BBA 12234

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

II. SUCCINATE AS HYDROGEN DONOR

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(Received February 8th, 1963)

SUMMARY

1. The synthesis of amino acids from α-oxoglutarate and ammonia in rat-liver mitochondria was studied with succinate as hydrogen donor and with arsenite added to prevent the oxidation of oxo acids. Glutamate and aspartate are synthesized under these conditions. The glutamate found represents the contribution of succinate, and the aspartate found the contribution of malate (derived from succinate), as hydrogen donor to α-oxoglutarate.

2. In the presence of succinate and oligomycin, the addition of α-oxoglutarate + ammonia results in an abrupt, 2.5-fold increase in rate of oxygen uptake. This increase only occurs in the presence of inorganic phosphate. Neither α-oxoglutarate nor ammonia, added separately, stimulates oxygen uptake.

3. The synthesis of glutamate is usually considerably stimulated by ATP and is always stimulated by oligomycin. This stimulation of glutamate synthesis is always accompanied by a stimulation of oxygen uptake.

4. When the aerobic oxidation of succinate is blocked by antimycin, glutamate synthesis is almost completely inhibited. ATP restores glutamate synthesis, but not if oligomycin or dinitrophenol is present.

5. It is concluded that the synthesis of glutamate from α-oxoglutarate, ammonia and succinate, in the presence of arsenite and oxygen, represents an energy-linked reversal of the respiratory chain and that a dinitrophenol-sensitive, oligomycin-insensitive intermediate of oxidative phosphorylation is required for the transfer of hydrogen from succinate to α-oxoglutarate (+ ammonia).

6. Possible reasons for the stimulation by ATP and oligomycin of the synthesis of glutamate coupled with the aerobic oxidation of succinate are discussed.

INTRODUCTION

In 1956, CHANCE[1] discovered that mitochondrial NAD^+ becomes reduced when succinate is added to aerobic mitochondria in the absence of phosphate acceptor. The mechanism of the reduction has since been extensively investigated, either by direct spectrophorometric[2–8] or fluorometric[4,5] measurement of reduced nicotinamide nucleotide in the mitochondrial suspension, or by specific spectrophotometric[6–9]

or fluorometric[9–12] determinations of the nicotinamide nucleotides present in deproteinized extracts. These studies have led to the conclusion that the reduction of NAD^+ is due to an energy-linked reversal of the respiratory chain, as originally suggested by CHANCE AND HOLLUNGER[2].

Another method of studying the succinate-induced reduction of mitochondrial NAD^+ is to couple the reaction with an oxido–reduction utilizing NADH. This approach has been adopted by KREBS *et al.*[13–16], ERNSTER[17–19] and KLINGENBERG[20,21], using the reduction of acetoacetate to β-hydroxybutyrate as a measure of the NADH formed. In this laboratory[22] and in others[20,21,23] the reduction of NAD^+ by succinate in rat-liver mitochondria has been coupled with the synthesis of glutamate from α-oxoglutarate + NH_3. As shown in the preceding paper[24], the amount of glutamate that is synthesized when rat-liver mitochondria are incubated with succinate, α-oxoglutarate, NH_3 and arsenite in the absence of phosphate acceptor is a quantitative measure of the rate of reduction of the NAD^+ by succinate. Any NAD^+ reduced by malate, which is derived from succinate and is the only other donor of reducing equivalents under these conditions, is recovered as aspartate. The finding[24] that the synthesis of glutamate coupled with the aerobic oxidation of succinate is inhibited by Amytal, dinitrophenol and antimycin suggested that it is due to an energy-linked reversal of the respiratory chain. This is examined further in the present paper. It is shown that high-energy intermediates formed during the aerobic oxidation of succinate are utilized for glutamate synthesis and that this leads to a relief of respiratory control imposed by the absence of phosphate acceptor. When the aerobic oxidation of succinate is blocked by antimycin, the high-energy intermediates can be formed from ATP. A preliminary report of part of this investigation has appeared[25].

