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SYNTHESIS OF GLUTAMATE FROM α-OXOGLUTARATE AND AMMONIA IN RAT-LIVER MITOCHONDRIA

IV. REDUCTION OF NICOTINAMIDE NUCLEOTIDE COUPLED WITH THE AEROBIC OXIDATION OF TETRAMETHYL-*p*-PHENYLENEDIAMINE

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SUMMARY

1. The synthesis of glutamate from α-oxoglutarate and ammonia in rat-liver mitochondria has been studied with succinate or malate as hydrogen donor and with the aerobic oxidation of tetramethyl-*p*-phenylenediamine, in the presence of antimycin to inhibit the oxidation of succinate or malate, as a source of energy.

2. Tetramethyl-*p*-phenylenediamine itself is a relatively inefficient hydrogen donor for glutamate synthesis in rat-liver mitochondria.

3. The synthesis of glutamate in the succinate–antimycin–tetramethyl-*p*-phenylenediamine system is inhibited by Amytal or dinitrophenol, requires inorganic phosphate and is not significantly affected by oligomycin. The addition of α-oxoglutarate (+ ammonia) in the presence of oligomycin stimulates oxygen uptake slightly.

4. It is concluded that the synthesis of glutamate in the succinate–antimycin–tetramethyl-*p*-phenylenediamine system represents an energy-linked reversal of the respiratory chain.

5. The energy necessary for the reduction of α-oxoglutarate (+ ammonia) by malate can also be generated during the aerobic oxidation of tetramethyl-*p*-phenylenediamine. In this case, Amytal can be used instead of antimycin to inhibit the aerobic oxidation of malate.

6. In rabbit-heart sarcosomes in the presence of oxygen, mitochondrial NAD^+ is reduced to a greater extent by tetramethyl-*p*-phenylenediamine than by succinate. The reduction of mitochondrial NAD^+ coupled with the aerobic oxidation of tetramethyl-*p*-phenylenediamine is inhibited by azide, antimycin, Amytal, dinitrophenol, arsenate and phosphate + phosphate acceptor, and is unaffected by oligomycin, arsenite and malonate. Oligomycin prevents the inhibition by arsenate or by phosphate + phosphate acceptor. In the presence of antimycin, mitochondrial NAD^+ becomes reduced when succinate and tetramethyl-*p*-phenylenediamine are added together .

7. The pathway of hydrogen transfer from tetramethyl-*p*-phenylenediamine to NAD^+ is discussed.

8. It is concluded that high-energy intermediates of oxidative phosphorylation

Abbreviation: TMPD, tetramethyl-*p*-phenylenediamine.

generated in the cytochrome oxidase region can probably react at the cytochrome *b*–cytochrome *c* and NAD^+–flavoprotein couples.

INTRODUCTION

In the first three papers of this series[1-3], it was shown that high-energy intermediates of oxidative phosphorylation are necessary for the synthesis of glutamate from α-oxoglutarate and ammonia in rat-liver mitochondria when either succinate or malate is the hydrogen donor. With succinate, energy is required in stoicheiometric amounts to reverse the respiratory chain between flavoprotein and NAD^+ (refs. 2, 4). In contrast, high-energy intermediates are required in less than stoicheiometric amounts for the transfer of hydrogens from malate to α-oxoglutarate + ammonia[3,4]. The high-energy intermediates can be generated during the aerobic oxidation of the hydrogen donor via the respiratory chain, even when oligomycin is present to block the formation of ATP[1-3].

Since there are two energy-conservation sites in the respiratory chain between succinate and O_2, it is clearly desirable to determine whether both or only one can provide the energy for glutamate synthesis. The report by JACOBS[5] that P:O ratios approaching 1 for the cytochrome oxidase step can be obtained by using TMPD to reduce cytochrome *c* suggested a direct method of testing whether high-energy intermediates generated in the cytochrome oxidase region could be used for glutamate synthesis. By using TMPD as oxidizable substrate in the presence of antimycin and succinate, the energy-generating reaction is separated from the reduction of NAD^+. The hydrogen atoms for the synthesis of glutamate from α-oxoglutarate + ammonia are provided by succinate and energy by the cytochrome oxidase step of the respiratory chain.

