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

I. COMPARISON OF DIFFERENT HYDROGEN DONORS

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SUMMARY

1. The synthesis of glutamate from α-oxoglutarate and ammonia in rat-liver mitochondria has been studied. Under conditions where oxaloacetate is formed, some of the glutamate synthesized transaminates with the oxaloacetate and gives rise to aspartate.

2. In the presence of α-oxoglutarate, ammonia and oxygen, the synthesis of amino acids is stimulated by phosphate acceptor and by succinate.

3. In the presence of α-oxoglutarate, ammonia, succinate and oxygen, and in the absence of phosphate acceptor, the synthesis of amino acids is stimulated by oligomycin, arsenite or dinitrophenol. When both arsenite and dinitrophenol are added, very little synthesis occurs.

4. These results are interpreted in terms of three different mechanisms for the synthesis of amino acids from α-oxoglutarate and ammonia, in which reducing equivalents are supplied by α-oxoglutarate, succinate, and the products of oxidation of succinate, respectively.

5. The synthesis of glutamate with α-oxoglutarate as hydrogen donor is stimulated by phosphate acceptor and is completely inhibited by arsenite. α-Oxoglutarate is a relatively inefficient hydrogen donor.

6. With succinate as hydrogen donor, in the presence of arsenite, the amounts of glutamate and aspartate found represent the contribution of succinate and of malate (derived from succinate), respectively, as hydrogen donor.

7. The synthesis of glutamate with succinate as hydrogen donor is greatly stimulated by oligomycin, and greatly inhibited by antimycin, malonate or Amytal. It is concluded that this synthesis represents an energy-linked reversal of the respiratory chain.

8. The synthesis of glutamate + aspartate with malate as hydrogen donor, in the presence of arsenite and glutamate, is markedly inhibited by antimycin or Amytal, and stimulated by oligomycin. This shows that energy is also required for this reaction.

9. When isocitrate is the hydrogen donor, the synthesis of glutamate is very little affected by antimycin, Amytal, oligomycin or dinitrophenol, indicating that no energy is required for this reaction.

10. Inorganic phosphate or arsenate is necessary for maximum synthesis of amino acids from α-oxoglutarate and ammonia with succinate, malate or isocitrate as hydrogen donor.

11. The possibility that glutamate dehydrogenase in the mitochondria reacts exclusively with NADP is discussed.

INTRODUCTION

Recent work from several laboratories[1–6] has shown that glutamate is oxidized by isolated mitochondria, not only by glutamate dehydrogenase (EC 1.4.1.3), but also by a transamination pathway in which the end-product of the oxidation is aspartate. This transamination pathway, first described by MÜLLER AND LEUTHARDT[7], is virtually the only pathway for the oxidation of glutamate by muscle mitochondria which contain little glutamate dehydrogenase. It also forms the main mechanism for the oxidation of glutamate by isolated rat-liver mitochondria, at least in the presence of phosphate and phosphate acceptor, even though these mitochondria contain an active glutamate dehydrogenase.

Thus the physiological role of this enzyme is still uncertain. It is possible that it is to catalyse the reduction of α-oxoglutarate and ammonia by NADH to glutamate, rather than the thermodynamically less favourable oxidation of glutamate by NAD^+ (*cf.* ref. 8).

In 1939, KREBS AND COHEN[9] showed that kidney cortex and heart muscle catalyse the dismutation of α-oxoglutarate and NH_3 to form glutamate, succinate and CO_2 (Reaction 1)

$$2\ \alpha\text{-oxoglutarate} + NH_3 \rightarrow \text{glutamate} + \text{succinate} + CO_2 \qquad (1)$$

The KREBS–COHEN dismutation has since been demonstrated in mitochondrial preparations from rat liver[4,10,11] and kidney[11] and rabbit kidney[12].

KREBS *et al.*[9] found that the dismutation reaction in rat-liver homogenates is only slightly stimulated by anaerobiosis (*cf.* refs. 14, 15) and that the synthesis of glutamate from α-oxoglutarate and NH_3 is considerably enhanced by the addition of β-hydroxybutyrate (*cf.* DEWAN[16]), malate and, in particular, isocitrate (*cf.* ADLER *et al.*[17]), indicating that α-oxoglutarate is a relatively inefficient donor of reducing equivalents for glutamate synthesis. A similar requirement for a hydrogen donor for maximum synthesis of glutamate has been emphasized by studies linking this process with the oxidation of succinate[18–20].

In this and other papers of this series, the transfer of reducing equivalents from several different substrates to α-oxoglutarate + NH_3 in rat-liver mitochondria is considered. Some of the results of this investigation, which arose from unpublished observations by BORST, have been reported in a preliminary form[18,21,22].

METHODS

Rat-liver mitochondria

Rat-liver mitochondria were prepared by the method of HOGEBOOM[23] as described by MYERS AND SLATER[24].

Standard reaction mixture

The standard reaction mixture used in the experiments described in this paper contained 15 mM KCl, 2 mM EDTA, 5 mM $MgCl_2$, 50 mM Tris–HCl buffer, 0.1 mM ADP, 25 mM sucrose (derived from the mitochondrial suspension), and the additions indicated in the legends to the tables and figure, in a final volume of 1 ml. The final pH of the reaction mixture was 7.5. The reaction was carried out at 25° in Warburg flasks with 0.1 ml 2.0 M KOH and a fluted filter paper in the centre well, or, in the anaerobic experiments, in Thunberg tubes. The reaction was stopped by the addition of 0.05 ml 70% or 0.1 ml 35% $HClO_4$. After removal of the protein by centrifugation, the $HClO_4$ was removed as $KClO_4$ in the cold.

O_2 uptake

O_2 uptake was measured with differential manometers having small capillaries; with the small flasks used, the gas volume was 6–8 ml. The O_2 uptake during the temperature-equilibration period was calculated by extrapolation.

