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

V. ENERGETICS AND MECHANISM

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SUMMARY

1. The value of n in the equation: succinate + α-oxoglutarate + H^+ + NH_3 + $n \sim \rightleftharpoons$ fumarate + glutamate, has been determined by measuring the ratio Δglutamate:$-\Delta\sim$, where Δglutamate is the amount of glutamate synthesized and $-\Delta\sim$ is the decrease in the amount of hexose monophosphate found when α-oxoglutarate (+ NH_3) is added to mitochondria oxidizing succinate in the presence of ADP, P_i hexokinase, glucose and arsenite.

2. The mean values for this ratio were 0.84 and 0.82 when the energy was provided by the aerobic oxidation of succinate and tetramethyl-*p*-phenylenediamine, respectively. Low concentrations of dinitrophenol lowered both Δglutamate and $-\Delta\sim$, without affecting the ratio.

3. Energy is also required for the NAD-catalysed amination to aspartate of the malate. Mean values of 3.8 and 1.7 were obtained for the ratio Δ(amino acid):$-\Delta\sim$ when the energy was provided by the aerobic oxidation of malate and tetramethyl-*p*-phenylenediamine, respectively.

4. When allowance is made for the $\sim$ required for the synthesis of aspartate, the value of n in the above equation becomes equal to 1.

5. When the aerobic oxidation of succinate is inhibited by low concentrations of antimycin, ATP can provide the energy for the reduction of α-oxoglutarate (+ NH_3) by succinate. Ratios of 0.25–0.68 were obtained for glutamate:ΔP_i, but side-reactions caused loss of ATP.

6. Unequivocal evidence that both energy-conserving steps in succinate oxidation are concerned in the reduction of NAD^+ by succinate could not be obtained, but glutamate:O ratios of 1.0 uncorrected for side-reactions make this likely.

7. A mechanism for the reduction of NAD^+ by succinate utilizing high-energy intermediates formed by the aerobic oxidation of succinate or tetramethyl-*p*-phenylenediamine or from ATP is proposed. This mechanism involves a high-energy compound of NAD^+.

8. Other theories which have been proposed to explain this reaction are discussed.

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

INTRODUCTION

In previous papers of this series[1-3] it has been shown that, in the presence of a source of energy, α-oxoglutarate (+ NH_3) is reduced to glutamate by the reaction sequence

$$\text{succinate} + NAD^+ + n \sim \rightleftharpoons \text{fumarate} + NADH + H^+ \quad (1)$$
$$NADH + \alpha\text{-oxoglutarate} + NH_3 + H^+ \rightleftharpoons NAD^+ + \text{glutamate} \quad (2)$$
$$\text{succinate} + \alpha\text{-oxoglutarate} + NH_3 + n \sim \rightleftharpoons \text{fumarate} + \text{glutamate} \quad (3)$$

The energy (~) used in Reaction 1 can be provided by the aerobic oxidation of succinate, by the aerobic oxidation of tetramethyl-*p*-phenylenediamine or by ATP. It is the purpose of the present paper to discuss the energetics of this reaction, *i.e.* the value of *n* in Reaction 1, and its mechanism.

It was found[4] that, under the conditions of these experiments, energy was also necessary for the NAD^+-catalysed amination of malate to aspartate (Reaction 6), which is made up of Reactions 4, 5 and 2.

$$\text{malate} + NAD^+ \rightleftharpoons \text{oxaloacetate} + NADH + H^+ \quad (4)$$
$$\text{oxaloacetate} + \text{glutamate} \rightleftharpoons \text{aspartate} + \alpha\text{-oxoglutarate} \quad (5)$$
$$NADH + \alpha\text{-oxoglutarate} + NH_3 + H^+ \rightleftharpoons NAD^+ + \text{glutamate} \quad (2)$$
$$\text{malate} + NH_3 \rightleftharpoons \text{aspartate} \quad (6)$$

The energetics of this reaction have also been studied.

METHODS

The methods, materials and experimental procedure used are described in the first paper of this series[1]. The standard reaction mixture used 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°.

Oxaloacetate was determined by the procedure described by TAGER[4].

Esterified P was determined by the method of SLATER[5]. Because of the low concentration of ADP used the amount of hexose monophosphate in the "myokinase control" was negligible and this control was usually omitted. Allowance was made for the oxaloacetate and pyruvate also present in the deproteinized reaction mixture[4], or when (oxaloacetate + pyruvate) was not separately determined, they were allowed to react with excess NADH in the presence of malate dehydrogenase (EC 1.1.1.37) and lactate dehydrogenase (EC 1.1.1.27) before addition of the rabbit-muscle fractions necessary to catalyse the oxidation of the NADH by hexose monophosphate + ~P (see ref. 5).

RATIONALE

According to the mechanism of oxidative phosphorylation which has served us as a working hypothesis for the last 10 years[6], high-energy compounds (~) are formed during the operation of the respiratory chain and are decomposed by P_i

and ADP in a reaction leading to synthesis of ATP. In the absence of P_i or ADP, respiration is limited by the rate of spontaneous decomposition of the high-energy compounds. This decomposition can be accelerated, with a corresponding stimulation of the respiration, by the addition of dinitrophenol. In a previous paper of this series[2], it has been shown that, when the reaction with ADP and P_i is inhibited by oligomycin, respiration can also be stimulated by the addition of α-oxoglutarate (+ NH_3), which, by oxidizing the NADH, promotes the energy-requiring reduction of NAD^+ by succinate. Thus, the $\sim$ compound(s) is able to be utilized for this energy-requiring reaction, even in the presence of oligomycin. When oligomycin is absent, and ADP, P_i, α-oxoglutarate and NH_3 are all present, there will be three competing reactions for the $\sim$ compound, as shown in Fig. 1. Pathway i is stimulated

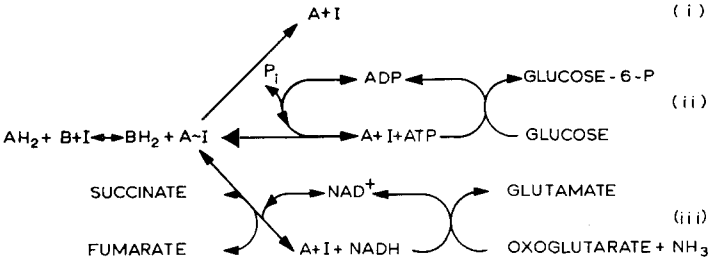


Fig. 1. Competing reactions of $\sim$ compound formed by oxidative phosphorylation. AH_2 and B represent two carriers (not necessarily adjacent, *cf.* SLATER AND COLPA-BOONSTRA[7]) of the respiratory chain, and I (*cf.* CHANCE AND WILLIAMS[8]) replaces C of the original formulation[6].

by dinitrophenol; Pathway ii requires P_i and ADP (and glucose and hexokinase (EC 2.7.1.12) to regenerate the ADP) and is inhibited by oligomycin; Pathway iii requires α-oxoglutarate, NH_3 and succinate, and is inhibited by Amytal or hexylguanidine[2].

The stimulation of the rate of respiration caused by α-oxoglutarate (+ NH_3) and that caused by ADP + P_i are not additive presumably because the decomposition by Pathway ii is sufficiently rapid for the oxidation reaction to become rate-limiting. Thus, as will be shown below, the addition of α-oxoglutarate + NH_3 to a system containing ADP and P_i does not stimulate the rate of oxidation, but by introducing Pathway iii the amount of A $\sim$ I available for the synthesis of ATP is decreased. The decrease in ATP synthesis (measured by the amount of Glc-6-*P* formed) brought about by the addition of α-oxoglutarate + NH_3 gives, then, the amount of A $\sim$ I used for the reduction of NAD^+ leading to the synthesis of glutamate. The yield of glutamate can be expressed by the ratio Δglutamate: $-\Delta\sim$, where $\Delta\sim$ = (Glc-6-*P* in presence of α-oxoglutarate + NH_3) minus (Glc-6-*P* in absence of α-oxoglutarate + NH_3).

According to this argument, addition of dinitrophenol by accelerating Pathway i should decrease the yield of both Glc-6-*P* and glutamate, but should not affect the competition between Pathways ii and iii. The ratio Δglutamate:$-\Delta\sim$ should, then, be the same in the absence and in the presence of dinitrophenol.

RESULTS

Energy requirement for reduction of α-oxoglutarate (+ NH_3) by succinate linked to the aerobic oxidation of succinate

The effects of adding α-oxoglutarate and NH_3 on the amount of esterified P formed during the oxidation of succinate are shown in Table I. In Expts. 103 and 107, the addition of α-oxoglutarate + NH_3 had little or no effect on the O_2 uptake. In Expts. 171 and 173, however, the addition of either NH_3 or α-oxoglutarate

TABLE I

MEASUREMENTS OF OXYGEN UPTAKE, PHOSPHORYLATION AND GLUTAMATE SYNTHESIS ASSOCIATED WITH THE AEROBIC OXIDATION OF SUCCINATE

The reaction mixture contained, in addition to the basic components, 60 mM succinate, 1 mM arsenite, 20 mM potassium phosphate buffer, 20 mM glucose, 150 units[9] hexokinase, 2% ethanol (except in Expts. 143 and 171), and 4.8–6.0 mg mitochondrial protein. The reaction time was 20 min except for Expt. 143 (22 min), Expt. 195 (30 min) and Expt. 210 (10 min).