A preliminary account of studies of this system has appeared[6]. A similar system in which NAD^+ reduction was measured directly instead of coupling the reaction with the synthesis of glutamate has been used by PACKER AND DENTON[7]. TMPD has also been used, in the absence of antimycin, as a donor of reducing equivalents for the energy-linked reversal of electron transfer from cytochrome *c* to NAD^+ (refs. 8–10) and to ubiquinone[11] in the respiratory chain. That the respiratory chain could be reversed over the whole span NADH–cytochrome *c* was first shown by CHANCE AND FUGMANN[12], using ATP as source of energy.

In this paper, it is shown that the high-energy intermediates necessary for the synthesis of glutamate from α-oxoglutarate and ammonia in rat-liver mitochondria when either succinate or malate is the hydrogen donor can be generated during the aerobic oxidation of TMPD. In the absence of antimycin, TMPD can also provide reducing equivalents for the synthesis of glutamate, although relatively inefficiently. On the other hand, NAD^+ in rabbit-heart sarcosomes is readily reduced by TMPD.

MATERIALS AND METHODS

Experiments with rat-liver mitochondria

The methods, materials and experimental procedure employed are described in the first paper of the series[1]. The reaction mixture contained 15 mM KCl, 2 mM EDTA, 5 mM $MgCl_2$, 50 mM Tris–HCl buffer, 0.1 mM ADP, 25 mM sucrose (derived

from the mitochondrial suspension) and the additions indicated in the legends to the tables in a final volume of 1 ml. The final pH was 7.5 and the reaction temperature was 25°.

Experiments with rabbit-heart sarcosomes

The methods and experimental procedure employed have been described by SNOSWELL[13].

Incubations of the sarcosomes were carried out at 0° for 90 sec. The reaction mixture contained 0.22 M sucrose, 0.01 M EDTA (pH 7.4), sarcosomes and the additions indicated in the legends to the tables in a final volume of 1.25 ml. The mixture was agitated vigorously in order to ensure that the suspension did not become anaerobic during the incubation.

RESULTS

Glutamate synthesis coupled with the oxidation of TMPD in rat-liver mitochondria

TMPD as hydrogen donor. In all the experiments with rat-liver mitochondria, arsenite was added to prevent the oxidation of α-oxoglutarate. In the two experiments shown in Table I, only 0.1 μmole glutamate was synthesized when no hydrogen donor was added, and 0.5–0.7 μmole when TMPD (kept in the reduced state by ascorbate) was present. Antimycin inhibited glutamate synthesis in one experiment,

TABLE I

SYNTHESIS OF GLUTAMATE COUPLED TO THE AEROBIC OXIDATION OF SUCCINATE AND OF TMPD IN RAT-LIVER MITOCHONDRIA

Reaction mixture contained, in addition to the basic components, 20 mM α-oxoglutarate, 20 mM NH_4Cl, 1 mM arsenite, 2% ethanol, 10 μg oligomycin, 20 mM potassium phosphate buffer and 5.4 mg (Expt. 131) or 4.7 mg (Expt. 132) mitochondrial protein. Reaction time, 30 min.

Additions	*Expt. 131*			*Expt. 132*		
	Δ O (μatoms)	*Δ Glutamate (μmoles)*	*Δ Aspartate (μmoles)*	*Δ O (μatoms)*	*Δ Glutamate (μmoles)*	*Δ Aspartate (μmoles)*
None	0.8	0.12	0	0.6	0.13	0
Antimycin (4 μg)	0.9	0.13	0	—	—	—
Succinate (60 mM)	11.6	7.64	4.66	9.8	4.49	1.02
Succinate + antimycin	0.8	0.58	0	0.5	0.22	0.15
TMPD (0.3 mM) + ascorbate (20 mM)	14.1	0.68	0	15.1	0.46	0
TMPD + ascorbate + antimycin	18.4	0.22	0	13.5	0.46	0
Succinate + antimycin + TMPD + ascorbate	15.3	2.02	0.05	13.7	1.41	0.23

as would be expected if TMPD were the hydrogen donor, but was without effect in the other (Table I, line 6; see also Table IV). Amytal inhibited slightly (Table IV).