Anaerobic experiments

In the anaerobic experiments the mitochondrial suspension was placed in the hollow stopper and the other components in the main compartment of a Thunberg tube. The tube was evacuated and filled with O_2-free N_2 6 times and finally evacuated. After sealing the Thunberg tubes, 0.05 ml 70% $HClO_4$ was pipetted into the side-tube used to evacuate the apparatus. The side-tube was then sealed with a piece of rubber tubing and a clamp. The reaction was started by tipping the mitochondria from the hollow stopper into the main compartment and stopped by turning the stopper so that the $HClO_4$ was expelled from the side-tube into the evacuated Thunberg tube.

Determination of glutamate

Glutamate was determined with glutamate decarboxylase (EC 4.1.1.15) by the method of GALE[25]. CO_2 production was measured manometrically under aerobic conditions at 25°, using differential manometers with a small capillary and small Warburg flasks. The medium contained 0.14 M acetate buffer (pH 5.0), an aliquot of the neutralized deproteinized reaction mixture containing up to 2.5 μmoles glutamate in a final volume, after addition of the enzyme from the side-arm of the Warburg flask, of 1.1 ml. The preparations of glutamate decarboxylase used were free of aspartate decarboxylase.

Determination of aspartate

Aspartate was determined spectrophotometrically by the method of PFLEIDERER *et al.*[26]. Malate dehydrogenase (EC 1.1.1.37) was present in the medium and the reaction was started by the addition of aspartate transaminase (see below) which was free of glutamate dehydrogenase and lactate dehydrogenase (EC 1.1.1.27).

Determination of protein

Protein was determined by the biuret method as described by CLELAND AND SLATER[27], with egg albumin as standard.

Hexokinase

Hexokinase (EC 2.7.1.1) was prepared as described by DARROW AND COLOWICK[28], omitting the final crystallization step.

Aspartate transaminase

Mitochondrial aspartate transaminase (L-aspartate:2-oxoglutarate aminotransferase, EC 2.6.1.1) with a low K_m for aspartate was prepared from a KEILIN AND HARTREE[29] horse-heart-muscle preparation by a modification of the procedure described by BORST AND PEETERS[30]. The heart-muscle preparation was taken up in 0.2 M phosphate buffer (pH 7.5) and solubilized by adding an equal volume of 2% sodium deoxycholate (pH 8–9). After centrifuging, the supernatant (pH 7.5) was fractionated with solid $(NH_4)_2SO_4$. The fraction precipitating between 65 and 90% saturation with $(NH_4)_2SO_4$ was taken up in 5 mM Tris–HCl buffer (pH 7.8), dialysed overnight against the same buffer and introduced onto a DEAE-cellulose column which had been regenerated with 0.1 N NaOH and equilibrated with 5 mM Tris–HCl buffer (pH 7.8), under these conditions aspartate transaminase is not adsorbed. The column was washed with 5 mM Tris–HCl buffer (pH 7.8) and a mixture of a aspartate transaminase and malate dehydrogenase free from glutamate and lactate dehydrogenase was recovered in the effluent*.

Special chemicals and enzymes

Special chemicals and enzymes were obtained from the following sources: ADP, NAD^+ and glutamate decarboxylase, Sigma Chemical Co,; α-oxoglutarate, sodium succinate, NAD^+ and malate dehydrogenase, Boehringer und Soehne; L-malate, Nutritional Biochemicals Corp.; L-glutamate and 2,4-dinitrophenol, British Drug Houses; Amytal, Amsterdamsche Chinine Fabriek; DEAE-cellulose, Serva, Heidelberg. Antimycin was kindly donated by the Kyowa Fermentation Company; oligomycin (a mixture of oligomycin A and B) by the Upjohn Chemical Company, and L-(+)isocitric acid by Dr. W. C. HÜLSMANN.

RESULTS

KREBS–COHEN *dismutation and effect of succinate*

Reaction 1, the KREBS–COHEN[9] dismutation, is the sum of Reactions 2 and 3:

$$\alpha\text{-oxoglutarate} + NAD^+ \rightarrow \text{succinate} + CO_2 + NADH + H^+ \quad (2)$$

$$\alpha\text{-oxoglutarate} + NADH + NH_4^+ \rightleftharpoons \text{glutamate} + NAD^+ \quad (3)$$

* The method of preparing aspartate transaminase has been improved. The heart-muscle preparation is freeze-dried and the dry powder extracted with phosphate buffer (p_H 7.4) for 30 min at roomtemperature. After cooling to 0°, the suspension is centrifuged at high speed and the supernatant further treated as described above.

Since Reaction 2 actually proceeds according to Reactions 4 and 5:

$$\alpha\text{-oxoglutarate} + NAD^+ + CoA \rightarrow \text{succinyl-CoA} + NADH + H^+ + CO_2 \quad (4)$$

$$\text{succinyl-CoA} + ADP + H_3PO_4 \rightleftharpoons \text{succinate} + ATP + CoA \quad (5)$$

the dismutation of α-oxoglutarate + NH_3 is accompanied by the synthesis of ATP, as was first shown by HUNTER AND HIXON[10]. Thus, in order to provide optimum conditions for the KREBS–COHEN dismutation, phosphate and phosphate acceptor should be present so that CoA can be regenerated.