Expt.	*α-Oxoglutarate (mM)*	*NH_4Cl (mM)*	*Dinitrophenol (μM)*	*Δ O (μatoms)*	*Δ Esterified P (μmoles)*	*P:O*	*Δ Glutamate (μmoles)*	*Δ Aspartate (μmoles)*	*Δ Oxaloacetate (μmole)*
103	0	0	0	10.1	14.4	1.43	0	0.04	—
	20	20	0	9.6	10.6	1.11	2.36	2.20	—
107	0	0	0	9.3	11.0	1.19	0.02	0.02	—
	20	20	0	9.3	7.5	0.80	2.65	1.84	—
143	0	20	0	12.0	13.4	1.12	0	—	—
	20	20	0	10.9	8.9	0.82	2.49	—	—
	0	20	13	13.9	8.8	0.63	0	—	—
	20	20	13	11.9	6.5	0.55	0.88	—	—
171	0	0	0	14.0	16.7	1.19	0	0	—
	0	20	0	9.5	11.5	1.21	0.05	0.31	—
	20	0	0	9.8	11.9	1.21	0.24	0	—
	20	20	0	9.7	9.4	0.97	2.71	2.03	—
173	0	0	0	13.0	16.8	1.29	0	0.07	—
	0	20	0	9.0	12.2	1.36	0	0.28	—
	20	0	0	8.8	11.8	1.34	0.19	0.08	—
	20	20	0	8.7	8.7	1.00	2.47	1.89	—
195	0	20	0	12.1	14.1	1.08	0.07	0	0.02
	20	20	0	13.1	10.7	0.82	3.60	3.49	0.32
	0	20	6	13.1	12.3	0.94	0.30	0	0.02
	20	20	6	13.4	9.7	0.73	3.04	2.91	0.31
210	0	20	0	5.1	7.0	1.37	0	0.08	0.07
	20	20	0	4.8	5.4	1.13	0.89	0.61	0.10

inhibited the respiration appreciably, without affecting the P:O ratio. The addition of α-oxoglutarate + NH_3 had no greater effect than that of α-oxoglutarate or NH_3 alone. In these experiments, therefore, and also in Expts. 143, 195 and 210, the control for the calculation of $\Delta\sim$ was the measurement in the presence of NH_3 but absence of α-oxoglutarate. The very small difference in the ΔO between this control and the flask in which glutamate was synthesized was allowed for by calculating an "expected" esterified P, which was equal to the ΔO obtained in the presence of α-oxoglutarate + NH_3 multiplied by the P:O ratio in the control.

$-\Delta\sim$ then equalled this "expected" esterified P *minus* the actual amount of esterified P found in the flask containing both α-oxoglutarate and NH_3. The calculations of the ratio Δglutamate:$-\Delta\sim$ are shown in Table II.

The ratio varied between 0.73 and 1.11 in 9 measurements, with a mean of 0.84 $\pm$ 0.04 (standard error). There was no obvious correlation with the P:O ratio of the control which varied between 1.08 and 1.43*. As predicted, the addition of

TABLE II

RELATION BETWEEN SYNTHESIS OF GLUTAMATE COUPLED TO THE AEROBIC OXIDATION OF SUCCINATE AND CONCOMITANT DECREASE IN PHOSPHORYLATION

Data obtained from Table I. Δ Glutamate = glutamate synthesized in presence of α-oxoglutarate + NH_3 *minus* glutamate found (blank determination) in absence of α-oxoglutarate + NH_3 or in absence of α-oxoglutarate. Δ Esterified P expected = (Δ O in presence of α-oxoglutarate + NH_3) multiplied by P:O ratio in absence of α-oxoglutarate + NH_3 or in absence of NH_3. Δ Esterified P found = P esterified in presence of α-oxoglutarate + NH_3.

Expt.	*Dinitrophenol (μM)*	*P:O in control*	*Δ Glutamate (μmoles)*	*Δ Esterified P (μmoles)*			*Δ Glutamate: decrease in Δ esterified P*
				Expected	*Found*	*Decrease*	
103	0	1.43	2.36	13.7	10.6	3.1	0.76
107	0	1.19	2.63	11.1	7.5	3.6	0.73
143	0	1.12	2.49	12.2	8.9	3.3	0.75
	13	0.63	0.88	7.5	6.5	1.0	0.88
171	0	1.19	2.66	11.8	9.4	2.4	1.11
173	0	1.29	2.47	11.8	8.7	3.1	0.80
195	0	1.08	3.53	15.2	10.7	4.5	0.78
	6	0.94	2.74	12.6	9.7	2.9	0.95
210	0	1.37	0.89	6.59	5.43	1.16	0.77
						Mean	0.84 $\pm$ 0.04**

** Standard error of the mean.

dinitrophenol, although inhibiting the synthesis of both esterified P and glutamate, had no appreciable effect on the ratio Δglutamate:$-\Delta\sim$.

As discussed in a previous paper[1] the amount of glutamate found is a measure of the rate of reduction of NAD^+ by succinate, and the amount of aspartate found is a measure of the rate of reduction of NAD^+ by the malate formed by the oxidation of succinate, when three assumptions are made: (a) all the oxaloacetate formed in Reaction 4 by the oxidation of the malate reacts with glutamate according to Reaction 5, (b) all the NADH formed in Reaction 4 reacts with α-oxoglutarate + NH_3 according to Reaction 2, (c) the glutamate formed is not further oxidized. We shall now consider in how far these assumptions are valid under the conditions of the experiments summarized in Tables I and II.

1. Expts. 195 and 210 show that some oxaloacetate is present at the end of the experiments, amounting to rather less than 10% of the aspartate found. The

* This was considerably lower than the P:O ratio (mean 1.62) found with 60 mM succinate by GREENGARD *et al.*[10]. It is probable that the 1 mM arsenite used to prevent the oxidation of the glutamate formed was slightly uncoupling (*cf.* ref. 11).

sum reaction describing the accumulation of oxaloacetate is Reaction 7, the sum of Reactions 4 and 2

$$\text{malate} + \alpha\text{-oxoglutarate} + NH_3 \rightleftharpoons \text{oxaloacetate} + \text{glutamate} \quad (7)$$

Thus, an amount of glutamate equal to the amount of oxaloacetate found is formed not by the reduction of NAD^+ by succinate, but by the reduction of NAD^+ by malate. Allowing for this error, the last three ratios become 0.73, 0.85 and 0.74, instead of 0.77, 0.95 and 0.77, respectively. The error, although not completely negligible, is small.

2. In most of the experiments reported in the previous papers[1-4], the aerobic oxidation of NADH by the phosphorylating pathway (Reaction 8)

$$NADH + H^+ + {}^1/_2\,O_2 \rightarrow NAD^+ + H_2O \quad (8)$$

was completely inhibited by omission of ADP, or by addition of oligomycin. Under the conditions of the experiments summarized in Table I, however, ADP is present and oligomycin is absent. The possibility that Reaction 8 is operating under these conditions was investigated by studying the products formed when malate or succinate was oxidized in the presence of arsenite and glutamate. The second line of Table III shows that, with malate, the formation of aspartate and α-oxoglutarate

TABLE III

OXIDATION OF SUCCINATE AND MALATE BY RAT-LIVER MITOCHONDRIA IN PRESENCE OF GLUTAMATE AND ARSENITE

The reaction mixture contained, in addition to the basic components, 1 mM arsenite, 20 mM potassium phosphate buffer, 20 mM glucose, 150 units hexokinase, 10 mM glutamate and 4.5 mg mitochondrial protein. The reaction time was 20 min.

Further additions	*Δ O (µatoms)*	*Δ Glutamate (µmoles)*	*Δ Aspartate (µmoles)*	*Δ α-oxo-glutarate (µmoles)*	*Δ Oxalo-acetate (µmole)*
None	1.15	−1.1	0.11	0.50	0
Malate (20 mM)	6.80	−5.4	6.00	5.94	0.13
Succinate (60 mM)	9.20	−0.3	0.41	0.41	0.02
Succinate + malate	9.05	−1.1	0.58	0.46	0.02

agrees closely with the disappearance of glutamate, in accordance with Reaction 9, which is the sum of Reactions 4, 5 and 8 (*cf.* ref. 4).

$$\text{malate} + \text{glutamate} + O \rightarrow \text{aspartate} + \alpha\text{-oxoglutarate} \quad (9)$$

The oxygen uptake was slightly in excess, probably due to oxidation of some endogenous substrate. With succinate, however, only 4% of the oxygen uptake can be accounted for by oxidation of malate by Reaction 9 (see third line of Table III). Even when 20 mM malate was present from the beginning (fourth line of Table III), only 6% of the oxygen uptake was due to Reaction 9. These results are in agreement with the findings of WHITTAM *et al.*[12] and GREENGARD *et al.*[10] that, when high (about

60 mM[10]) concentrations of succinate are used, the amount of (fumarate + malate) found at the end of the reaction equalled the oxygen uptake (in μatoms). Thus, succinate almost completely inhibits the aerobic oxidation of malate, presumably because it inhibits the oxidation of NADH, either by saturating the respiratory chain with reducing equivalents (*cf.* WU AND TSOU[13] and KREBS[14]), or by a mechanism which will be discussed below. When α-oxoglutarate and NH_3 are also present, as in the experiments summarized in Tables I and II, the NADH will be oxidized exclusively by Reaction 2.

The ineffectiveness of oxygen in comparison with α-oxoglutarate (+ NH_3) as oxidant for the NADH in these experiments is also indicated by the fact that the amounts of glutamate and aspartate formed are not affected by the presence or absence of phosphate acceptor[1,2].

3. Table III (first line) shows, in agreement with BORST[15], that the oxidation of glutamate to aspartate is completely blocked by arsenite, while some oxidation of glutamate to α-oxoglutarate takes place. This is due to glutamate dehydrogenase, which can catalyse the oxidation of glutamate when the transamination pathway is blocked[15]. However, the position of the equilibrium of Reaction 2 is such that, in the presence of the high concentrations of NH_3 and α-oxoglutarate used in the experiments summarized in Tables I and II, no oxidation of glutamate is to be expected.