METHODS AND MATERIALS

The methods, materials and experimental procedure used are described in the preceding paper[24]. The standard reaction mixture in the experiments carried out in Warburg flasks contained 15 mM KCl, 2 mM EDTA, 5 mM $MgCl_2$, 50 mM Tris–HCl buffer, 0.1 mM ADP and 25 mM sucrose (derived from the mitochondrial suspension) in a final volume of 1.0 ml. Other additions are indicated in the legends to the tables. The final pH of the reaction mixture was 7.5. The reaction temperature was 25°.

Hexylguanidine sulphate was kindly donated by Dr. J. B. CHAPPELL.

In some experiments oxygen uptake was measured polarographically with a Gilson Medical Electronics Oxygraph. The standard reaction mixture contained the same basic components as in the manometric experiments, except that the sucrose concentration was 12.5 mM, in a final volume of 2.0 ml. Other additions to the reaction mixture are shown in the legends to the figures. The pH was 7.5 and the reaction temperature was 25°.

RESULTS

Stimulation of succinate oxidation by α-oxoglutarate + NH_3

In the presence of oligomycin succinate is oxidized relatively slowly by rat-

liver mitochondria. Fig. 1 shows that the addition of α-oxoglutarate + NH_3 to mitochondria in the presence of succinate, arsenite and oligomycin resulted in a marked stimulation (2.5-fold) of oxygen uptake. This "uncoupling" of succinate oxidation by α-oxoglutarate + NH_3 took place only in the presence of inorganic

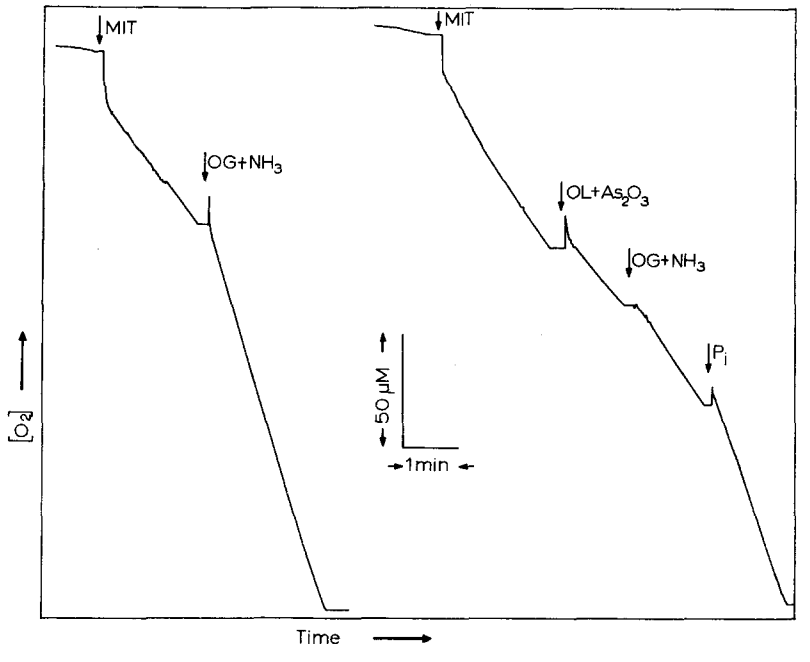

Fig. 1. Effect of α-oxoglutarate + NH_3 on succinate oxidation. Tracings of Gilson Medical Electronics Oxygraph records. Mitochondria (4.6 mg protein) were added to a solution containing, in addition to the basic components, 10 mM glutamate, 20 mM glucose, 150 units hexokinase, 30 mM succinate and (left-hand trace only) 1 mM arsenite, 10 µg oligomycin and 5 mM P_i. Additions made as indicated by the arrows. Abbreviations: MIT, mitochondria; OG + NH_3, 18 µmoles α-oxoglutarate + 18 µmoles NH_4Cl; OL + As_2O_3, 10 µg oligomycin + 2 µmoles arsenite; P_i, 10 µmoles P_i.

phosphate (Fig. 1; *cf.* Table XI of ref. 24). Neither α-oxoglutarate nor NH_3, added separately, had any effect on succinate oxidation in these experiments (Fig. 2).