In Table I, the results of experiments are presented in which the synthesis of glutamate and aspartate from α-oxoglutarate + NH_3 in rat-liver mitochondria was measured under anaerobic and aerobic conditions. As was to be expected, under anaerobic conditions the formation of glutamate was enhanced by the presence of phosphate acceptor, and little aspartate was found. Unexpectedly, the exclusion of oxygen, which by preventing the oxidation of NADH by the respiratory chain would be expected to make more available for glutamate synthesis, did not lead to an enhancement of glutamate synthesis. This was not due to damage of the mitochondria during the evacuation procedure used to obtain anaerobic conditions, since mitochondria subjected to this procedure behaved similarly to untreated mitochondria under subsequent aerobic conditions. Moreover, BORST[3] obtained a value of 27 mμmoles/mg protein/min for the KREBS–COHEN[9] dismutation in rat-liver mitochondria in the presence of phosphate acceptor, when he allowed the reaction to proceed in Warburg flasks gassed with nitrogen; this value falls within the range for anaerobic conditions reported in Table I.

Under aerobic conditions, the addition of succinate, one of the products of α-oxoglutarate oxidation, markedly stimulated the synthesis of both glutamate and aspartate from oxoglutarate and NH_3, especially when phosphate acceptor was also present.

TABLE I

EFFECT OF PHOSPHATE ACCEPTOR, SUCCINATE AND ANAEROBIOSIS ON THE SYNTHESIS OF AMINO ACIDS FROM α-OXOGLUTARATE + NH_3

Experimental conditions described under METHODS. Reaction mixture contained the basic components plus 20 mM α-oxoglutarate, 20 mM NH_4Cl, 20 mM potassium phosphate buffer and 1.1–6.9 mg mitochondrial protein. In anaerobic experiments, the reaction was carried out in Thunberg tubes. Reaction time, 15 or 30 min. The phosphate acceptor was glucose (20 mM) + hexokinase (150 units)[60].

Additions	*Δ Glutamate (mμmoles/mg protein/min)*			*Δ Aspartate (mμmoles/mg protein/min)*		
	Mean	*Range*	*Number of measurements*	*Mean*	*Range*	*Number of measurements*
Anaerobic conditions						
None	9	8–11	3	3	3–5	3
Phosphate acceptor	17	7–34	5	1	0–3	4
Aerobic conditions						
None	11	2–17	15	3	2–7	11
Phosphate acceptor	22	10–46	20	11	3–22	10
Succinate (60 mM)	27	11–55	20	8	2–18	18
Succinate + phosphate acceptor	51	33–77	24	25	13–31	14

TABLE II

EFFECT OF INHIBITORS ON THE AEROBIC SYNTHESIS OF AMINO ACIDS FROM α-OXOGLUTARATE + NH_3 + SUCCINATE, IN THE ABSENCE OF PHOSPHATE ACCEPTOR

Reaction mixture contained basic components plus 20 mM α-oxoglutarate, 20 mM NH_4Cl, 60 mM succinate, 20 mM potassium phosphate buffer and 8.1 mg mitochondrial protein. Reaction time, 20 min. (Expt. 246).

Additions	*Δ O (μatoms)*	*Δ Glutamate (μmoles)*	*Δ Aspartate (μmoles)*
None	7.10	3.32	1.02
Oligomycin (1.2 μg/mg protein)	7.90	6.52	1.38
Dinitrophenol (50 μM)	11.03	3.74	1.21
Oligomycin + dinitrophenol	11.49	2.78	0.57
Arsenite (1 mM)	8.51	5.15	2.67
Arsenite + dinitrophenol	6.37	0.88	0.14
Oligomycin + arsenite	9.64	7.89	5.41
Oligomycin + arsenite + dinitrophenol	7.49	0.63	0.22

Table II shows the effect of various inhibitors on the succinate-stimulated synthesis of amino acids* from α-oxoglutarate and NH_3, measured in the absence of phosphate acceptor. Oligomycin, LARDY's[31] inhibitor of oxidative phosphorylation, markedly stimulated the synthesis of both glutamate and aspartate. This can not be due to suppression of the aerobic oxidation of NADH by the respiratory chain, since this was already inhibited by lack of phosphate acceptor. Indeed, oligomycin had little effect on the oxygen uptake.

Dinitrophenol, the uncoupler of oxidative phosphorylation, slightly stimulated the synthesis of both glutamate and aspartate and prevented the stimulation by oligomycin.

Arsenite, the inhibitor of α-oxoglutarate and pyruvate oxidation (*cf.* ref. 32), stimulated the synthesis of glutamate and especially of aspartate, both in the presence and absence of oligomycin.

Although both dinitrophenol and arsenite are on their own stimulatory, the addition of both compounds together brought the synthesis of glutamate and aspartate to low levels, both in the presence and absence of oligomycin. This is partly, but not mainly, accounted for by the inhibition of the oxygen uptake**. The specific inhibitory effect of dinitrophenol on glutamate synthesis in the presence of arsenite is clearly seen by comparing the ratios Δglutamate:ΔO. These are 0.60 (-oligomycin) and 0.82 (+oligomycin) in the absence of dinitrophenol and 0.14 (-oligomycin) and 0.08 (+oligomycin) in its presence.

In the presence of phosphate acceptor, and absence of oligomycin or dinitrophenol, arsenite inhibited the synthesis of both glutamate and aspartate (Table III).

As will be described in DISCUSSION, these results are interpreted in terms of three different mechanisms for the synthesis of glutamate and aspartate, in which

* Only glutamate and aspartate, and in some cases traces of alanine, were detected by paper chromatography[3]. In particular, glutamine was absent.

** The rate of O_2 uptake in the presence of succinate arsenite and dinitrophenol declines with time. This is believed to be due to inhibition of succinate dehydrogenase by oxaloacetate accumulating under these conditions[33], since it does not occur in the presence of Amytal.

TABLE III

ARSENITE INHIBITION OF AMINO ACID SYNTHESIS FROM α-OXOGLUTARATE + NH_3 + SUCCINATE IN THE PRESENCE OF PHOSPHATE ACCEPTOR

Experimental conditions described under METHODS. Reaction mixture contained basic components plus 20 mM α-oxoglutarate, 20 mM NH_4Cl, 60 mM succinate, 20 mM potassium phosphate buffer, 20 mM glucose, 150 units hexokinase and 3.2 mg (Expt. 66) or 3.7 mg (Expt. 69) mitochondrial protein. Reaction time, 15 min.