A further assumption which has been made in calculating the yield Δglutamate:$-\Delta\sim$ is that the $\sim$ is only required for the reduction of NAD^+ by succinate. In fact, it has already been shown by TAGER[4] (*cf.* KLINGENBERG[16]) that energy is also required for Reaction 6 which also takes place under the conditions of the experiments described in Tables I and II. The amount of energy required for this reaction will be discussed in the following section.

TABLE IV

MEASUREMENTS OF OXYGEN UPTAKE, PHOSPHORYLATION AND (GLUTAMATE + ASPARTATE) SYNTHESIS ASSOCIATED WITH THE AEROBIC OXIDATION OF MALATE

The reaction mixture contained, in addition to the basic components, 10 mM (Expt. 157) or 20 mM α-oxoglutarate, 20 mM malate, 1 mM arsenite, 20 mM potassium phosphate buffer, 5 mM (Expt. 157) or 10 mM glutamate, 20 mM glucose, 150 units hexokinase, and 8.8 mg (Expt. 157), 6.7 mg (Expt. 172) or 4.8 mg (Expt. 200) mitochondrial protein. 1% ethanol was also present in Expt. 157.

Expt.	*Reaction time (min)*	*NH_4Cl (mM)*	*Δ O (μatoms)*	*Δ Esterified P (μmoles)*	*P:O*	*Δ Glutamate (μmoles)*	*Δ Aspartate (μmoles)*	*Δ (Glutamate + aspartate) (μmoles)*	*Δ Oxaloacetate (μmole)*
157	20	0	7.0	12.2	1.74	−4.69	5.02	0.33	—
	20	20	5.5	9.1	1.64	−3.89	7.12	3.23	—
172	20	0	5.8	11.8	2.04	−4.34	4.64	0.30	—
	20	20	4.5	7.4	1.65	−2.77	6.29	3.52	—
200	8	0	2.27	4.06	1.79	−1.96	1.77	−0.19	0.21
	8	20	1.86	2.87	1.54	−1.48	2.87	1.39	0.25
	16	0	3.91	6.63	1.69	−2.87	3.29	0.42	0.40
	16	20	3.08	4.36	1.41	−1.91	4.50	2.59	0.55
	24	0	5.13	8.37	1.63	−4.29	3.94	−0.35	0.64
	24	20	3.38	5.12	1.32	−2.20	5.55	3.35	0.87

Energy requirement for aspartate synthesis from malate and NH_3, linked to the aerobic oxidation of malate

Tables IV and V summarize the results of experiments similar to those shown in Tables I and II, but in which succinate was replaced by malate. In the absence of ammonia, glutamate was converted into aspartate, as in Table III. In the presence of NH_3, Reaction 8 is partially replaced by Reaction 2, so that the sum Reaction 9 is partly replaced by Reaction 6. The rate of Reaction 6 is given by Δ(glutamate + aspartate), since the sum of the two amino acids is not changed by Reaction 9.

The values of the ratio Δ(glutamate + aspartate):$-\Delta\sim$ range between 2.1 and 6.5 (Table V). The ratio did not change during the course of the experiment (Expt. 200).

TABLE V

RELATION BETWEEN SYNTHESIS OF GLUTAMATE + ASPARTATE COUPLED TO THE AEROBIC OXIDATION OF MALATE AND CONCOMITANT DECREASE IN PHOSPHORYLATION

Data obtained from Table IV. Δ (Glutamate + aspartate) = (glutamate + aspartate) synthesized in presence of α-oxoglutarate + NH_3. Δ Esterified P expected = Δ O in presence of α-oxoglutarate + NH_3 multiplied by P:O ratio in absence of NH_3. Δ Esterified P found = P esterified in presence of α-oxoglutarate + NH_3.

Expt.	*Time (min)*	*P:O in control*	*Δ (Glutamate + aspartate) (µmoles)*	*Δ Esterified P (µmoles)*			*Δ (Glutamate + aspartate): decrease in Δ esterified P*		
				Expected	*Found*	*Decrease*			
157	20	1.74	3.23	9.6	9.1	0.5			6.5
172	20	2.04	3.52	9.1	7.4	1.7			2.1
200	8	1.79	1.39	3.35	2.87	0.48	2.9		
	16	1.69	2.59	5.23	4.36	0.87	3.0	Mean	2.9
	24	1.63	3.35	6.31	5.12	1.19	2.8		
							Mean		3.8

Expt. 200 of Table IV shows that some oxaloacetate is present in the reaction mixture at the end of the experiment. This does not, however, affect the measurement of the yield of amino acid, since the glutamate formed by Reaction 7 which has not reacted with oxaloacetate is included in the (glutamate + aspartate).

Although too much quantitative significance should not be given to the ratios in Table V, since they are based on rather small differences between the "expected" and the actual amounts of esterified P, it is clear that considerably more than one molecule of amino acid is synthesized for each molecule of $\sim$ consumed.

Energy requirement for aspartate synthesis from malate and NH_3, linked to the aerobic oxidation of TMPD

When the aerobic oxidation of malate is inhibited by Amytal, the synthesis of aspartate is restored by adding TMPD (+ ascorbate)[4]. Since this synthesis is not inhibited by oligomycin, it appears that the $\sim$ compounds formed during the aerobic oxidation of TMPD are also able to provide the energy necessary for Reaction 6. The stoicheiometry of this energy utilization is summarized in Tables VI and VII. The amount of glutamate synthesized under these conditions is presumably a measure

TABLE VI

MEASUREMENTS OF OXYGEN UPTAKE, PHOSPHORYLATION AND SYNTHESIS OF GLUTAMATE + ASPARTATE FROM MALATE, α-OXOGLUTARATE AND NH_3 IN PRESENCE OF AMYTAL, TMPD AND ASCORBATE

The reaction mixture contained, in addition to the basic components, 20 mM α-oxoglutarate, 20 mM malate, 10 mM glutamate, 1 mM arsenite, 2 mM Amytal, 0.1 mM TMPD, 20 mM ascorbate, 20 mM glucose, 150 units hexokinase, 1% ethanol, 20 mM potassium phosphate buffer, and 6.9 mg (Expt. 170) or 7.2 mg (Expt. 181) mitochondrial protein. Reaction time, 20 min.

Expt.	NH_4Cl (mM)	Δ O (μatoms)	Δ Esterified P (μmoles)	P:O	Δ Glutamate (μmoles)	Δ Aspartate (μmoles)	Δ (Glutamate + aspartate) (μmoles)
170	0	6.2	3.5	0.57	+0.19	0.02	0.21
	20	5.6	1.1	0.20	+0.99	2.34	3.33
181	0	7.8	5.3	0.69	+0.25	0.36	0.61
	20	7.3	2.1	0.29	+1.35	5.06	6.41

of the amount of oxaloacetate which has not reacted with glutamate. However, no determinations of oxaloacetate were made since, as already mentioned, this is not a source of error when Δ(aspartate + glutamate) is measured.

Again, more than 1 mole of (aspartate + glutamate) was synthesized for each ~ consumed.

TABLE VII

RELATION BETWEEN SYNTHESIS OF GLUTAMATE + ASPARTATE COUPLED TO THE AEROBIC OXIDATION OF TMPD IN THE SYSTEM TMPD–AMYTAL–MALATE AND CONCOMITANT DECREASE IN PHOSPHORYLATION

Data obtained from Table VI. Δ (Glutamate + aspartate) = (glutamate + aspartate) in presence of α-oxoglutarate + NH_3 *minus* (glutamate + aspartate) synthesized in absence of NH_3. Esterified P expected = Δ O in presence of α-oxoglutarate + NH_3 multiplied by P:O ratio in absence of α-oxoglutarate + NH_3. Esterified P found = P esterified in presence of α-oxoglutarate + NH_3.

Expt.	Δ (Glutamate + aspartate) (μmoles)	Δ Esterified P (μmoles)			Δ (Glutamate + aspartate): decrease in Δ esterified P
		Expected	*Found*	*Decrease*	
170	3.12	3.2	1.1	2.1	1.5
181	5.80	5.1	2.1	3.0	1.9

Energy requirement for reduction of α-oxoglutarate (+ NH_3) by succinate, linked to the aerobic oxidation of TMPD

Tables VIII and IX summarize the results of experiments similar to those shown in Tables I and II, but in which antimycin was added to inhibit the aerobic oxidation of succinate, and TMPD (+ ascorbate) was the oxidizable substrate. The aspartate which was formed, at an increasing rate during the course of Expt. 209, was derived from the malate formed from the fumarate by Reaction 1.

TABLE VIII

MEASUREMENTS OF OXYGEN UPTAKE, PHOSPHORYLATION AND GLUTAMATE SYNTHESIS ASSOCIATED WITH THE AEROBIC OXIDATION OF TMPD

The reaction mixture contained, in addition to the basic components, 60 mM succinate, 20 mM potassium phosphate buffer, 1 mM arsenite, 20 mM glucose, 150 units hexokinase, 2% (Expt. 135) or 1% ethanol, 2 μg (Expt. 135) or 0.3 μg antimycin, 0.3 mM (Expt. 135) or 0.1 mM TMPD, 15 mM (Expt. 135) or 20 mM ascorbate, and 4.9 mg (Expt. 135), 6.9 mg (Expt. 208) or 7.0 mg (Expt. 209) mitochondrial protein.