Effect of ATP and oligomycin on the aerobic synthesis of glutamate

The synthesis of glutamate coupled with the aerobic oxidation of succinate was usually considerably stimulated by ATP (Table I) and was always stimulated by oligomycin (Table II; *cf.* Table V of ref. 24). A stimulation of glutamate synthesis was always accompanied by an increase in oxygen uptake and in aspartate synthesis. Glutamate synthesis was stimulated to a greater extent by oligomycin than by ATP (Table II). The stimulation was the same with 21 µg oligomycin/mg as with 0.2 µg/mg protein, which is sufficient for maximal inhibition of the O_2 uptake in the presence of phosphate acceptor (Table III).

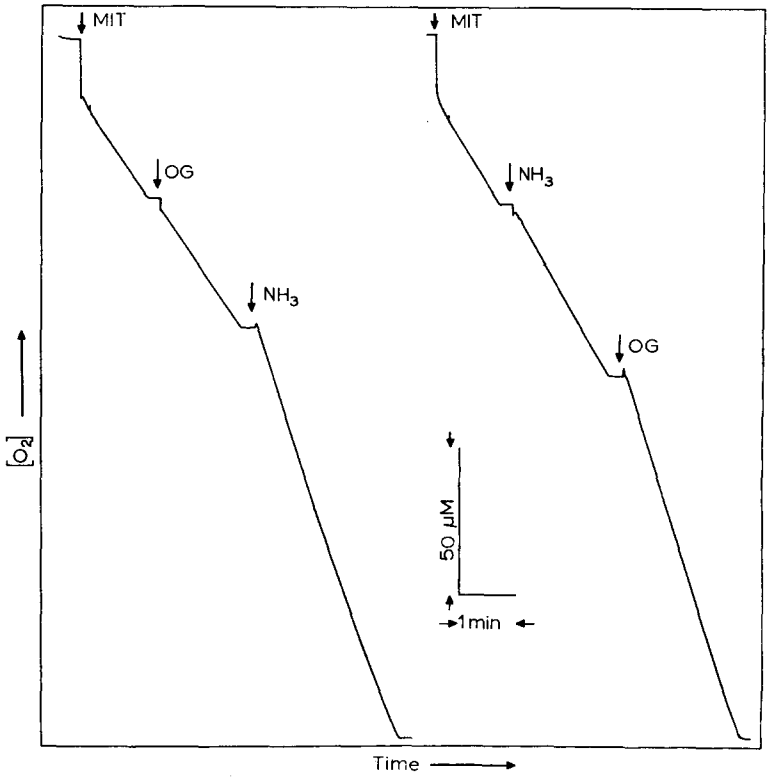

Fig. 2. Effect of α-oxoglutarate and NH_3 on succinate oxidation. Tracings of Gilson Medical Electronics Oxygraph records. Mitochondria (4.6 mg protein) were added to a solution containing, in addition to the basic components, 5 mM glutamate, 1 mM arsenite, 30 mM succinate, 5 mM potassium phosphate buffer, 20 mM glucose, 150 units hexokinase, and 10 µg oligomycin. Additions made as indicated by the arrows. Abbreviations: MIT, mitochondria; OG, 18 µmoles α-oxoglutarate; NH_3, 18 µmoles NH_4Cl.

TABLE I

EFFECT OF ATP ON THE SYNTHESIS OF GLUTAMATE COUPLED WITH THE AEROBIC OXIDATION OF SUCCINATE

Reaction mixture contained, in addition to the basic components, 20 mM α-oxoglutarate, 20 mM NH_4Cl, 1 mM arsenite, 60 mM succinate, 2% ethanol and 1.3 mM (Expt. 190), 2 mM (Expts. 187, 188, 192 and 196) or 20 mM (Expts. 119 and 120) potassium phosphate buffer. In Expt. 187, 10 mM glutamate was also present. ATP was added where indicated at a concentration of 10 mM. Reaction time, 20 min (Expts. 187 and 188) or 30 min (Expts. 119, 120, 190, 192 and 196).