Expt.	*Arsenite (mM)*	*Δ O (μatoms)*	*Δ Glutamate (μmoles)*	*Δ Aspartate (μmoles)*
66	0	6.1	2.02	1.18
	1	5.7	0.95	0.94
69	0	6.7	2.25	1.69
	1	6.9	0.93	1.15

reducing equivalents are supplied respectively by α-oxoglutarate, succinate and the products of oxidation of succinate. In the following section, the efficiencies of different hydrogen donors for amino acid synthesis are compared.

Comparison of different hydrogen donors for glutamate and aspartate synthesis

α-Oxoglutarate, succinate, malate and isocitrate as hydrogen donors are compared directly in Table IV. Although hexokinase was added, oligomycin was added to prevent phosphorylation of the phosphate acceptor, so that oxidation of the

TABLE IV

HYDROGEN DONORS FOR THE SYNTHESIS OF GLUTAMATE AND ASPARTATE FROM α-OXOGLUTARATE + NH_3 IN RAT-LIVER MITOCHONDRIA

Reaction mixture contained basic components plus 13 mM α-oxoglutarate, 20 mM NH_4Cl, 20 mM potassium phosphate buffer, 20 mM glucose, 150 units hexokinase, 1% ethanol, 10 μg oligomycin and 8.4 mg mitochondrial protein. Reaction time, 20 min.

Additions	*Δ O (μatoms)*	*Δ Glutamate (μmoles)*	*Δ Aspartate (μmoles)*
None	2.60	3.10	1.14
Arsenite (1 mM)	0.41	0	0.01
Arsenite + succinate (60 mM)	9.80	6.37	5.04
Arsenite + malate (20 mM) + glutamate (10 mM)	2.27	−0.74	12.82
Arsenite + isocitrate (20 mM)	0.61	5.07	0.01

NADH by the respiratory chain was inhibited. The comparison of the last three donors is made possible by the fact that the oxidation of α-oxoglutarate, which is of necessity added as hydrogen acceptor, can be prevented by the addition of arsenite*. Under these conditions, no amino acids are found (second line of Table IV) unless

* Experiments with the oxygen polarograph have shown that arsenite does not inhibit the oxidation of glutamate or α-oxoglutarate instantaneously. However, although this can cause difficulties in short-time experiments with the oxygen polarograph, it introduces only negligible errors in the more prolonged experiments described in this paper.

other hydrogen donors are added. The value obtained in the absence of arsenite (first line of Table IV) represents an upper limit for the contribution of the KREBS–COHEN dismutation, since under these conditions reducing equivalents can also be provided by succinate formed from the oxidation of α-oxoglutarate and by further oxidation products, and perhaps by endogenous substrate[34].

With succinate as donor, both glutamate and aspartate were found. It will be argued below that the amounts of these two amino acids formed under these conditions represent the contribution of succinate and malate, respectively, to the reduction of the α-oxoglutarate (+NH_3). The effectiveness of malate as hydrogen donor is shown directly in the fourth line of Table IV. Glutamate was added in order to favour the operation of the malate dehydrogenase by removing by transamination the oxaloacetate formed. The glutamate concentration remained almost constant, while large amounts of aspartate were formed.

In agreement with the work of KREBS *et al.*[13] and ADLER *et al.*[17], isocitrate was also found to be an effective donor of reducing equivalents for glutamate synthesis. Since, in the presence of arsenite, no malate can be formed by oxidation of isocitrate in the Krebs cycle, the absence of aspartate formation is to be expected. Since any oxaloacetate formed from isocitrate via SRERE AND LIPMANN'S citrate-cleavage enzyme[35] would be expected to react with the glutamate formed to give aspartate, it may be concluded that the reaction catalysed by this enzyme is not proceeding to any significant extent under the conditions of this experiment.

Succinate as hydrogen donor

The addition of phosphate acceptor had no effect on the amount of glutamate synthesized, either in the presence or absence of oligomycin, but stimulated the aspartate synthesis in the absence of oligomycin (Table V). Oligomycin considerably stimulated the synthesis of glutamate with succinate as hydrogen donor, both in the presence and absence of phosphate acceptor, and also stimulated the synthesis of aspartate in the absence of phosphate acceptor. It is noteworthy that, in the

TABLE V

STIMULATION BY OLIGOMYCIN OF GLUTAMATE SYNTHESIS COUPLED TO THE AEROBIC OXIDATION OF SUCCINATE

Reaction mixture contained the basic components plus 20 mM α-oxoglutarate, 20 mM NH_4Cl, 60 mM succinate, 1 mM arsenite, 20 mM potassium phosphate buffer, and 2.5–6.9 mg mitochondrial protein. In most experiments, 1 or 2% ethanol was also present. Reaction time, 15, 20 or 30 min. The phosphate acceptor was glucose (20 mM) + hexokinase (150 units).

Phosphate acceptor	*Oligomycin (10 μg/ml)*	*Δ Glutamate (mμmoles/mg protein/min)*			*Δ Aspartate (mμmoles/mg protein/min)*		
		Mean	*Range*	*Number of measurements*	*Mean*	*Range*	*Number of measurements*
Absent	Absent	23	9–49	13	9	2–21	13
Present	Absent	23	17–29	12	21	8–29	12
Absent	Present	49	32–67	14	22	7–33	14
Present	Present	44	37–47	13	24	18–30	13

TABLE VI

INHIBITION BY MALONATE AND ANTIMYCIN OF GLUTAMATE SYNTHESIS COUPLED TO THE AEROBIC OXIDATION OF SUCCINATE

Reaction mixture contained basic components plus 20 mM potassium phosphate buffer, 20 mM NH_4Cl, 20 mM α-oxoglutarate, 60 mM succinate, 1 mM arsenite, 2% ethanol and 5.5 mg (Expt. 90), 4.0 mg (Expt. 104) or 4.2 mg (Expt. 119) mitochondrial protein. Reaction time, 15 min (Expt. 104), 20 min (Expt. 90) or 30 min. (Expt. 119).