Expt.	*Reaction time (min)*	*NH_4Cl (mM)*	*α-Oxoglutarate (mM)*	*Δ O (μatoms)*	*Δ Esterified P (μmoles)*	*P:O*	*Δ Glutamate (μmoles)*	*Δ Aspartate (μmoles)*	*Δ Oxaloacetate (μmole)*
135	20	10	0	12.5	7.7	0.62	0.17	—	—
		10	20	12.0	5.7	0.47	1.74	—	—
208	30	0	20	14.1	9.1	0.64	0	0.05	—
		20	20	14.1	3.8	0.28	2.61	0.37	—
209	10	0	20	5.9	4.4	0.74	0	0.07	0.01
		20	20	6.2	3.2	0.52	1.49	0.34	0.07
	20	0	20	11.8	8.3	0.70	0	0.06	0.04
		20	20	12.5	6.0	0.48	2.98	0.95	0.29
	30	0	0	17.8	11.2	0.63	0	0.07	0.02
		20	20	18.4	7.8	0.42	4.01	1.57	0.44

Of the 3 experiments which were carried out, 2 gave Δglutamate:$-\Delta\sim$ ratios of near 1, similar to the results in Table II, and the third gave what appeared to be an abnormally low ratio.

Small amounts of oxaloacetate which escaped reaction with glutamate were also found at the end of the reaction, and, if correction is made for this, the ratios in Expt. 209 become 1.01, 0.99 and 0.93 instead of 1.06, 1.08 and 1.04, respectively.

ATP as energy donor

When respiration is inhibited by antimycin, the energy required for the reduction of α-oxoglutarate (+ NH_3) by succinate[2], or for the NAD+-catalysed amination

TABLE IX

RELATION BETWEEN SYNTHESIS OF GLUTAMATE COUPLED TO THE AEROBIC OXIDATION OF TMPD IN THE SYSTEM TMPD–ANTIMYCIN–SUCCINATE AND CONCOMITANT DECREASE IN PHOSPHORYLATION

Data obtained from Table VIII. Δ Glutamate = glutamate synthesized in presence of α-oxoglutarate + NH_3 minus glutamate synthesized in absence of NH_3. Δ Esterified P expected = Δ O in presence of α-oxoglutarate + NH_3 multiplied by P:O ratio in absence of NH_3. Δ Esterified P found = P esterified in presence of α-oxoglutarate + NH_3.

Expt.	*Reaction time (min)*	*P:O in control*	*Δ Glutamate (μmoles)*	*Δ Esterified P (μmoles)*			*Δ Glutamate:decrease in Δ esterified P*		
				Expected	*Found*	*Decrease*			
135	20	0.62	1.57	7.4	5.7	1.7			0.92
208	30	0.64	2.61	9.1	3.8	5.3			0.49
209	10	0.74	1.49	4.64	3.23	1.41	1.06		
	20	0.70	2.98	8.71	5.95	2.76	1.08	Mean	1.06
	30	0.63	4.01	11.60	7.75	3.85	1.04		
								Mean	0.82

TABLE X

STOICHEIOMETRY OF GLUTAMATE SYNTHESIS AND ATP BREAKDOWN IN THE ATP-INDUCED REDUCTION OF α-OXOGLUTARATE (+ NH_3) BY SUCCINATE

The reaction mixture contained, in addition to the basic components, 60 mM succinate, 1 mM arsenite, 10 mM ATP, 1.6 mM (Expt. 201) or 2.0 mM potassium phosphate, 1% ethanol, 0.16, 0.09, 0.07 or 0.12 μg antimycin/mg protein in Expts. 201, 204, 205 and 206, respectively, and 3.1–8.1 mg mitochondrial protein. Reaction time, 30 min.

Expt.	*NH_4Cl (mM)*	*α-Oxo-glutarate (mM)*	*Δ P_i (μmoles)*	*ΔGlutamate (μmoles)*	*Δ Glutamate: Δ P_i*	*Δ Aspartate (μmoles)*
201	0	0	1.7	0.02		0
	0	20	1.8	0.22		0.01
	20	0	1.9	0		0.01
	20	20	3.0	0.74	0.25	0.07
204	0	0	2.1	0.03		0
	0	20	2.4	0		0.03
	20	0	2.1	0.38		0
	20	20	3.6	1.24	0.34	0.19
205	0	0	3.1	0		0
	0	20	2.5	0		0
	20	0	2.9	0.07		0
	20	20	4.6	2.96	0.64	1.38
206	0	0	3.1	0		0.01
	0	20	2.6	0		0.01
	20	0	2.9	0		0.08
	20	20	4.6	3.11	0.68	1.63

of malate to aspartate[4], can be provided by ATP. This reaction is accompanied by the liberation of P_i from the ATP, so that it is theoretically possible to calculate the ratio Δglutamate:$-\Delta\sim$ from the amount of P_i formed.

Data on the ATP-induced reduction of α-oxoglutarate (+ NH_3) by succinate are given in Table X. Addition of either NH_3 or α-oxoglutarate had no significant effect on the amount of P_i liberated, but the latter was increased by the addition of both NH_3 and α-oxoglutarate. The ratio Δglutamate:ΔP_i varied widely, between 0.25 and 0.68, probably because of variable contributions by ATP-splitting reactions not connected with the reduction of NAD^+ by succinate.

Table XI shows that the ratio Δ(glutamate + aspartate):ΔP_i exceeded 1 for the ATP-induced amination of malate to aspartate.

TABLE XI

STOICHEIOMETRY OF GLUTAMATE AND ASPARTATE SYNTHESIS AND ATP BREAKDOWN IN ATP-INDUCED REDUCTION OF α-OXOGLUTARATE (+NH_3) BY MALATE

The reaction mixture contained, in addition to the basic components, 20 mM α-oxoglutarate, 20 mM malate, 10 mM glutamate, 1 mM arsenite, 2 mM potassium phosphate buffer, 1% ethanol, 0.5 μg antimycin, 10 mM ATP, and 4.3 mg mitochondrial protein. Reaction time, 30 min (Expt. 202).

NH_4Cl (mM)	*Δ P_i (μmoles)*	*Δ Glutamate (μmoles)*	*Δ Aspartate (μmoles)*	*Δ (Glutamate + aspartate) (μmoles)*	*Δ (Glutamate + aspartate): Δ P_i*
0	3.00	−0.42	0.35	−0.07	
20	4.21	0.67	4.46	5.13	1.22

DISCUSSION

Energetics

Malate as donor. Two NAD-catalysed syntheses of amino acids have been studied in this paper, the reduction of α-oxoglutarate (+ NH_3) by succinate to glutamate (Reaction 3) and the amination of malate to aspartate (Reaction 6). As sources of energy, the aerobic oxidation of succinate or malate, the aerobic oxidation of TMPD, and ATP have been used. The yields of amino acid per molecule of ~ expended are summarized in Table XII.

When malate was hydrogen donor, more than 1 mole of amino acid was formed per mole of ~ expended, even when ATP was the energy source. In one experiment

TABLE XII

YIELDS OF AMINO ACID PER ~ EXPENDED

Hydrogen donor	*Energy source*	*Moles amino acid*/mole ~ expended*		
		No. of measurements	*Range*	*Mean*
Succinate	Succinate → O_2	9	0.73–1.11	0.84
	TMPD → O_2	3	0.49–1.06	0.82
	ATP	4	0.25–0.68	0.48
Malate	Malate → O_2	3	2.1 –6.5	3.8
	TMPD → O_2	2	1.5 –1.9	1.7
	ATP	1	1.2	1.2

* Glutamate with succinate as donor, (glutamate + aspartate) with malate. The yields of glutamate formed have not been corrected for the small amounts of oxaloacetate present at the end of the reaction (see text).

when the aerobic oxidation of malate was the energy source more than 6 moles of amino acid were synthesized per mole of ~. This result is most simply explained by assuming that ~ is necessary as a catalyst and not in stoicheiometric amounts for Reaction 6. The fact that there is, nevertheless, a distinct consumption of ~, although less than stoicheiometric, could be explained in three ways: (a) there are two mechanisms, one requiring ~ in catalytic amounts, the other in stoicheiometric amounts, (b) there is only one mechanism, catalysed by ~, but there are side-reactions leading to the loss of ~, (c) ~ does not participate in the reactions, the sum of which is Reaction 6, but is concerned indirectly. Possible mechanisms are discussed by TAGER[4].

In experiments carried out with malate in the presence of oligomycin[4], a mean of 4.2 μmoles (range 2.9–6.5 in 16 experiments) amino acids was synthesized per atom of oxygen consumed.

Succinate as donor. Three possible sources of error in assessing the yield with succinate as donor and the aerobic oxidation of succinate or TMPD as the energy source have been discussed. That due to the presence of unreacted oxaloacetate leads to the over-estimation of the yield by less than 10%. It has been shown that the other possible sources of error cause negligible errors. A further source of error which remains to be discussed is the requirement of energy for the synthesis of

asparate by the malate formed by the oxidation of the succinate. An assessment of this error can be made by subtracting from the decrease in esterified P in Table II the amount of aspartate found divided by 3.8 (from Table V). When this correction is applied to the data of Table II (except in the case of Expt. 143 where aspartate was not determined) the mean value of the ratio is increased from 0.84 to 1.04.

It appears reasonable, then, to conclude that the value of n in Eqn. 3 is 1. Two of the three experiments summarized in Table V, when the aerobic oxidation of TMPD provided the energy, gave values in close agreement. Yields were lower when ATP was the energy source, but Table X shows that ATP is used for reactions other than synthesis of amino acids.