Succinate as hydrogen donor. Table I shows that when the aerobic oxidation of succinate was blocked by antimycin, glutamate synthesis was also inhibited (*cf.* ref. 2). In the presence of antimycin the amount of glutamate found when

succinate and TMPD were added together considerably exceeded the sum of the amounts found when the succinate and TMPD were added separately*. In Table II, it can be seen that the synthesis of glutamate in the succinate–antimycin–TMPD system is inhibited by Amytal or dinitrophenol, but is not significantly affected by oligomycin. In the experiments of Tables I and II, the amounts of aspartate found

TABLE II

EFFECT OF INHIBITORS ON GLUTAMATE SYNTHESIS COUPLED WITH THE AEROBIC OXIDATION OF TMPD IN THE PRESENCE OF SUCCINATE AND ANTIMYCIN IN RAT-LIVER MITOCHONDRIA

Reaction mixture contained, in addition to the basic components, 20 mM α-oxoglutarate, 20 mM NH_4Cl, 20 mM potassium phosphate buffer, 1 mM arsenite, 20 mM ascorbate, 0.3 mM TMPD, 60 mM succinate, 4 μg (Expt. 125), 3.3 μg (Expt. 133) or 2 μg (Expt. 134) antimycin, 2% ethanol, and 5.7 mg (Expt. 125), 4.4 mg (Expt. 133) or 6.0 mg (Expt. 134) mitochondrial protein. Reaction time, 30 min (Expt. 125) or 20 min (Expts. 133 and 134).

Expt.	*Additions*	*Δ O (μatoms)*	*Δ Glutamate (μmoles)*	*Δ Aspartate (μmoles)*
125	None	14.2	2.07	0.28
	Amytal (2 mM)	16.9	0.61	0
	Dinitrophenol (50 μM)	16.4	0.30	0
133	None	11.6	2.02	0.46
	Amytal (2 mM)	10.5	0.34	0
	Dinitrophenol (50 μM)	10.7	0.16	0.01
	Oligomycin (2.3 μg/mg protein)	10.6	1.94	0.50
	Oligomycin + Amytal	9.3	0.25	0.04
	Oligomycin + dinitrophenol	12.3	0.55	0.04
134	None	11.0	3.37	—
	Oligomycin (1.7 μg/mg protein)	9.7	3.70	—

represent the contribution of malate (derived from succinate) as hydrogen donor for the synthesis of amino acids from α-oxoglutarate and ammonia[1].

In the first paper of this series[1], it was shown that inorganic phosphate (or arsenate) was necessary for the synthesis of glutamate and aspartate coupled to the

TABLE III

EFFECT OF INORGANIC PHOSPHATE ON THE SYNTHESIS OF GLUTAMATE IN THE SUCCINATE–ANTIMYCIN–TMPD SYSTEM

Reaction mixture contained, in addition to the basic components, 20 mM α-oxoglutarate, 20 mM NH_4Cl, 60 mM succinate, 0.3 mM TMPD, 2–4 kg antimycin, 15–20 mM ascorbate, 1 mM arsenite, 2% ethanol, 10 μg oligomycin, and 5.1–6.4 mg mitochondrial protein. Reaction time, 20 min (Expt. 118) or 30 min.

Expt.	*Δ Glutamate (μmoles)*		*Δ Aspartate (μmoles)*	
	No P_i	*20 mM P_i*	*No P_i*	*20 mM P_i*
117	2.8	4.3	0.6	1.5
118	1.3	1.9	—	—
124	2.0	2.4	0	0.1
129	2.0	4.2	0.2	1.2

* Similar results were obtained when 2-heptyl-4-hydroxyquinoline-*N*-oxide (kindly provided by Dr. J. LIGHTBOWN) was used instead of antimycin to inhibit the respiratory chain in the cytochrome *b* region.

aerobic oxidation of succinate. The same is true of the succinate–antimycin–TMPD system (Table III), although quantitatively the effects of phosphate were less in this system.

Malate as hydrogen donor. The results of two experiments with malate as hydrogen donor for the synthesis of amino acids from α-oxoglutarate and ammonia are presented in Table IV. Antimycin or Amytal inhibited the synthesis of amino acids markedly (*cf.* ref. 3). This inhibition by either antimycin or Amytal could be overcome by adding TMPD.

TABLE IV

SYNTHESIS OF GLUTAMATE + ASPARTATE COUPLED WITH THE AEROBIC OXIDATION OF TMPD IN THE PRESENCE OF MALATE + ANTIMYCIN OR MALATE + AMYTAL IN RAT-LIVER MITOCHONDRIA

Reaction mixture contained, in addition to the basic components, 20 mM α-oxoglutarate, 20 mM NH_4Cl, 20 mM potassium phosphate buffer, 5 mM glutamate, 1 mM arsenite, 2% (Expt. 160) or 1% (Expt. 169) ethanol, 10 μg oligomycin and 4.5 mg (Expt. 160) or 7.1 mg (Expt. 169) mitochondrial protein. Reaction time, 20 min.