Expt.	*Mitochondrial protein (mg)*	*Δ O. (µatoms)*		*Δ Glutamate (µmoles)*		*Δ Aspartate (µmoles)*	
		−ATP	*+ATP*	*−ATP*	*+ATP*	*−ATP*	*+ATP*
119	3.3	6.75	10.4	2.74	5.50	0.71	2.99
120	4.2	6.4	8.7	1.37	4.15	0.65	2.86
187	7.7	9.2	9.0	4.55	4.26	4.20	5.27
188	6.5	10.3	10.4	4.46	4.66	2.15	2.84
190	6.7	10.3	11.3	5.16	7.06	2.74	4.64
192	5.1	7.2	8.7	2.26	4.26	0.65	2.15
196	7.1	10.4	12.4	3.86	6.06	1.58	6.22

TABLE II

EFFECT OF ATP AND OLIGOMYCIN ON GLUTAMATE SYNTHESIS COUPLED WITH THE OXIDATION OF SUCCINATE IN THE PRESENCE AND ABSENCE OF ANTIMYCIN

Reaction mixture contained, in addition to the basic components, 20 mM α-oxoglutarate, 20 mM NH_4Cl, 60 mM succinate, 1 mM arsenite, 2 mM potassium phosphate buffer, 2% ethanol and 5.1 mg (Expt. 192) or 8.3 mg (Expt. 189) mitochondrial protein. Reaction time, 30 min. Where indicated, 0.5 μg (Expt. 192) or 1.0 μg (Expt. 189) antimycin was added.

Additions	*Expt. 192*			*Expt. 189*		
	Δ O (μatoms)	*Δ Glutamate (μmoles)*	*Δ Aspartate (μmoles)*	*Δ O (μatoms)*	*Δ Glutamate (μmoles)*	*Δ Aspartate (μmoles)*
None	7.2	2.26	0.65	13.8	7.05	4.57
ATP (10 mM)	8.7	4.26	2.15	—	—	—
Oligomycin (10 μg)	8.6	5.58	3.16	—	—	—
Oligomycin + ATP	8.4	6.24	3.17	—	—	—
Antimycin	0.7	0.66	0.08	1.2	0.98	0.14
Antimycin + ATP	0.7	1.64	0.52	1.3	2.33	0.54
Antimycin + oligomycin	0.7	0.46	0.05	1.2	0.74	0.03
Antimycin + ATP + oligomycin	0.6	0.54	0.06	0.9	1.22	0.07
Antimycin + ATP + 2,4-dinitrophenol (50 μM)	—	—	—	0.9	0.85	0.11

Effect of ATP on glutamate synthesis in the presence of antimycin

When the aerobic oxidation of succinate was blocked by antimycin, glutamate synthesis was almost completely inhibited (Table II; *cf.* Tables VI and VII of ref. 24). The addition of ATP restored glutamate synthesis under these conditions, but not if oligomycin was present as well (Table II). Dinitrophenol also blocked the ATP-induced synthesis of glutamate (Table II, Expt. 189). The ATP-induced synthesis of glutamate that occurred in the presence of sufficient antimycin to block the aerobic

TABLE III

EFFECT OF OLIGOMYCIN CONCENTRATION ON THE OXIDATION OF SUCCINATE AND SYNTHESIS OF GLUTAMATE IN THE PRESENCE AND ABSENCE OF α-OXOGLUTARATE AND AMMONIA

Reaction mixture contained, in addition to the basic components, 60 mM succinate, 1 mM arsenite, 2% ethanol, 20 mM potassium phosphate buffer, 20 mM glucose, 150 units hexokinase and 4.8 mg mitochondrial protein. Reaction time, 20 min. (Expt. 103).

Oligomycin (μg/mg protein)	*Δ O (μatoms)*	*Δ Glutamate (μmoles)*
α-Oxoglutarate and NH_3 absent		
0	10.1	0
0.2	3.9	—
2.1	4.6	—
21.0	5.2	—
α-Oxoglutarate and NH_3 present		
0	9.6	2.36
0.2	6.8	4.84
2.1	7.0	4.33
21.0	6.5	4.30

TABLE IV

FACTORS AFFECTING THE STIMULATION BY ATP OF GLUTAMATE SYNTHESIS COUPLED TO THE OXIDATION OF SUCCINATE IN THE PRESENCE OF ANTIMYCIN

Reaction mixture contained, in addition to the basic components, 20 mM α-oxoglutarate, 20 mM NH_4Cl, 60 mM succinate, 1 mM arsenite, 2% ethanol and 8.3 mg (Expt. 189), 6.7 mg (Expt. 190) or 5.7 mg (Expt. 191) mitochondrial protein. Reaction time, 30 min. Values given are per vessel.