ERNSTER[17] has determined the stoichiometry of the utilization of $\sim$ for the reduction of NAD^+ by calculating the ratio [increase of oxygen (μatoms) uptake]:[acetoacetate (μmoles) disappearing] when acetoacetate is added to rat-liver mitochondria oxidizing succinate in the absence of phosphate or phosphate acceptor. He found a ratio of 0.57 in one experiment and 0.50 in a second, corresponding to about 1 high-energy bond equivalent per NAD^+ molecule reduced by succinate, allowing for the fact that 2 high-energy bond equivalents are produced for each atom of oxygen absorbed in succinate oxidation. From our data (Table XIII), values of 0.53–0.67 (mean 0.57) can be calculated for the ratio $\Delta O:\Delta$glutamate obtained on the addition of α-oxoglutarate (+ NH_3) in the presence of oligomycin.

Although ERNSTER's method for calculation of the stoichiometry gives approximately the same results as ours, whether applied to his data or to ours, there are three basic assumptions in his method which should be discussed.

1. Since, when acetoacetate is used as acceptor, no distinction is made between

TABLE XIII

OXYGEN: AMINO ACID STOICHEIOMETRY CALCULATED BY ERNSTER'S METHOD

The reaction mixture contained, in addition to the basic components, 20 mM potassium phosphate buffer, 1 mM arsenite, 60 mM succinate, 20 mM α-oxoglutarate, 20 mM NH_4Cl, 10 μg oligomycin, 20 mM glucose, 150 units hexokinase, 1% ethanol, 3.5–7.0 mg mitochondrial protein, and, where TMPD was the energy source, 0.3 mM TMPD and 20 mM ascorbate. The reaction time was 20–30 min.

Energy source	*Expt.*	*ΔO (μatoms)*			*Glutamate (μmoles)*			*Increment in ΔO:increment in glutamate*
		*(a)**	*(b)***	*Increment*	*(a)**	*(b)***	*Increment*	
Succinate $\rightarrow O_2$	103	4.6	7.0	2.4	—	4.33	4.33	0.55
	107	3.6	5.7	2.1	0.02	3.94	3.92	0.53
	112	3.8	7.5	3.7	0.27	5.81	5.54	0.67
	114	2.5	4.2	1.7	0	3.24	3.24	0.52
	173	3.8	6.6	2.8	0.48	5.20	4.72	0.59
							Mean	0.57
TMPD $\longrightarrow O_2$	111	14.4	15.0	0.6	0.10	2.55	2.45	0.24
	112	10.6	12.1	1.5	0.21	2.75	2.54	0.59
	114	6.6	8.4	1.8	0	1.55	1.55	1.16
	118	11.6	12.8	1.2	0.11	1.88	1.77	0.68
	135	10.3	11.3	1.0	0.17	2.81	2.64	0.38

* In absence of α-oxoglutarate and NH_3, or in absence of α-oxoglutarate, or in absence of NH_3.

** In presence of both α-oxoglutarate and NH_3.

the succinate and the malate (formed from succinate) as hydrogen donor, it is assumed that none of the acetoacetate is reduced by malate. KLINGENBERG[16,18] has recently published balance studies which support this assumption.

2. It is assumed that both ~ compounds formed by the aerobic oxidation of succinate are utilized for the reduction of NAD+ by succinate. This point is further discussed below.

3. It is assumed that the increase in respiratory rate associated with the utilization of the ~ compounds for the reduction of NAD+ by succinate is not accompanied by any suppression of the "basal" rate measured before the addition of acetoacetate. In other words, the introduction of Pathway iii for the breakdown of A ~ I does not affect the rate of Pathway i. This assumption is unconventional; it is not made in calculations of the stoicheiometry of oxidative phosphorylation (P:O ratio), since it is assumed that when Pathway ii is brought into play, Pathway i is suppressed. Thus, P:O ratios are calculated on the basis of the total O_2 uptake, not just the increment. The general non-validity of the "increment" method is illustrated by the behaviour of "loosely coupled" preparations when addition of ADP does not stimulate the O_2 uptake but is accompanied by synthesis of ATP. The increment method would give P:O ratios of infinity with these preparations.

The agreement in the results obtained by ERNSTER's method of calculation of the stoicheiometry and by ours suggest that these assumptions might be valid for succinate oxidation. The variable results (0.24–1.16) obtained for the ratio ΔO: Δglutamate when TMPD is substrate (Table XIII) show, however, that the third assumption is certainly not valid in this case. On the assumption that one ~ is required for the synthesis of each molecule of glutamate, it can be calculated that in Expt. 135, for example, the addition of α-oxoglutarate (+ NH_3) has suppressed Pathway i from 10.3 μatoms to 11.3 − 2.6 = 8.7 μatoms. The fact that the same yield of amino acid per ~ is obtained with TMPD as with succinate as oxidizable substrate (Table XII) could not have been revealed by ERNSTER's method.

CHANCE AND HOLLUNGER's[19] method of determining the stoicheiometry of the energy requirement for NAD+ reduction by succinate also depends upon measurement of the increment of the O_2 uptake brought about when succinate reduces NAD+, but a very much more sensitive method of measuring the O_2 uptake is used, and the calculation of the stoicheiometry does not require the assumptions implicit in ERNSTER's method. When succinate is added to rat-liver mitochondria, the NAD+ becomes more reduced, and simultaneously cytochrome *a* goes through an oxidation–reduction cycle, first becoming more reduced before returning to its original level. This oxidation–reduction cycle, which is due to utilization of ~, is also found after ADP addition. The area under the curve relating cytochrome *a* reduction with time is a measure of the amount of electron transfer along the chain to oxygen, *i.e.* of the O_2 uptake, and is proportional to the amount of ADP added. Thus, it is possible to calculate from the area under the curve obtained when succinate is added the amount of ~ utilized. In this way a stoicheiometry of NADH:~ of 0.4 was obtained. When corrected for the utilization of ~ for reactions other than the reduction of NAD+ the value became 0.6.

This method of estimating the amount of ~ expended is ingenious and appears to be free from any error. However, it seems possible that the increased reduction of NAD+, which really represents a change in the steady state, underestimates the

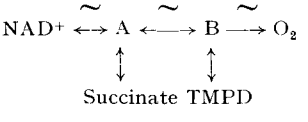
number of molecules of NAD^+ reduced by succinate. If this is so, the value of 0.6 for the stoicheiometry found by CHANCE AND HOLLUNGER represents a lower limit.

CHANCE AND HOLLUNGER[19] found that NAD^+ in pigeon-heart mitochondria was not reduced by succinate unless ATP was added. By determination of the amount of ADP formed from the ATP, NADH:$\sim$ ratios of 0.2–0.53 were obtained. By adding limiting amounts of ATP, a ratio of 0.3 was found. CHANCE AND ITO[20] obtained a ratio of 0.32 when the hydrolysis of ATP was measured by the pH change. These values are comparable with the ratios of 0.25–0.68 obtained for rat-liver mitochondria shown in Table X. As explained above (*cf.* CHANCE AND ITO[20]) these results are likely to be an under-estimate of the true stoicheiometry of the reduction of NAD^+ linked to the hydrolysis of ATP, since they do not take account of other reactions leading to hydrolysis of ATP.

All the evidence taken together, it seems justified to conclude that 1 $\sim$ is expended in the reduction of NAD^+ by succinate.

Mechanism

The mechanism of the reduction of NAD^+ by succinate will be discussed in terms of an abbreviated form of the respiratory chain.



and the theory of oxidative phosphorylation discussed above*. A and B represent two carriers in the respiratory chain, A in the flavoprotein–ubiquinone region, B in the region of cytochrome *c*. One energy-conservation site is located between A and B and one between B and O_2.

The experiments with TMPD show clearly that the energy-conservation site** between B and O_2 can be utilized for the reduction of NAD^+ by succinate. The question will now be discussed whether the site between A and B is also utilized.

The determination of the energetics of the process discussed above does not give an unequivocal answer to this question. Our method and that of CHANCE AND HOLLUNGER[19] determines the NADH:$\sim$ ratio, without regard to the source of the $\sim$. In a preliminary note [23], we concluded from the fact that the glutamate:O ratio in the presence of oligomycin, α-oxoglutarate and NH_3 was one-half that of the P:O ratio in the absence of these additions that only one of the energy-conserving sites

* We should like to draw attention to the fact that all the data available on the energy-linked reduction of NAD^+ can also be explained by the theory of MITCHELL[55] in which no high-energy intermediates of oxidative phosphorylation are involved.

** For purposes of this discussion, it has been assumed that there is only one phosphorylation step in the region between TMPD and oxygen. Recent work in this laboratory by HOWLAND[21] has confirmed RAMIREZ'S[22] suggestion that there is more than one phosphorylation step in this region, and has shown that one of these is sensitive to low concentrations of antimycin. Thus, in the experiments in which the oxidation of succinate provides the energy for the reduction of NAD^+ by succinate, three energy-conserving sites might be available for this purpose, one between A and B, and two between B and O_2. When the oxidation of TMPD provides the energy in the presence of antimycin only one site above B is available.

was involved. Further experiments[24], however, showed that no such conclusion can be drawn from a comparison of the glutamate:O and P:O ratios. This is illustrated by the fact that the mean glutamate:O ratio for the TMPD system is only 0.20 (range 0.10–0.38 in 16 experiments), compared with a P:O ratio of 0.62–0.74 in Table IX. The low glutamate:O ratio is due to the high activity of Pathway i for the decomposition of the $\sim$ compound in this step. Indeed, the Glu:O ratio in the presence of oligomycin is a measure of the ratio $v_{iii}/(v_i + v_{iii})$, where v_i and v_{iii} are the rates of Pathways i and iii, respectively. This ratio is not related in a simple manner to the P:O ratio $(v_{ii}/(v_i + v_{ii}))$.