Expt.	*Substrate*	*Δ O (μatoms)*	*Δ Glutamate (μmole)*	*Δ Aspartate (μmoles)*	*Δ (Glutamate + aspartate) (μmoles)*
160	Malate (20 mM)	1.0	0.50	3.68	4.18
	Malate + antimycin (1 μg)	0.4	0.61	0.92	1.53
	TMPD (0.1 mM) + ascorbate (20 mM)	3.6	0.48	0.15	0.63
	TMPD + ascorbate + antimycin	4.0	0.50	0.08	0.58
	Malate + antimycin + TMPD + ascorbate	3.8	0.76	2.49	3.25
169	Malate (20 mM)	1.6	−0.32	5.77	5.45
	Malate + Amytal (2 mM)	0.1	0.56	0.22	0.78
	TMPD (0.1 mM) + ascorbate (20 mM)	3.7	0.78	0.14	0.92
	TMPD + ascorbate + Amytal	3.8	0.54	0.24	0.78
	Malate + Amytal + TMPD + ascorbate	4.8	0.96	4.88	5.84

The reduction of mitochondrial NAD$^+$ coupled with the oxidation of TMPD in rabbit-heart sarcosomes

In Table V the results of experiments are summarized in which the extent of reduction of NAD$^+$ was measured in fresh sarcosomes and after incubation with TMPD or with succinate. Both substrates brought about the reduction of NAD$^+$, but the extent of reduction was appreciably greater with TMPD.

The effect of inhibitors and of phosphate acceptor on the reduction of NAD$^+$ coupled with the aerobic oxidation of TMPD is shown in Table VI. The reduction was completely inhibited by azide, antimycin, dinitrophenol and arsenate and strongly inhibited by Amytal and phosphate acceptor. Oligomycin alone had no effect but partially prevented the inhibition by arsenate and by phosphate + phosphate acceptor. Table VI also shows that the reduction of NAD$^+$ coupled with the oxidation of TMPD was not inhibited by arsenite or malonate.

Table VII shows that, in the presence of antimycin, which inhibits the reduction of NAD$^+$ coupled with the aerobic oxidation of either succinate or TMPD, an extensive reduction of NAD$^+$ could be obtained when succinate and TMPD were added together. The reduction of NAD$^+$ that occurred in the presence of antimycin, succinate and TMPD was stimulated by oligomycin.

TABLE V

THE REDUCTION OF NAD^+ COUPLED WITH THE AEROBIC OXIDATION OF TMPD AND OF SUCCINATE IN RABBIT-HEART SARCOSOMES

The reaction mixture contained, in addition to the basic components, 4–8 mg sarcosomal protein and, where indicated, 0.24 mM TMPD + 12 mM ascorbate, or 40 mM succinate. The values given are the mean's with the range in brackets.

Experimental conditions	*No. of Expts.*	*NAD^+ (μmoles/g protein)*	*NADH (μmoles/g protein)*	*$NAD^+ + NADH$ (μmoles/g protein)*	$\frac{NADH}{NAD^+ + NADH}$
Fresh sarcosomes	10	4.5 (3.2–7.7)	2.9 (1.8–4.6)	7.4 (5.7–9.5)	0.39 (0.19–0.55)
Incubated with TMPD	10	0.7 (0.5–1.4)	6.4 (4.8–8.1)	7.1 (5.4–8.6)	0.91 (0.81–0.98)
Incubated with succinate	4	1.2 (0.8–1.7)	5.1 (3.9–6.4)	6.3 (5.0–7.7)	0.80 (0.73–0.88)

TABLE VI

EFFECT OF INHIBITORS AND OF PHOSPHATE ACCEPTOR ON THE REDUCTION OF NAD^+ COUPLED WITH THE AEROBIC OXIDATION OF TMPD IN RABBIT-HEART SARCOSOMES

The reaction mixture contained, in addition to the basic components, 0.24 mM TMPD, 12 mM ascorbate and 4–8 mg sarcosomal protein. The reaction time was 90 sec at 0°. When oligomycin was used, the sarcosomes were preincubated with the inhibitor for 2 min at room temperature before the addition of TMPD + ascorbate.