Expt.	*Additions*	*Δ O (μatoms)*	*Δ Glutamate (μmoles)*	*Δ Aspartate (μmoles)*
90	Oligomycin (1.8 μg/mg protein)	7.42	6.23	3.09
	Oligomycin + malonate (20 mM)	1.17	0.61	0.17
104	Oligomycin (2.5 μg/mg protein)	4.51	4.22	2.42
	Oligomycin + antimycin (0.5 μg/mg protein)	0.37	0.49	0
119	None	6.75	2.74	0.71
	Antimycin (0.95 μg/mg protein)	0.61	0.25	0

absence of phosphate acceptor or oligomycin, the amount of glutamate synthesized varied considerably from preparation to preparation.

The synthesis of both glutamate and aspartate was inhibited by at least 90% by antimycin or malonate (Table VI). There was a close correlation between the degree of inhibition of the aerobic oxidation of succinate by antimycin and the degree of inhibition of glutamate and aspartate synthesis (Table VII).

TABLE VII

EFFECT OF ANTIMYCIN CONCENTRATION ON THE SYNTHESIS OF GLUTAMATE COUPLED TO THE AEROBIC OXIDATION OF SUCCINATE IN RAT-LIVER MITOCHONDRIA

Reaction mixture contained basic components plus 20 mM α-oxoglutarate, 20 mM NH_4Cl, 60 mM succinate, 1 mM arsenite, 20 mM potassium phosphate buffer, 2% ethanol, 10 μg oligomycin and 7.1 mg mitochondrial protein. Reaction time, 28 min. (Expt. 151).

Antimycin (μg/mg protein)	*Δ O*		*Δ Glutamate*		*Δ Aspartate*	
	μatoms	*Inhibition (%)*	*μmoles*	*Inhibition (%)*	*μmoles*	*Inhibition (%)*
0	13.8		10.1		5.84	
0.014	13.0	6	8.85	12	6.06	−4
0.028	11.1	20	7.65	24	4.94	15
0.042	5.60	59	3.09	69	1.77	70
0.070	1.10	92	0.57	94	0.60	90

Amytal also inhibited the synthesis of glutamate and aspartate (Table VIII). The inhibition of the oxygen uptake when oligomycin and Amytal were both present is discussed in another paper[36].

Malate as hydrogen donor

The net synthesis of glutamate + aspartate with malate as hydrogen donor,

TABLE VIII

INHIBITION BY AMYTAL OF GLUTAMATE SYNTHESIS COUPLED TO THE AEROBIC OXIDATION OF SUCCINATE

Reaction mixture contained basic components plus 20 mM potassium phosphate buffer, 20 mM NH_4Cl, 10 mM (Expt. 78) or 20 mM (Expt. 97) α-oxoglutarate, 60 mM succinate, 1 mM arsenite, 2% ethanol and 4.0 mg (Expt. 78) or 3.4 mg (Expt. 97) mitochondrial protein. Reaction time, 15 min.

Expt.	*Additions*	*Δ O (μatoms)*	*Δ Glutamate (μmoles)*	*Δ Aspartate (μmoles)*
78	None	3.44	0.69	0.24
	Oligomycin (0.5 μg/mg protein)	4.47	3.24	1.44
	Amytal (2 mM)	4.13	0.69	0.38
	Oligomycin + Amytal	2.71	0.54	0.90
97	None	4.16	2.68	1.14
	Oligomycin (1.7 μg/mg protein)	4.20	3.62	1.78
	Amytal (2 mM)	4.10	0.65	0.48
	Oligomycin + Amytal	2.60	0.69	0.39

in the presence of arsenite and glutamate, was partially inhibited by the addition of phosphate acceptor (Table IX, line 4) and this inhibition was relieved by oligomycin (line 5). In the absence of phosphate acceptor also, oligomycin stimulated the synthesis of amino acids. Antimycin and Amytal were markedly inhibitory. Since these two inhibitors prevent the oxidation of NADH by the respiratory chain, this inhibition of amino acid synthesis was unexpected. It will be further discussed below.

TABLE IX

EFFECT OF INHIBITORS AND OF PHOSPHATE ACCEPTOR ON THE SYNTHESIS OF GLUTAMATE + ASPARTATE COUPLED TO THE AEROBIC OXIDATION OF MALATE IN RAT-LIVER MITOCHONDRIA

Reaction mixture contained basic components plus 20 mM α-oxoglutarate, 20 mM NH_4Cl, 20 mM malate, 10 mM glutamate, 1 mM arsenite, 2% ethanol and 6.9 mg mitochondrial protein. Reaction time, 20 min. (Expt. 168).

Additions	*Δ O (μatoms)*	*Δ Glutamate (μmoles)*	*Δ Aspartate (μmoles)*	*Δ (Glutamate + aspartate) (μmoles)*
None	3.26	−0.32	7.28	6.96
Oligomycin (10 μg)	2.13	−0.11	9.16	9.05
Antimycin (5 μg)	0.73	0.86	1.69	2.55
Glucose (20 mM) + hexokinase (150 units)	4.87	−2.41	6.92	4.51
Glucose + hexokinase + oligomycin	2.09	0.21	7.70	7.91
Amytal (2 mM)	0.23	0.54	0.89	1.43

Isocitrate as hydrogen donor

In contrast with the results obtained with succinate or malate as hydrogen donor, oligomycin, antimycin or Amytal had little effect on glutamate synthesis with isocitrate as hydrogen donor in the presence of arsenite (Table X). The addition of dinitrophenol or phosphate acceptor also had little effect.