ERNSTER's[17] method of measuring the value of n is based on the assumption that both energy-conserving sites can be utilized for the reduction of NAD^+ by succinate, so that the results obtained by this method cannot formally be used to deduce the number of energy-conserving sites involved. At first sight, it might appear that the values of about 0.5 for (increment in ΔO):(increment in glutamate) obtained by this method (Table XIII) give strong support to this assumption. However, as already explained, this method is based on a second assumption, *viz.* that Pathway i is not suppressed by the introduction of Pathway iii. If only one of the energy-conserving sites were utilized for the reduction of NAD^+, the value of the ratio (ΔO in Pathway iii):glutamate would be 1.0. On this assumption, Pathway iii in Expt. 103 of Table XIII would account for 4.3 μatoms oxygen, leaving 2.7 for Pathway i, in comparison with 4.6 in the absence of α-oxoglutarate and NH_3. Since this would not appear unreasonable, it must be concluded that the data assembled in Table XIII do not give unequivocal evidence that both energy-conserving steps are utilized.

It could be argued that both steps must be utilized to explain the stimulation in respiration brought about by the addition of α-oxoglutarate and NH_3 in the presence of oligomycin[2]. However, it is not certain that both energy-conserving steps contribute to the respiratory control. Moreover, in our preliminary note[23], we suggested that the requirement for relatively high concentrations of phosphate or arsenate for this stimulation of respiration and for glutamate synthesis is due to an uncoupling by these compounds of the energy-conserving site between A and B. This explanation became less likely when it was found that phosphate and arsenate also stimulate the reduction of α-oxoglutarate (+ NH_3) by malate or isocitrate, as well as by succinate[1].

Completely unequivocal evidence for the concept that both energy-conserving steps are concerned in the reduction of NAD^+ by succinate would be the demonstration that glutamate:O ratios clearly above 1 can be regularly obtained. The results of a recent study of this question are shown in Table XIV. As discussed above (see p. 281), the amounts of glutamate should be corrected for the small amounts of oxaloacetate present at the end of the experiment to allow for glutamate derived from reduction of α-oxoglutarate (+ NH_3) by malate.

Thus, the data in Table XIV are expressed as (glutamate—oxaloacetate) rather than glutamate. The mean of 25 measurements with between 24 and 72 mM* succinate was 0.94. However, in view of the fact that Pathway i is certainly operating (*cf.* the

* The fact that there is no clear effect of succinate concentration on the glutamate:O ratio supports the conclusion of GREENGARD *et al.*[10] that the low P:O ratio with high succinate concentrations is not due to uncoupling by succinate.

TABLE XIV

GLUTAMATE: OXYGEN RATIO WITH SUCCINATE AS HYDROGEN DONOR*

Reaction mixture contained, in addition to the basic components, 20 mM potassium phosphate buffer, 20 mM α-oxoglutarate, 20 mM NH_4Cl, 1 mM arsenite, 1% ethanol, 10 μg oligomycin, and 2.5–9.7 mg mitochondrial protein. The reaction time was 20 min.

Succinate (mM)	(Glutamate—oxaloacetate):O			$\frac{\text{Aspartate}}{\text{(glutamate—oxaloacetate)}} \times 100$
	Mean	Range	No. of measurements	Mean
24	0.95	0.75–1.11	4	43
36	1.01	0.85–1.16	5	49
48	0.97	0.84–1.11	5	47
60	0.90	0.83–1.10	7	42
72	0.90	0.82–1.01	4	37

* Expts. 260–267.

P:O ratios in Table II) and that some energy is required for the concomitant synthesis of aspartate from malate and NH_3, it seems very likely, although not absolutely certain, that more than one energy-conservation site is utilized in the reduction of NAD^+ by succinate. This provisional conclusion will be assumed to be correct in the discussion of the mechanism which follows. In fact, no fundamental changes in the mechanism are required if only one energy-conservation step is involved (*cf.* ref. 23).

The following reaction scheme is proposed for the reduction of NAD^+ coupled to the aerobic oxidation of succinate.

$$3 \text{ succinate} + 3 \text{ A} \rightleftharpoons 3 \text{ fumarate} + 3 \text{ AH}_2 \quad (10)$$
$$\text{AH}_2 + \text{B} + \text{I}_2 \rightleftharpoons \text{A} \sim \text{I}_2 + \text{BH}_2 \quad (11)$$
$$\text{BH}_2 + {}^1/_2\,\text{O}_2 + \text{I}_3 \rightarrow \text{B} \sim \text{I}_3 + \text{H}_2\text{O} \quad (12)$$
$$\text{A} \sim \text{I}_2 + \text{NAD}^+ + \text{I}_1 \rightleftharpoons \text{A} + \text{I}_2 + \text{NAD} \sim \text{I}_1 \quad (13)$$
$$\text{B} \sim \text{I}_3 + \text{NAD}^+ + \text{I}_1 \rightleftharpoons \text{B} + \text{I}_3 + \text{NAD} \sim \text{I}_1 \quad (14)$$
$$2 \text{ AH}_2 + 2 \text{ NAD} \sim \text{I}_1 \rightleftharpoons 2 \text{ A} + 2 \text{ NADH} + 2 \text{ I}_1 + 2 \text{ H}^+ \quad (15)$$
$$3 \text{ succinate} + {}^1/_2\,\text{O}_2 + 2 \text{ NAD}^+ \rightarrow 3 \text{ fumarate} + 2 \text{ NADH} + 2 \text{ H}^+$$

I_1, I_2 and I_3 represent components required for hydrogen or electron transfer in the three phosphorylation steps of the respiratory chain (*cf.* SLATER[25]). If I_1, I_2 and I_3 are identical (see SLATER[26] for a discussion of this point) Reactions 13 and 14 are simply an I transfer, *e.g.*

$$\text{B} \sim \text{I} + \text{NAD}^+ \rightleftharpoons \text{B} + \text{NAD} \sim \text{I}$$

If they are not identical the following type of transfer seems one possibility

$$\text{B} \sim \text{I}_3 + \text{NAD}^+ \rightleftharpoons \text{B} + \text{NAD} \sim \text{I}_3$$
$$\text{NAD} \sim \text{I}_3 + \text{I}_1 \rightleftharpoons \text{NAD} \sim \text{I}_1 + \text{I}_3$$

It should be noted that these transfers cannot proceed through the oligomycin-sensitive component of oxidative phosphorylation or through components on the

ATP side of the oligomycin-sensitive block, since the reduction of NAD^+ by succinate is oligomycin-insensitive.

A could conceivably be ubiquinone. In this case Reaction 10 would proceed through the succinate dehydrogenase flavoprotein and Reaction 15 through the NADH dehydrogenase flavoprotein. Alternatively, A could be succinate dehydrogenase, and Reaction 16 involve both ubiquinone and NADH dehydrogenase.

Reactions 10–14 of our proposed mechanism are in accordance with an earlier suggestion[25,27] that the energy-conservation reactions linked with the oxidation of succinate may be used to synthesize the high-energy compound NAD ~ I from its constituents. This proposal was based on the observation of PURVIS[28] in 1958 that, on addition of succinate to rat-liver mitochondria, NAD^+ disappeared without concomitant appearance of NADH. PURVIS[28,29] suggested that the "Extra NAD" which was formed in this reaction was NAD ~ I. Although later work from this laboratory[30] did not confirm the non-appearance of NADH when succinate was added to freshly prepared rat-liver mitochondria, qualitatively similar results to those of PURVIS were obtained when the mitochondria were incubated with succinate in the absence of P_i. Moreover, it has been repeatedly observed in this laboratory[30,31] that when succinate is added to heart mitochondria in the presence of oxygen, the amount of NADH which is formed is appreciably less than the amount of NAD^+ which disappears. This is to be expected according to our mechanism, since Reaction 15 is reversible. Whether or not NADH will be formed on addition of succinate to mitochondria will depend upon many factors, including the relative concentrations of NAD^+, NADH and NAD ~ I originally present. This may be the explanation for the different results obtained by PURVIS and by other authors[30,32,33]. As already mentioned[25,27,34], an increased absorption at 340 mμ and an increased fluorescence would be obtained in any case, since NAD ~ I would be expected to resemble NADH with respect to ultraviolet absorption and fluorescence.

The idea that the NAD^+ reduced by succinate is different from that reduced by NAD-linked dehydrogenases is supported by KLINGENBERG AND SLENCZKA'S[32] finding that, in the controlled state, much more of the mitochondrial NAD is in the form of NADH when succinate or glycerol-1-phosphate is the oxidizable substrate than with NAD-linked substrates such as β-hydroxybutyrate or malate.

Reaction 15 is the reverse of the first reaction in the aerobic oxidation of NADH. This may be the reason why, when succinate is present, NADH is not oxidized by the respiratory chain.

