would promote the oxidation of NADPH by NAD^+. Thus, the stimulation by dicoumarol of hydrogen transfer from isocitrate to acetoacetate is probably due to promotion of the reaction *via* the NADP-linked isocitrate dehydrogenase.

4. *Oxidoreductions with α-oxoglutarate (plus ammonia) as hydrogen acceptor*. In intact, isolated rat-liver mitochondria, glutamate dehydrogenase reacts preferentially with NADPH rather than with NADH (refs. 30, 31; see also refs. 28, 29 and 37). Thus maximal reduction of α-oxoglutarate (*plus* ammonia) by NAD-linked substrates, like that of α-oxoglutarate (*plus* CO_2), would be favoured by operation of the energy-linked transhydrogenase. Indeed, with α-oxoglutarate as the hydrogen donor, and the substrate-linked phosphorylation step as the source of energy, dicoumarol and oligomycin inhibit the reduction of α-oxoglutarate (*plus* ammonia). Furthermore, with β-hydroxybutyrate as hydrogen donor, ATP stimulates the transfer of hydrogens to

α-oxoglutarate (*plus* ammonia) in an oligomycin-sensitive reaction. As expected, inhibition of the energy-linked transhydrogenase during hydrogen transfer from NAD-linked substrates to α-oxoglutarate (*plus* ammonia) led to an increased reduction of NAD (Table VI).

With isocitrate as the hydrogen donor, there is no effect of ATP on the reduction of α-oxoglutarate (*plus* ammonia). This suggests that the formation of NADPH *via* the NADP-linked isocitrate dehydrogenase is sufficient to support maximal reduction of α-oxoglutarate (*plus* ammonia).

5. *Oxidoreductions with glutamate as hydrogen donor.* When the energy-linked transhydrogenase is operating, the oxidation of NADPH by NAD^+ is inhibited. Thus, as VAN DAM[45] has shown, on the addition of an NAD-linked substrate like acetoacetate to coupled rat-liver mitochondria, intramitochondrial NADPH remains reduced, and only NADH is oxidized. In the presence of uncoupler, however, both nucleotides become oxidized.

In intact, isolated rat-liver mitochondria, glutamate dehydrogenase acts like an $NADP^+$-linked enzyme[28–31]. When glutamate is the hydrogen donor for the reduction of acetoacetate and oxaloacetate, there is an oligomycin-sensitive inhibition by ATP of the hydrogen transfer. It seems likely that the inhibition is due to activation of the energy-linked transhydrogenase; indeed, the inhibition is accompanied by an increase in the percentage reduction of NADP (Table VII and Fig. 1).

The energy-linked transhydrogenase is not the only reaction in the KREBS–COHEN dismutation requiring the energy-rich bond. Indeed, when the intramitochondrial nicotinamide nucleotides were exposed to a cycle of oxidation and reduction in the presence of dicoumarol and oligomycin, the oxidation of NAD(P)H by α-oxoglutarate (*plus* ammonia) was strongly inhibited, suggesting that the energy-rich bond is also required for the latter step.

Recent studies (see refs. 25–27) have led to the proposal that the inhibition by uncouplers of the oxidation of succinate (*plus* rotenone), and possibly of other anionic substrates, is due to inhibition of the penetration of the substrates into the mitochondria. However, the data of Figs. 2 and 4 show that the inhibition in the uncoupled mitochondria of the oxidation of NAD(P)H by α-oxoglutarate (*plus* ammonia) can not be simply explained by inhibition of α-oxoglutarate penetration.

Experiments designed to throw light on the energy-rich bond requirement for this reaction of nicotinamide nucleotides with glutamate dehydrogenase, as well as to clarify the nature of the preferential reaction of glutamate dehydrogenase with NADPH, are reported in the accompanying paper[37].

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