Additions	*Concentration*	*Inhibition* (%)*
Azide	4 mM	100
Antimycin	3.4 μg/mg protein	100
Amytal	0.4 μmole/mg protein (2 mM)	49
Amytal	1.3 μmole/mg protein (4 mM)	83
Malonate	4 mM	0
Arsenite	1 mM	0
2,4-Dinitrophenol	0.1 mM	100
2,4-Dinitrophenol + Amytal	0.1 mM, 4 mM	100
Oligomycin	4.3 μg/mg protein	0
Arsenate	24 mM	100
Arsenate + oligomycin	24 mM, 3.9 μg/mg protein	35
ADP, P_i, glucose, hexokinase	0.1 mM, 20 mM, 20 mM and 150 units	79
ADP, P_i, glucose, hexokinase + oligomycin	0.1 mM, 20 mM, 20 mM, 150 units and 3.9 μg/mg protein	26

* The method of calculating the degree of inhibition may be illustrated with the example of Amytal as shown in line 4. $\frac{NADH}{NAD^+ + NADH}$ in the absence of substrate = 0.32 and with TMPD = 0.93. Therefore the reduction by TMPD = 0.61. $\frac{NADH}{NAD^+ + NADH}$ in the presence of Amytal = 0.30 and in the presence of TMPD plus Amytal = 0.40. Therefore the reduction produced by TMPD + Amytal = 0.40 − 0.30 = 0.10, or $\frac{0.10}{0.61} \times 100 = 17\%$ of that produced by TMPD alone, *i.e.* Amytal inhibited the TMPD reduction by 83%.

TABLE VII

REDUCTION OF NAD^+ BY TMPD AND BY SUCCINATE IN RABBIT-HEART SARCOSOMES AND THE EFFECT OF ANTIMYCIN

Experimental conditions as in Table V. Each value is the mean of two determinations.

Additions	$\frac{NADH}{NAD^+ + NADH}$
None	0.29
Antimycin (5.0 μg/mg protein)	0.24
Succinate (40 mM)	0.77
Succinate + antimycin	0.23
TMPD (0.24 mM) + ascorbate (12 mM)	0.88
TMPD + ascorbate + antimycin	0.24
Succinate + TMPD + ascorbate + antimycin	0.70
Succinate + TMPD + ascorbate + antimycin + + oligomycin (3.2 μg/mg protein)	0.79

DISCUSSION

The synthesis of glutamate from α-oxoglutarate and ammonia in rat-liver mitochondria with succinate as hydrogen donor can take place in the presence of antimycin by coupling the reduction with the aerobic oxidation of TMPD. The properties of the succinate–antimycin–TMPD system are similar to those of the system in which glutamate synthesis is coupled with the aerobic oxidation of succinate[1,2]. In both systems, the synthesis of glutamate is inhibited by dinitrophenol and Amytal, and the addition of α-oxoglutarate (+ ammonia) in the presence of phosphate acceptor and absence of oligomycin leads to a lowering of the P:O ratio, and to an increase in the oxygen uptake in the presence of oligomycin[4,6]. However, the increased O_2 uptake is smaller with the succinate–antimycin–TMPD system than that observed in the succinate system[2]. These results indicate that the transfer of hydrogens from succinate to α-oxoglutarate + ammonia in the succinate–antimycin–TMPD system represents an energy-linked reversal of the respiratory chain[14].

There is one quantitative difference between the two systems. In the succinate system, the synthesis of glutamate is greatly stimulated by oligomycin even in the absence of phosphate acceptor[2], while in the succinate–antimycin–TMPD system, oligomycin has only a slight effect. The reason for the lack of an effect of oligomycin in the latter system may be that the maximal synthesis of glutamate is limited by other factors, for instance by the presence of antimycin. It has been shown that antimycin at high concentrations has an inhibitory effect on the synthesis of glutamate with succinate as hydrogen donor and ATP as energy source which is not obviously related to its inhibitory action on electron transport in the cytochrome *b* region of the respiratory chain[2]. Table VIII shows that this inhibition is found in the succinate–antimycin–TMPD system as well. The requirement for phosphate (Table III) was also less marked in the succinate–antimycin–TMPD system than in the succinate system.

TABLE VIII

EFFECT OF ANTIMYCIN CONCENTRATION ON THE SYNTHESIS OF GLUTAMATE WITH SUCCINATE AS HYDROGEN DONOR IN RAT-LIVER MITOCHONDRIA

Reaction mixture contained, in addition to the basic components, 20 mM α-oxoglutarate, 20 mM NH_4Cl, 1 mM arsenite, 60 mM succinate, 2 mM potassium phosphate buffer, 1% ethanol and 6.7 mg mitochondrial protein. Reaction time, 20 min. (Expt. 251).