The reduction of NAD^+ in the system succinate–antimycin–TMPD could be explained by the sequence

succinate + A	$\rightleftharpoons$ fumarate + AH_2	(10)
TMPD + B	$\rightleftharpoons$ oxidized TMPD + BH_2	(16)
$BH_2 + {}^1/_2 O_2 + I_3$	$\rightarrow B \sim I_3 + H_2O$	(12)
$B \sim I_3 + NAD^+ + I_1$	$\rightleftharpoons B + I_3 + NAD \sim I_1$	(14)
$AH_2 + NAD \sim I_1$	$\rightleftharpoons A + NADH + I_1 + H^+$	(15)

succinate + TMPD + $^1/_2 O_2$ + NAD^+ → fumarate + oxidized TMPD + NADH + H^+

The reduction of NAD^+ by TMPD (ref. 3) could be explained by the sequence

$$\begin{array}{lll} 3\ TMPD + 3\ B & \rightleftharpoons 3 \text{ oxidized TMPD} + 3\ BH_2 & (16) \\ 2\ BH_2 + O_2 + 2\ I_3 & \rightarrow 2\ B \sim I_3 + 2\ H_2O & (12) \\ B \sim I_3 + A + I_2 & \rightleftharpoons B + I_3 + A \sim I_2 & (17) \\ B \sim I_3 + NAD^+ + I_1 & \rightleftharpoons B + I_3 + NAD \sim I_1 & (14) \\ BH_2 + A \sim I_2 & \rightleftharpoons B + AH_2 + I_2 & (11) \\ AH_2 + NAD \sim I_1 & \rightleftharpoons A + NADH + I_1 + H^+ & (15) \\ \hline 3\ TMPD + O_2 + NAD^+ & \rightarrow 3 \text{ oxidized TMPD} + NADH + H^+ & \end{array}$$

Inhibition by dinitrophenol or arsenate (in the absence of oligomycin) of NAD^+ reduction is explained by the ability of these uncouplers to promote the hydrolysis of the high-energy intermediates. Reaction 11 is the site of action of antimycin. Reaction 15 is the site of action of Amytal*. Reduction of NAD^+, promoted by the continuous removal of NADH by the addition of α-oxoglutarate + NH_3, results in less of the $A \sim I_2$ and $B \sim I_3$ being available for synthesis of ATP, with a consequent lowering of the P:O ratio. Either (ADP + P_i) or NAD^+ (formed by addition of α-oxoglutarate and NH_3) can stimulate respiration of mitochondria in the controlled state, since both cause the splitting of $A \sim I_2$ and $B \sim I_3$. Oligomycin, which inhibits the reaction of $A \sim I_2$ and $B \sim I_3$ with P_i and ADP, thereby completely blocking oxidative phosphorylation, does not inhibit the reduction of NAD^+ by succinate. In fact, it substantially increases the degree of reduction[2].

Since there is reason to believe that Reaction 18

$$B \sim I_3 + P_i + ADP \rightleftharpoons B + I_3 + ATP \qquad (18)$$

is difficultly reversible (see HEMKER[36]), the ATP-induced reduction of NAD^+ by succinate probably either involves $A \sim I_2$ according to the reaction sequence

$$\begin{array}{lll} \text{succinate} + A & \rightleftharpoons \text{fumarate} + AH_2 & (10) \\ ATP + A + I_2 & \rightleftharpoons ADP + P_i + A \sim I_2 & (19) \\ A \sim I_2 + NAD^+ + I_1 & \rightleftharpoons A + I_2 + NAD \sim I_1 & (13) \\ AH_2 + NAD \sim I_1 & \rightleftharpoons A + NADH + I_1 + H^+ & (15) \\ \hline \text{succinate} + NAD^+ + ATP & \rightleftharpoons \text{fumarate} + NADH + ADP + P_i & \end{array}$$

or involves $NAD \sim I_1$ formed by Reaction 20

$$ATP + NAD^+ + I_1 \rightleftharpoons ADP + P_i + NAD \sim I_1 \qquad (20)$$

Both Reactions 19 and 20 are oligomycin-sensitive. No choice can be made between these possibilities at the moment (*cf.* CHANCE[38]).

Although the essential feature of our mechanism—the utilization of the energy of intermediates of oxidative phosphorylation to drive the respiratory chain in reverse between succinate and NAD^+—is the same as that in mechanisms proposed by CHANCE AND HOLLUNGER[19] and ERNSTER[17] (*cf.* KLINGENBERG AND SCHOLLMEYER[39]) there are some important differences of detail which must now be discussed:

* HÜLSMANN'S[35] suggestion in 1958 that Amytal reacts, directly or indirectly, with $NAD \sim I$, to form Amytal-I (see also refs. 25, 27, 28) has been supported by HEMKER[36] in this laboratory and by CHANCE, HOLLUNGER AND HAGIHARA'S[37] recent demonstration that the inhibition by Amytal of the oxidation of NAD-linked substrates can be partially reversed by uncouplers.

1. In our mechanism, the high-energy intermediates necessary for the reduction of NAD^+ by succinate are internally generated during the aerobic oxidation of succinate. CHANCE AND HOLLUNGER[19] and KLINGENBERG AND SCHOLLMEYER[39], on the other hand, have emphasized in some of their earlier papers experiments in which the high-energy intermediates were already present in the freshly prepared mitochondria, or were generated in aged mitochondria by prior incubation with glutamate or with ATP*. Since high-energy intermediates are normally generated immediately on addition of succinate to mitochondria, we do not understand why pre-treatment is necessary to generate these intermediates. We suspect that the activation, by the addition of ATP or glutamate, of the reduction of NAD^+ by succinate is not due to generation of high-energy intermediates but to the promotion of the oxidation of succinate (*e.g.* by removal of inhibiting oxaloacetate, *cf.* CHANCE AND HAGIHARA[41]) or to "tightening" of the coupling between oxidation and energy conservation (*cf.* refs. 39, 42). The fact that serum albumin could replace ATP in KLINGENBERG AND SCHOLLMEYER'S[39] experiments strongly suggests that ATP was acting in this indirect fashion in these experiments, since both serum albumin and ATP can remove uncoupling unsaturated fatty acids in aged mitochondrial preparations[43]. The fact that the ATP-induced reduction of NAD^+ by succinate in CHANCE'S[44] experiments with pigeon-heart mitochondria was only partly oligomycin-sensitive suggests that ATP was acting at least partly indirectly in this experiment. In other experiments, in which the aerobic oxidation of succinate is inhibited by respiratory inhibitors, both CHANCE[44] and KLINGENBERG AND SCHOLLMEYER[45] have clearly demonstrated an ATP-dependent reduction of NAD^+ by succinate, and CHANCE[44] demonstrated that this was completely oligomycin sensitive (*cf.* ref. 2).

2. In our mechanism, NADH is formed by the reduction of a NAD $\sim$ I compound. CHANCE AND ITO[20] write the reduction by the equation

$$X \sim I + NAD^+ + \text{succinate} \rightleftharpoons \text{fumarate} + NADH + H^+ + X + I \qquad (21)$$

in which no intermediate form of NAD is specified. NADH $\sim$ I, which has always been preferred to NAD $\sim$ I by CHANCE[8] (see also ESTABROOK AND NISSLEY[46]), is formed in a subsequent reaction

$$X \sim I + NADH \rightleftharpoons NADH \sim I + X \qquad (22)$$

Since, in our experiments, the NADH formed is rapidly oxidized by the glutamate dehydrogenase reaction, the subsequent formation of NADH $\sim$ I by CHANCE'S mechanism would be irrelevant to our experiments. This possibility is, therefore, not excluded by our results. Although we have chosen NAD $\sim$ I as an intermediate in the reduction, we are well aware that we have produced no more than suggestive evidence for its existence, and that Reaction 23, which is the sum of Reactions 13 and 15

$$AH_2 + NAD^+ + A \sim I_2 \rightleftharpoons 2\,A + I_2 + NADH + H^+ \qquad (23)$$

could just as well involve compounds of the type $AH_2 \sim I$ as NAD $\sim$ I (see below).

* For example, CHANCE[40] wrote "The mitochondria must be sufficiently free of endogenous high-energy intermediates that succinate alone causes no measurable reduction".

However, the intermediate formation of NADH $\sim$ I in Reaction 23 does not seem feasible, since two $\sim$ equivalents would be required, one for the endergonic reduction of NAD^+, one for the formation of NADH $\sim$ I. CHANCE[47] has recently suggested that energy can be conserved in the NAD^+ (written NAD^*) formed when NADH is oxidized by flavoprotein. There is, in the present context, no fundamental difference between what we write as NAD $\sim$ I and what CHANCE writes as NAD^*.

CHANCE AND ITO[20] find support for the formation of NADH $\sim$ I in their important experiments on the inhibition, after an initial stimulation, of the endogenous ATPase of pigeon-heart mitochondria by succinate. Since I is required for the ATPase the formation of NADH $\sim$ I would be expected to inhibit the ATPase. However, these experiments can also be explained by our theory, if it is assumed that the endogenous ATPase is mainly contributed by the first phosphorylation step[36,48] and that it involves NAD $\sim$ I_1.

$$ATP + NAD^+ + I_1 \rightleftharpoons NAD \sim I_1 + ADP + P_i \qquad (24)$$

$$NAD \sim I_1 \xrightarrow{(H_2O)} NAD^+ + I_1 \qquad (25)$$

Reduction of NAD $\sim$ I_1 to NADH by Reaction 15 would remove NAD^+ from the system thereby preventing Reaction 24 taking place. The initial stimulation of the rate of hydrolysis observed by CHANCE AND ITO would suggest, on this view, that Reaction 15 is more rapid than Reaction 25, which does not appear unreasonable.

3. ERNSTER (ref. 17, p. 136) has proposed a mechanism for oxidative phosphorylation and for reduction of NAD^+ by succinate involving high-energy compounds of reduced carrier. As in our mechanism, the high-energy compounds formed in that part of the respiratory chain lying between flavoprotein and oxygen are used to drive the chain in reverse between flavoprotein and NAD^+. However, ERNSTER makes the novel proposal that DPNH dehydrogenase (fp_D) is involved not only in the reduction of NAD^+ by succinate, but also in the reduction of O_2 by succinate. For both reactions it is necessary to form a high-energy compound of fp_DH_2 ($fp_DH \sim X$) by linking the reduction of fp_D by reduced succinate dehydrogenase (fp_sH_2) with energy-conserving reactions in that part of the chain lying between flavoprotein and oxygen. The $fp_DH \sim X$ is used either to reduce NAD^+ by Reaction 26

$$NAD^+ + fp_DH \sim X + OH^- \rightleftharpoons NADH + fp_D + X \qquad (26)$$

or is oxidized by cytochrome *b*

$$fp_DH \sim X + 2\,Fe_b^{3+} + P_i \rightleftharpoons fp_D + 2\,Fe_b^{2+} + X \sim P + 2\,H^+ \qquad (27)$$

(Reaction 26 is ERNSTER's Reaction 4 written in reverse, Reaction 27 is ERNSTER's Reaction 5 in which Fe_b^{3+} and Fe_b^{2+} represent ferri- and ferrocytochrome *b*.) Oxidation of ferrocytochrome *b* gives rise to carrier H $\sim$ X compounds which are utilized for the formation of $fp_DH \sim X$.