Expt.	*Antimycin (μg/mg protein)*	*Inorganic phosphate (mM)*	*ATP (mM)*	*Δ O (μatoms)*	*Δ Glutamate (μmoles)*	*Δ Aspartate (μmoles)*
190	0	1.3	0	10.3	5.16	2.74
	0.07	1.3	0	0.9	0.49	0.06
	0.15	1.3	0	0.9	0.45	0.09
	0.75	1.3	0	0.7	0.54	0.07
	0.07	1.3	10	0.8	2.60	0.57
	0.15	1.3	10	0.9	2.31	0.38
	0.75	1.3	10	0.9	1.35	0.18
191	0	2	0	9.4	6.50	4.03
	0.09	2	0	0.7	0.63	0.05
	0.09	20	0	0.7	0.25	0
	0.09	2	10	0.9	2.12	0.51
	0.09	20	10	0.7	1.41	0.19
189	0	2	0	13.8	7.05	4.57
	0.12	2	0	1.2	0.98	0.14
	0.12	2	10	1.3	2.33	0.54
	0.12	2	18	1.0	2.68	0.72

oxidation of succinate maximally was diminished if the antimycin concentration was increased still further (Table IV, Expt. 190). Maximal glutamate synthesis in the presence of antimycin occurred when the ATP concentration was high and the inorganic phosphate concentration low (Table IV).

DISCUSSION

The following lines of evidence show that the synthesis of glutamate from α-oxoglutarate, NH_3 and succinate in the presence of arsenite and oxygen in rat-liver mitochondria represents an energy-linked reversal of the respiratory chian. (a) The only sources* of reducing equivalents in these experiments are succinate and malate which give rise to glutamate and aspartate respectively[24]. The transfer of reducing equivalents from succinate to α-oxoglutarate + NH_3, resulting in the synthesis of glutamate, is an endergonic reaction. (b) Little glutamate synthesis occurs when succinate oxidation is blocked by malonate[24]. (c) When the aerobic oxidation of succinate is blocked by antimycin, little glutamate synthesis occurs unless a source of energy is supplied, either ATP (Tables II and III) or the aerobic oxidation of tetramethyl-*p*-phenylenediamine[26,27]. (d) Amytal, a known inhibitor of reversed electron transfer from succinate to nicotinamide nucleotide[2], inhibits glutamate

* Endogenous substrate and α-oxoglutarate make a negligible contribution to glutamate synthesis under our experimental conditions. In the absence of succinate, there is little or no glutamate synthesis (*cf.* Table IV of ref. 24). In the presence of succinate, the α-oxoglutarate that disappears is almost completely recovered as glutamate. In a typical experiment, 6.0 μmoles of α-oxoglutarate disappeared and 5.7 μmoles of glutamate were found.

synthesis[24]. (e) The glutamate synthesis is inhibited by dinitrophenol[24]. (f) The synthesis of glutamate brings about an "uncoupling" of succinate oxidation (Fig. 1).

Oligomycin, which prevents the formation of ATP from a dinitrophenol-sensitive high-energy intermediate of oxidative phosphorylation[28,29], enhances the synthesis of glutamate. This and the inhibition of glutamate synthesis by dinitrophenol indicate that the dinitrophenol-sensitive intermediate of oxidative phosphorylation, or another intermediate in equilibrium with it, is required for the transfer of reducing equivalents from succinate to α-oxoglutarate + NH_3. Antimycin, by blocking electron transfer to oxygen, prevents the generation of the high-energy intermediate and thus prevents the synthesis of glutamate (*cf.* refs. 4, 5, 17).