Additions	*Antimycin (μg/mg protein)*	*Δ O (μatoms)*	*Δ Glutamate (μmoles)*	*Δ Aspartate (μmoles)*
ATP (10 mM)	0.07	0.7	1.85	0.49
ATP	0.75	0.6	1.07	0.21
TMPD (0.1 mM) + ascorbate (20 mM)	0.07	8.6	3.43	1.27
TMPD + ascorbate	0.75	8.6	2.45	0.37

These results and those of PACKER[7–9] and LÖW AND VALLIN[10] provide direct evidence that high-energy intermediates of oxidative phosphorylation generated in the cytochrome oxidase region can react at the NAD+–flavoprotein couple and bring about a reversal of the respiratory chain.

In the experiments with rabbit-heart sarcosomes, it was found that the re-

duction of NAD^+ coupled with the aerobic oxidation of TMPD was inhibited by azide, antimycin, Amytal, dinitrophenol and (in the absence of oligomycin) by arsenate, and was not affected by arsenite, malonate or oligomycin. The lack of inhibition by arsenite or malonate shows that endogenous substrate does not play a role (*cf.* ref. 13). These results show that TMPD can provide reducing equivalents for an energy-linked reversal of the respiratory chain from cytochrome *c* to NAD^+ (*cf.* refs. 9, 12, 15 and 16). The pathway followed by the reducing equivalents from TMPD is shown in Fig. 1. Ubiquinone, which is also reduced by TMPD in a reaction

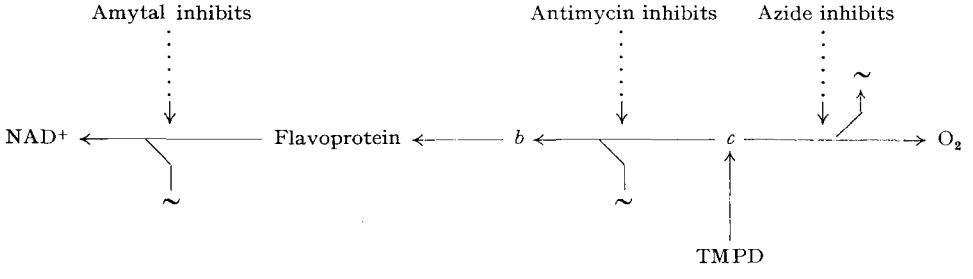

Fig. 1. Pathway for the reduction of NAD^+ by TMPD.

coupled to the oxidation of TMPD[11], may lie between cytochrome *b* and the flavoprotein. Since the reduction of NAD^+ by TMPD is inhibited by antimycin* and Amytal, the pathway probably involves the cytochrome *c*–cytochrome *b* and flavoprotein–NAD^+ couples, and it is at these couples that energy must be invested to reverse the respiratory chain. The energy is provided by the aerobic oxidation of TMPD, which explains why azide inhibits the reduction of NAD^+ in this system. Thus high-energy intermediates of oxidative phosphorylation generated in the cytochrome oxidase region are probably able to react not only at the NAD^+–flavoprotein couple, as the studies on glutamate synthesis in the succinate–antimycin–TMPD system had already indicated, but at the cytochrome *b*–cytochrome *c* couple as well. The mechanism of the reversal of the respiratory chain will be further discussed in the following paper[4]. The uncouplers, dinitrophenol and arsenate, inhibit by reacting with the high-energy intermediate (see ref. 17). In agreement with previous work[13,18,19], oligomycin prevents uncoupling by arsenate.

Evidence has been presented that the reduction of α-oxoglutarate and ammonia by malate in rat-liver mitochondria requires the participation of high-energy intermediates of oxidative phosphorylation. As shown in Table IV of the present paper, these intermediates can be generated in the cytochrome oxidase region.

* It is not known why antimycin is relatively ineffective in inhibiting the synthesis of glutamate linked with the aerobic oxidation of TMPD by rat-liver mitochondria in contrast with the complete inhibition of the reduction of sarcosomal NAD^+. The reduction of ubiquinone in beef-heart sarcosomes coupled with the aerobic oxidation of TMPD was also almost completely inhibited by antimycin[11]. It is possible that the antimycin-insensitive pathway (*cf.* ref. 17) is involved in the liver-mitochondria experiments, but it would not be expected that this would be reversible, since it is not phosphorylative. Alternatively, it is possible that the antimycin-sensitive site was not completely inhibited, and the residual activity was sufficient not to be rate-limiting in the very slow reduction of NAD^+ by TMPD. There was always a small antimycin-resistant glutamate synthesis in the succinate system (see Table I and refs. 1 and 2).

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