TABLE X

EFFECT OF INHIBITORS, DINITROPHENOL, PHOSPHATE ACCEPTOR AND ABSENCE OF INORGANIC PHOSPHATE ON GLUTAMATE SYNTHESIS COUPLED TO THE AEROBIC OXIDATION OF ISOCITRATE IN RAT-LIVER MITOCHONDRIA

Reaction mixture contained basic components and 20 mM α-oxoglutarate, 20 mM NH_4Cl, 20 mM potassium phosphate buffer, 1 mM arsenite, 10 mM isocitrate, 2% ethanol and 3.4 mg (Expt. 162) or 6.1 mg (Expt. 167) mitochondrial protein. Reaction time, 20 min.

Additions or omissions	*Δ Glutamate (μmoles)*	
	Expt. 162	*Expt. 167*
None	2.22	4.18
+ oligomycin (10 μg)	2.10	4.20
+ antimycin (1 μg)	2.01	—
+ Amytal (2 mM)	1.91	3.85
+ dinitrophenol (50 μM)	2.04	—
+ glucose (20 mM) + hexokinase (150 units)	1.92	—
− inorganic phosphate	—	3.00

Phosphate requirement for the synthesis of glutamate and aspartate

When inorganic phosphate was omitted from the reaction mixture, the synthesis of glutamate and aspartate coupled with the aerobic oxidation of succinate was markedly inhibited, both in the presence and in the absence of oligomycin (Table XI). Phosphate could be replaced by arsenate, but not by borate or nitrate.

TABLE XI

INORGANIC PHOSPHATE REQUIREMENT FOR THE SYNTHESIS OF GLUTAMATE COUPLED TO THE OXIDATION OF SUCCINATE

Reaction mixture contained basic components plus 20 mM NH_4Cl, 20 mM α-oxoglutarate, 60 mM succinate, 1 mM arsenite and 6.4 mg (Expt. 88) or 3.4 mg (Expt. 97) mitochondrial protein. In Expt. 88, 2% ethanol was present. Reaction time, 15 min.

Expt.	*Additions*	*Δ O (μatoms)*	*Δ Glutamate (μmoles)*	*Δ Aspartate (μmoles)*
88	None	3.30	1.08	0.07
	Phosphate (20 mM)	6.44	2.62	0.88
	Arsenate (10 mM)	10.1	2.50	1.52
	Oligomycin (0.94 μg/mg protein)	3.31	1.91	0.29
	Oligomycin + phosphate	6.70	4.83	1.81
	Oligomycin + arsenate	6.12	4.58	1.80
97	None	2.76	1.05	0.26
	Phosphate (20 mM)	4.46	2.68	1.14
	Borate (20 mM)	2.62	0.72	0.20
	Nitrate (20 mM)	2.98	0.89	0.22

Fig. 1 shows the effect of varying concentrations of inorganic phosphate on the synthesis of amino acids and on the oxygen uptake. The synthesis of glutamate and uptake of oxygen increased as the concentration of added inorganic phosphate was increased from 0 to 3 mM, and then remained constant. Maximum aspartate syn-

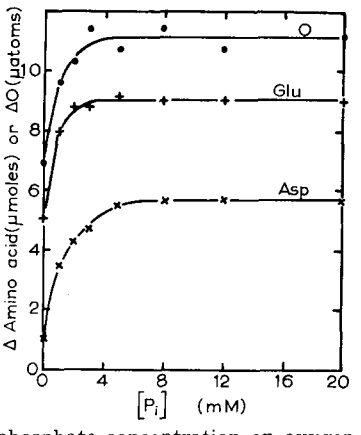

Fig. 1. Influence of inorganic phosphate concentration on oxygen uptake and synthesis of glutamate and aspartate coupled to succinate oxidation in presence of oligomycin. Reaction mixture contained basic components plus 20 mM α-oxoglutarate, 20 mM NH_4Cl, 60 mM succinate, 1 mM arsenite, 1% ethanol, 10 μg oligomycin and 5.9 mg mitochondrial protein. Reaction time, 30 min.

thesis was reached at a slightly higher concentration of added inorganic phosphate. The reason for the increased oxygen uptake is discussed in another paper[36].

That the effect of omission of inorganic phosphate is not due to irreversible structural damage to the mitochondria is shown by the experiment presented in Table XII. Mitochondria were incubated with α-oxoglutarate, NH_3, arsenite, succinate and oligomycin in the presence and in the absence of added inorganic phosphate.

TABLE XII

REVERSIBILITY OF EFFECT OF ABSENCE OF INORGANIC PHOSPHATE ON GLUTAMATE SYNTHESIS COUPLED TO THE AEROBIC OXIDATION OF SUCCINATE

Reaction mixture contained basic components plus 20 mM NH_4Cl, 20 mM α-oxoglutarate, 60 mM succinate, 1 mM arsenite, 2% ethanol, 6 μg oligomycin and 3.0 mg mitochondrial protein. 20 mM potassium phosphate (pH 7.5) present from start of reaction where indicated or tipped into main compartment of Warburg vessel from side arm 15 min after addition of mitochondria. (Expt. 86).