The high-energy intermediate, instead of being used to form ATP from ADP and inorganic phosphate, as in actively respiring mitochondria, or accumulating in the absence of ADP or in the presence of oligomycin, is utilized in the reduction of NAD^+. Thus, when the mitochondrial oxidation of succinate is inhibited by the absence of phosphate acceptor or by the presence of oligomycin, the addition of α-oxoglutarate + NH_3 provides an alternative pathway for utilization of the high-energy intermediate and leads to a stimulation of respiration, in the same way as "uncoupling" by dinitrophenol, which is also thought to be due to splitting of a high-energy intermediate[30], stimulates the oxidation in the absence of ADP or in the presence of oligomycin. ERNSTER[19] found that acetoacetate has a similar stimulating effect on succinate oxidation, and also concluded that this is due to a utilization of high-energy intermediates for NAD^+ reduction (*cf.* ref. 21).

The "uncoupling" by α-oxoglutarate + NH_3 only takes place if glutamate is being synthesized. For instance maximal synthesis of glutamate is dependent on the presence of inorganic phosphate[24]. Similarly, the stimulation of succinate oxidation by α-oxoglutarate + NH_3 is dependent on the presence of inorganic phosphate (Fig. 1). Further, when the synthesis of glutamate coupled with the aerobic oxidation of succinate is inhibited by Amytal, oxygen uptake is lowered as well (see Table VIII of ref. 24; *cf.* ref. 19), although Amytal has no effect on succinate oxidation in the absence of α-oxoglutarate + NH_3. Hexylguanidine sulphate, whose mode of action appears to be similar to that of Amytal[31], also brings about an inhibition of glutamate synthesis and a concomitant diminution of the oxygen uptake*. The "uncoupling" by α-oxoglutarate + NH_3 differs in one respect from that brought about by a compound such as dinitrophenol. Although both inhibit the synthesis of ATP, energy is utilized for the synthesis of glutamate from α-oxoglutarate and NH_3, but it is dissipated when dinitrophenol is added.

The availability of oligomycin[28] has enabled a test of the suggestion made in 1953 (see ref. 30) that the energy of intermediates of oxidative phosphorylation could be directly utilized without first being converted to ATP[29]. This has now been demonstrated for the following energy-requiring processes: the reduction of mitochondrial[10,11] or added NAD^+ (see refs. 32–34) by succinate, the reduction of acetoacetate by succinate[19], the reduction of α-oxoglutarate (+NH_3) by succinate[22,25] and by malate[35], the reduction of NAD^+ (ref. 27) and ubiquinone[36] by tetramethyl-*p*-phenylenediamine, the uptake of K^+ and extrusion of Na^+ by rat-liver slices[37] and uptake of phosphate and Mn^{2+} (see ref. 38) or Mg^{2+} (see ref. 39) in mitochondria.

* Unpublished experiments carried out in collaboration with Dr. R. GUILLORY.

The reduction of α-oxoglutarate (+NH_3) by succinate is markedly stimulated by oligomycin, even in the absence of ADP, and, in most experiments, by ATP. ADP, on the other hand, has very little effect[24]. ATP has very little effect in the presence of oligomycin. In contrast, ERNSTER[23] has reported that the succinate-induced synthesis of glutamate (as measured by α-oxoglutarate disappearance) is markedly dependent on the presence of both ATP and oligomycin. The reason for this discrepancy is unknown. It may be due to the fact that ERNSTER used aged mitochondria. The effect of oligomycin has also been studied by ERNSTER[19] in the acetoacetate system. Here oligomycin has only a slight stimulating effect in the absence of phosphate acceptor. In earlier publications[17,19], ERNSTER reported that low concentrations of oligomycin stimulated acetoacetate reduction in the presence of phosphate acceptor, but that the extent of reduction decreased very markedly with increasing concentrations of oligomycin. However, in a subsequent report[40], the decreased reduction at higher concentrations is not shown. Our experience with the α-oxoglutarate system agrees with this latter report. Oligomycin in amounts up to 21 μg/mg protein had no inhibitory effect (Table IV; *cf.* ref. 25).