According to this mechanism, $fp_DH \sim X$ cannot be oxidized in the absence of P_i or in the presence of oligomycin which inhibits Reaction 27. In fact, as ERNSTER[49] and SNOSWELL[31] have shown, P_i is not necessary for reduction of NAD^+ by succinate linked to the aerobic oxidation of succinate, nor is the reduction inhibited by oli-

gomycin. The mechanism proposed by ERNSTER requires modification to explain these findings. The fact that oligomycin does not inhibit an energy-requiring reaction compulsorily linked with the oxidation of succinate in the respiratory chain shows quite unequivocally that oligomycin must act subsequent to all oxidation reactions.

The minimum modification of the ERNSTER theory required to explain these findings is to split Reaction 27 into an oligomycin-insensitive oxidation reaction (Reaction 28) and an oligomycin-sensitive energy-transfer reaction (Reaction 29)

$$fp_DH \sim X + 2\,Fe_b^{3+} + Y \rightleftharpoons fp_D + 2\,Fe_b^{2+} + X \sim Y + 2\,H^+ \quad (28)$$

$$X \sim Y + P_i \rightleftharpoons X \sim P + Y \quad (29)$$

The modified ERNSTER mechanism may then be written

$$3\ \text{Succinate} + 3\ fp_s \rightleftharpoons 3\ \text{fumarate} + 3\ fp_sH_2 \quad (30)$$

$$fp_sH_2 + X \sim Y + fp_D \rightleftharpoons fp_s + fp_DH \sim X + Y \quad (31)$$

$$fp_DH \sim X + 2\,Fe_b^{3+} + Y \rightleftharpoons fp_D + 2\,Fe_b^{2+} + X \sim Y + 2\,H^+ \quad (28)$$

$$2\,Fe_b^{2+} + \text{carrier} + X + 2\,H^+ \rightleftharpoons 2\,Fe_b^{3+} + \text{carrier H} \sim X \quad (32)$$

$$fp_sH_2 + fp_D + \text{carrier H} \sim X + 2\,Fe_c^{3+} \rightarrow fp_s + fp_DH \sim X + \text{carrier} + 2\,Fe_c^{2+} \quad (33)$$

$$2\,Fe_c^{2+} + \text{carrier} + X + 2\,H^+ \rightleftharpoons 2\,Fe_c^{3+} + \text{carrier H} \sim X \quad (34)$$

$$fp_sH_2 + fp_D + \text{carrier H} \sim X + 2\,Fe_a^{3+} \rightarrow fp_s + fp_DH \sim X + \text{carrier} + 2\,Fe_a^{2+} \quad (35)$$

$$2\,Fe_a^{2+} + 2\,H^+ + {}^1/_2\,O_2 \rightarrow 2\,Fe_a^{3+} + H_2O \quad (36)$$

$$2\,fp_DH \sim X + 2\,NAD^+ + 2\,OH^- \rightleftharpoons 2\,NADH + 2\,fp_D + 2\,X \quad (37)$$

$$3\ \text{Succinate} + {}^1/_2\,O_2 + 2\,NAD^+ \rightarrow 3\ \text{fumarate} + H_2O + 2\,NADH$$

None of the evidence produced in this paper, nor in our opinion brought forward by ERNSTER[17], allows us to make a final choice between mechanisms involving high-energy compounds of NAD^+ and those involving high-energy compounds of fp_DH_2. The antimycin-insensitive reduction of acetoacetate by succinate, linked to the oxidation of succinate by ferricyanide, reported by ERNSTER[17], is unexpected and appears to be difficult to reconcile with an ERNSTER-like mechanism. In the presence of antimycin, only Reactions 30, 31 and 28 would be expected, and these would lead to reduction of ferricyanide by succinate without production of high-energy compounds. On the other hand, it is conceivable that ferricyanide could replace B in Reaction 11 of our mechanism leading to the formation of $A \sim I_2$.

ERNSTER[17] brings in support of his mechanism the finding of KLINGENBERG AND BÜCHER[50] that inhibition by Amytal of the reduction of NAD^+ by glycerol-1-phosphate was accompanied by reduction of mitochondrial flavine, believed to be associated with both NADH dehydrogenase and glycerol-1-phosphate dehydrogenase, and that the addition of uncoupler inhibited the reduction of this NADH dehydrogenase while leaving the reduction of glycerol-1-phosphate dehydrogenase unaffected. In terms of the modified ERNSTER mechanism given above, the sum reaction in the presence of Amytal, which inhibits Reaction 37, would be

$$3\ \text{Succinate} + {}^1/_2\,O_2 + 2\,fp_D + 2\,X \rightarrow 3\ \text{fumarate} + H_2O + 2\,fp_DH \sim X \quad (38)$$

in agreement with this interpretation. However, the results of KLINGENBERG AND

BÜCHER are also consistent with our mechanism, since in the presence of Amytal and absence of uncoupler the sum reaction is

$$(x+1)\text{succinate} + \tfrac{x}{2}\,O_2 + x\,A + (x-1)B + (x-1)I_2 + (x-1)I_3 + 2\,I_1 + 2\,\text{Amytal} \rightarrow$$
$$\rightarrow (x+1)\text{fumarate} + (x-1)A \sim I_2 + (x-1)B \sim I_3 + 2\,\text{Amytal} - I_1 + AH_2 \qquad (39)$$

if it is assumed that Amytal reacts with NAD $\sim$ I_1. As already explained it is possible that A is ubiquinone, in which case Reaction 14 should be written

$$UQH_2 + fp_D \rightleftharpoons UQ + fp_DH_2 \qquad (40)$$
$$fp_DH_2 + NAD \sim I_1 \rightleftharpoons fp_D + NADH + I_1 \qquad (41)$$

In the presence of Amytal, Reaction 41 cannot take place, so that the sum reaction now becomes

$$(x+1)\text{succinate} + \tfrac{x}{2}\,O_2 + (x-1)UQ + (x-1)B + (x-1)I_2 + (x-1)I_3 + 2\,I_1 +$$
$$2\,\text{Amytal} + fp_D \rightarrow (x+1)\text{fumarate} + (x-1)UQ \sim I_2 + (x-1)B \sim I_3 + 2\,\text{Amytal} - I_1 +$$
$$+ fp_DH_2 \qquad (42)$$

which would explain the reduction of fp_D in the presence of Amytal. Uncouplers by decomposing A $\sim$ I_2 and B $\sim$ I_3 would make the sum reaction

$$(x+1)\text{succinate} + \tfrac{x}{2}\,O_2 + 2\,I_1 + 2\,\text{Amytal} \rightarrow (x+1)\text{fumarate} + 2\,\text{Amytal} - I_1$$

in which the steady-state reduction of intermediates, such as succinate dehydrogenase (corresponding to glycerol-1-phosphate in KLINGENBERG AND BÜCHER's experiments), would depend upon the relative activities of the dehydrogenase and the rest of the respiratory chain.

ERNSTER[51] has criticized the concept of NAD $\sim$ I as an intermediate on the grounds that it does not explain the ability of mitochondria to catalyse the NAD-linked reduction of acetoacetate by pyruvate + malate in the absence of phosphate acceptor. We cannot accept this criticism as valid. Under these conditions, we have, according to our mechanism (SH_2 = hydrogen donor)

$$(x+1)SH_2 + (x+1)NAD^+ \rightleftharpoons (x+1)S + (x+1)NADH + (x+1)H^+ \qquad (43)$$
$$NADH + A + I_1 \rightleftharpoons NAD \sim I_1 + AH_2 \qquad (44)$$
$$AH_2 + B + I_2 + I_3 + {}^1/_2\,O_2 \rightarrow A \sim I_2 + B \sim I_3 + H_2O \qquad (45)$$

$$(x+1)SH_2 + (x+1)NAD^+ + A + B + I_1 + I_2 + I_3 + {}^1/_2\,O_2 \rightarrow (x+1)S + x\,NADH +$$
$$(x+1)H^+ + NAD \sim I_1 + A \sim I_2 + B \sim I_3 + H_2O$$

if the concentration of NAD exceeds that of A, B, I_1, I_2 or I_3, whichever is present in the lowest concentration, which does not appear to be unreasonable. Thus, NADH would be available for reduction of acetoacetate.

In the absence of evidence which we find convincing for the existence of a high-energy form of reduced flavin as an intermediate in oxidative phosphorylation, or of evidence which compels us to abandon the concept of a high-energy form of NAD^+ used in previous formulations, we prefer to retain the mechanism given in Eqns. 10–15.

One at least of the two theories—NAD $\sim$ I or fpH_2 $\sim$ I—is certainly "überflüssig" (KLINGENBERG AND SCHOLLMEYER[45]). Both require the isolation of the

postulated intermediates for their support. In this respect, we are encouraged by the demonstration of a form of NAD+ different from NADH or NAD+ which is formed during the reduction of NAD+ by succinate[27,29–31,52,53] or during the oxidation of NADH[54].

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