Conditions	*Δ Glutamate (μmoles)*		*Δ Aspartate (μmoles)*	
	0–15 min	*15–30 min*	*0–15 min*	*15–30 min*
Phosphate absent	0.61	0.60	0.20	0.07
Phosphate tipped in 15 min after mitochondria	—	2.20	—	0.91
Phosphate present from start	2.64	2.57	0.83	0.75

Glutamate and aspartate synthesis were measured after 15 and 30 min. In one flask, inorganic phosphate was tipped into the reaction mixture 15 min after the mitochondria were added. When inorganic phosphate was added at the beginning of the incubation, equal amounts of glutamate were synthesized in the first and second 15-min periods. In the absence of added phosphate, the glutamate synthesis was 77% less, but when inorganic phosphate was added after 15 min the synthesis

TABLE XIII

COMPARISON OF EFFECT OF ADDED INORGANIC PHOSPHATE ON GLUTAMATE AND ASPARTATE SYNTHESIS WITH DIFFERENT HYDROGEN DONORS

Reaction mixture contained basic components plus 20 mM α-oxoglutarate, 20 mM NH_4Cl, 1 mM arsenite, 10 μg oligomycin, 1% ethanol and 6.8 mg mitochondrial protein (Expt. 165) or mitochondria from approximately 400 mg (wet weight) of liver (Expt. 166). Reaction time, 20 min.

Expt.	*Additions*	*Δ Glutamate (μmoles)*	*Δ Aspartate (μmoles)*	*Δ (Glutamate + aspartate) (μmoles)*
165	Succinate (60 mM)	2.53	0.65	3.11
	Succinate + phosphate (20 mM)	4.58	2.11	6.69
	Isocitrate (20 mM)	2.44	0.10	2.54
	Isocitrate + phosphate	3.30	0.05	3.35
166	Malate (20 mM) + glutamate (5 mM)	−0.43	5.07	4.64
	Malate + glutamate + phosphate (20 mM)	0.35	5.70	6.05

in the next 15 min was almost the same as that found when inorganic phosphate had been present from the start of the incubation. Similar results were obtained for the aspartate synthesis.

When the hydrogen donor was malate or isocitrate, amino acid synthesis was also decreased by omission of inorganic phosphate, but the effects were less than when succinate was the hydrogen donor (Tables X and XIII).

DISCUSSION

When α-oxoglutarate, succinate or malate was used as hydrogen donor for the reduction of α-oxoglutarate ($+NH_3$) to glutamate, aspartate was also formed (*cf.* WORCEL AND ERECINSKA[15]). In the presence of phosphate acceptor and absence of inhibitors, this aspartate could have been formed by oxidation of glutamate, since aspartate is the end-product of glutamate oxidation by rat-liver mitochondria under these conditions[1-6]. This cannot be the explanation, however, of the considerable amounts of aspartate formed in the absence of phosphate acceptor, or in the presence of oligomycin or arsenite, which inhibit the oxidation of glutamate to aspartate. Since the only known mechanism of aspartate synthesis is by transamination of oxaloacetate from glutamate (Reaction 6)

$$\text{oxaloacetate} + \text{glutamate} \rightleftharpoons \text{aspartate} + \alpha\text{-oxoglutarate} \quad (6)$$

the amount of aspartate found at the end of the reaction is a reflection of the amount of oxaloacetate formed during the course of the reaction. At least in the presence of arsenite, the only known reaction leading to formation of oxaloacetate is Reaction 7

$$\text{malate} + NAD^+ \rightleftharpoons \text{oxaloacetate} + NADH + H^+ \quad (7)$$

In the presence of oligomycin (or absence of phosphate acceptor), NADH is not oxidized by the phosphorylating respiratory chain, but it is readily available for the glutamate dehydrogenase catalysing Reaction 8

$$NADH + H^+ + \alpha\text{-oxoglutarate} + NH_3 \rightleftharpoons NAD^+ + \text{glutamate} \quad (8)$$

The sum of Reactions 6–8 is Reaction 9

$$\text{malate} + NH_3 \rightleftharpoons \text{aspartate} \qquad (9)$$

Thus, in the absence of phosphate acceptor or in the presence of oligomycin, the amount of aspartate found is a measure of the rate of reduction of NAD^+ by malate*.

This interpretation is supported by the findings that, when malate was hydrogen donor, in the presence of oligomycin and with glutamate added at the beginning of the reaction, aspartate was synthesized with little change in the amount of glutamate (Tables IV, IX, XIII), whereas when isocitrate was hydrogen donor, in the presence of arsenite which prevents the formation of malate, glutamate but no aspartate was found at the end of the reaction (Tables IV, XIII).

Thus, the hydrogen donor for glutamate synthesis linked with the oxidation of succinate cannot be the malate formed by oxidation of the succinate. The NADH necessary for glutamate synthesis (Reaction 8) must have been formed in a reaction other than Reaction 7. In the presence of arsenite, α-oxoglutarate cannot serve as a hydrogen donor and the oxidation of any oxaloacetate escaping Reaction 6 is inhibited. This leaves succinate as the only possible donor of hydrogen equivalents for the reduction of α-oxoglutarate ($+NH_3$) in the experiment described in line 3 of Table IV and in similar experiments. Since the reduction of NAD^+ by succinate requires energy[38] (Reaction 10)

$$\text{succinate} + NAD^+ + \sim \rightleftharpoons \text{fumarate} + NADH + H^+ \qquad (10)$$

the sensitivity of glutamate synthesis to dinitrophenol (Table II) is understandable, as is also the sensitivity to inhibitors of the aerobic oxidation of succinate (Tables VI–VIII), which is necessary to provide the ~. Similarly, since in the presence of arsenite, the oxidation of isocitrate is confined to the one-step oxidation to α-oxoglutarate, isocitrate is the only possible donor of hydrogen equivalents in the experiment described in line 5 of Table IV, NADPH being formed by Reaction 11

$$\text{isocitrate} + NADP^+ \rightarrow \alpha\text{-oxoglutarate} + NADPH + H^+ + CO_2 \qquad (11)$$

which does not require energy. Thus, the reduction of α-oxoglutarate ($+NH_3$) by isocitrate is not inhibited by dinitrophenol or respiratory inhibitors (Table X).