The mechanism of the reduction of α-oxoglutarate (+NH_3) by succinate will be discussed in greater detail in another paper in this series[41]. The present discussion will be restricted to a consideration of the effects of ATP, ADP and oligomycin. According to the mechanism which we shall propose, an intermediate of oxidative phosphorylation (A ~ I) can either react with ADP and P_i to form ATP in an oligomycin-sensitive reaction, or can promote the reduction of NAD^+ by succinate, as shown in Fig. 3.

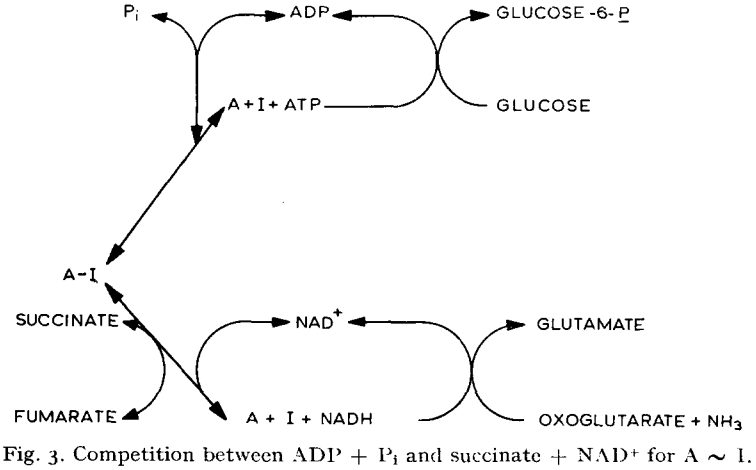

Fig. 3. Competition between ADP + P_i and succinate + NAD^+ for A ~ I.

SNOSWELL[11] showed that glucose + hexokinase completely inhibited the reduction of NAD^+ by succinate in rabbit-heart sarcosomes as could be expected according to this scheme. In contrast, glucose + hexokinase had no effect on the synthesis of glutamate[24]. A possible explanation for this difference may lie in the fact that, in the absence of phosphate acceptor, the two reactions of A ~ I in SNOSWELL's system are in equilibrium. The addition of glucose and hexokinase

makes the phosphorylation reaction irreversible, and consequently leads to the oxidation of all the NADH. In our experiments, however, the NADH is rapidly oxidized by α-oxoglutarate and NH_3 in an essentially irreversible reaction. When hexokinase and glucose are added, both reactions of A ~ I become irreversible, so that there is a simple competition between ADP + P_i and succinate + NAD^+ for A ~ I. A later paper of this series[41] will show that, in fact, the pathway leading to synthesis of ATP is the more active (the addition of α-oxoglutarate + NH_3 causes a decline of the P:O ratio of 17–33%), so that one might expect that the addition of phosphate acceptor would lead to a decrease in glutamate synthesis. However, it has been known for some time[42–44] that oxidative phosphorylation has a very high affinity for ADP, so that the equilibrium of the reaction

$$A \sim I + ADP + P_i \rightleftharpoons A + I + ATP \qquad (1)$$

probably lies very far to the right. Under these conditions, it is quite conceivable that the removal of ATP from the right-hand side of this equation would not lead to any appreciable decline in the concentration of A ~ I, so that glutamate synthesis would not be inhibited.

The effects of oligomycin and ATP are consistent with this explanation. Oligomycin by completely inhibiting Reaction 1 would be expected to lead to an increased concentration of A ~ I, thereby promoting the synthesis of glutamate. A high concentration of ATP would be expected to have the same effect, although if the equilibrium of Reaction 1 is far to the right, ATP would be expected to be less effective than oligomycin. That ATP has no effect in the presence of oligomycin is also to be expected.

According to ERNSTER's[19] data, the reduction of acetoacetate by succinate behaves quite differently. It is inhibited by ADP, is only slightly stimulated by oligomycin and is not affected by the ratio [ATP]/[ADP]·[P_i] (see ref. 45). It seems possible that the difference between acetoacetate and α-oxoglutarate (+NH_3) lies in the lower potential of the acetoacetate ⇌ β-hydroxybutyrate (E_0' at pH 7.0 = —266 mV)[55] compared with glutamate ⇌ α-oxoglutarate in the presence of 20 mM NH_3 (E_0' at pH 7.0 = −190 mV).

Our interpretation of the effect of ATP is a special case of the influence of what KLINGENBERG AND SCHOLLMEYER[46–48] call the phosphate potential, $\frac{[P_i]\,[ADP]}{[ATP]}$, which they consider plays an important role in determining redox equilibria in mitochondria. However, we believe that the phosphate potential is only of importance in influencing the redox state of the respiratory carriers when electron transfer to oxygen is inhibited, either by agents such as antimycin or KCN (*cf.* ref. 49), or by the absence of ADP. In our experiments it probably plays a decisive role in reversed electron transfer only in the presence of antimycin, when there is a response to changes in the concentration of at least two components of the adenine nucleotide system, ATP and inorganic phosphate. Oligomycin inhibits, as is to be expected, since oligomycin inhibits the formation of A ~ I from ATP.

In the fourth paper of this series[27], it is shown that high-energy intermediates generated during the oxidation of tetramethyl-*p*-phenylenediamine, which reacts with the respiratory chain at about the level of cytochrome *c* (see ref. 50), can also be used for succinate-induced glutamate synthesis in the presence of antimycin.

These results indicate that the antimycin-sensitive site is not obligatorily involved in the reduction of NAD^+ by succinate, in agreement with the conclusions of LÖW *et al.*[32], ERNSTER[19], KLINGENBERG AND HAEFEN[20] (see also ref. 47) and PACKER AND DENTON[51]. ERNSTER[19] based his conclusion on the fact that the reduction of acetoacetate by succinate in the presence of cyanide and ferricyanide was insensitive to antimycin. In contrast to these reports CHANCE AND HOLLUNGER[1-4] (*cf.* also refs. 33, 52, 53) have been unable to demonstrate the succinate-induced reduction of NAD^+ in the presence of antimycin and with ATP as an energy source, and have therefore concluded that the antimycin-sensitive site must be on the pathway of electron transfer from succinate to NAD^+. These negative results may be due to secondary effects of antimycin, such as a stimulation of ATPase activity[54]. Indeed, in our system, and in that used by LÖW AND VALLIN[34], increasing concentrations of antimycin inhibit the ATP-dependent reduction of NAD^+.

The results presented in this paper, which show that ATP can serve as an energy source for reversed electron transfer in the α-oxoglutarate–NH_3 system, are in agreement with those obtained by KLINGENBERG[21], who has demonstrated that ATP can be used in both the α-oxoglutarate ($+NH_3$) and in the acetoacetate systems (contrast refs. 17, 19).

The α-oxoglutarate–NH_3 system has the following advantages over the acetoacetate system for studying the succinate-linked reduction of NAD^+:

1. A clear distinction can be made between succinate and malate as donors of reducing equivalents for NAD^+ reduction, since in the presence of arsenite, hydrogens derived from succinate are recovered as glutamate and those from malate as aspartate. In the acetoacetate system it is not possible to make this distinction directly, since the only product of the reduction is β-hydroxybutyrate, regardless of the source of reducing equivalents. However, KLINGENBERG AND HAEFEN[20,21] by measuring oxygen uptake, succinate and acetoacetate disappearance and malate formation, have shown unequivocally that succinate provides reducing equivalents for acetoacetate reduction in liver mitochondria (contrast refs. 13–16).

2. Glutamate synthesis coupled with the aerobic oxidation of succinate takes place even in the presence of phosphate acceptor. As described in the last paper of this series[41], this can be made use of to determine the stoicheiometry of energy utilization for reversed electron transfer. In the acetoacetate system, the addition of phosphate acceptor results in a complete inhibition of acetoacetate reduction[19].

3. The synthesis of glutamate is considerably more favourable thermodynamically than the synthesis of β-hydroxybutyrate.

ACKNOWLEDGEMENTS

The authors wish to thank Miss B. KELDER for her technical assistance.

This study was supported by a grant from The Life Insurance Medical Research Fund.

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