To summarize the situation in the presence of arsenite: When succinate is added the aspartate found is derived from malate-reduced NAD^+ (Reaction 7) and the glutamate found is derived from succinate-reduced NAD^+ (Reaction 10). When isocitrate is added, the glutamate found is derived from isocitrate-reduced $NADP^+$ (Reaction 11) and no aspartate is found.

The situation is much more complicated when arsenite is not present, since α-oxoglutarate can then act both as donor and acceptor (the KREBS–COHEN dismutation). The activity of α-oxoglutarate as donor is best measured under anaerobic conditions (Table I). In the presence of phosphate acceptor, necessary for Reaction 5, an average of 17 mμmoles glutamate/mg protein/min could be synthesized by this

* There are two possible sources of error in equating the aspartate synthesized with the NAD^+ reduced by malate. The first is due to any NADH which is oxidized by the respiratory chain even in the absence of phosphate acceptor, or in the presence of oligomycin. This is, however, very small even in presence of phosphate acceptor[37]. The second possible error is due to the small amount of oxaloacetate remaining at the end of the experiment. This can be allowed for by measuring the oxaloacetate (see refs. 36, 37).

pathway, compared with 49 mμmoles glutamate/mg protein/min with succinate as donor in the presence of oligomycin and oxygen (Table V). Under aerobic conditions, the succinate formed from the oxidation of α-oxoglutarate and further oxidation products can also provide reducing equivalents. Thus, the expected decline of the amount of glutamate synthesized by the KREBS–COHEN dismutation by introducing the possibility for the aerobic oxidation of the NADH is more than compensated by the increased rate of reduction of NAD^+ by the additional hydrogen donors formed (Table I). With both succinate and α-oxoglutarate in the absence of arsenite, as in Table III, Reactions 2, 7 and 10 are all available for the reduction of NAD^+. Moreover, any oxaloacetate not reacting with glutamate can be converted to isocitrate, introducing Reaction 11 as a fourth possibility. In the presence of arsenite, Reaction 2 is eliminated and isocitrate can no longer be formed from oxaloacetate. The effect of removal of Reaction 2 (and Reaction 11) by arsenite is indicated by the inhibition of glutamate and aspartate synthesis in the experiments shown in Table III.

In the absence of phosphate acceptor, as in Table II, Reaction 2 will contribute much less to the synthesis of glutamate and aspartate. Thus, the addition of arsenite might be expected not to cause much inhibition of amino acid synthesis. In fact, it caused a stimulation of glutamate synthesis and especially of aspartate synthesis, both in the presence and absence of oligomycin. The reason for this is not clear.

Dinitrophenol in the absence of arsenite, by inducing the hydrolysis of ATP[39], will promote Reaction 2. Moreover, by stimulating the oxidation of succinate to malate, it would be expected to promote Reaction 7 and to provide more isocitrate for Reaction 11. Thus, the inhibition of Reaction 10 might possibly be more than compensated for by the stimulation of Reactions 2 and 11, which appears to have been the case in the experiment illustrated in line 3 of Table II. In the absence of arsenite, dinitrophenol will also stimulate the oxidation of glutamate to aspartate. This reaction and the stimulation of Reaction 7 would be expected to lead to greatly increased amounts of aspartate. This was not the case. Moreover, in the presence of arsenite and dinitrophenol, practically no aspartate was formed. These findings were puzzling until it was shown by the experiment summarized in Table IX that energy, provided in this case by the aerobic oxidation of malate, is necessary for the reduction of α-oxoglutarate ($+ NH_3$) by malate (see further refs. 22, 40, 41). This energy could not be available in the presence of dinitrophenol.

To summarize, the results in Table II can be explained on the assumption that there are at least three and probably four mechanisms concerned in the amino acid synthesis: (a) the arsenite-sensitive KREBS–COHEN dismutation which is stimulated by dinitrophenol, (b) the arsenite-insensitive dinitrophenol-sensitive reduction of α-oxoglutarate ($+NH_3$) by succinate, (c) the arsenite-stimulated and dinitrophenolsensitive amination of malate to aspartate, via the reduction of α-oxoglutarate ($+NH_3$) by malate, (d) the dinitrophenol-insensitive reduction of α-oxoglutarate ($+NH_3$) by isocitrate formed from oxaloacetate in an arsenite-sensitive reaction*.

* It is not excluded that pyruvate formed by the decarboxylation of oxaloacetate can also provide hydrogen equivalents for glutamate synthesis.

In the presence of both arsenite and dinitrophenol, no amino acids are formed. The existence of these different mechanisms explain the apparently paradoxical finding of BORST (see also ref. 42) which initiated this investigation, that the addition of phosphate acceptor or dinitrophenol, in the presence of α-oxoglutarate and succinate, led to an increased synthesis of glutamate.

The original postulate of CHANCE AND HOLLUNGER[38] that the succinate-induced reduction of mitochondrial NAD^+ was due to an energy-requiring reversal of the respiratory chain, in which reducing equivalents are supplied by the succinate, has been strongly supported by the investigations of KLINGENBERG[20,40,43], ERNSTER[44,45] and SNOSWELL[34]. This interpretation has, however, been challenged by KREBS and colleagues[46–49] who offered the alternative explanation that succinate promoted the reduction of the mitochondrial NAD^+ first by "saturating" the respiratory chain which would lead to inhibition of the oxidation of the NADH (*cf.* WU AND TSOU[50]), secondly by providing reducing equivalents for NAD^+ reduction in the form of malate formed by the oxidation of the succinate.

The results reported in Tables I–III indicate that both CHANCE's and KREBS' mechanisms can be operating. In the presence of dinitrophenol, the energy-requiring reduction by succinate is inhibited, but glutamate can still be synthesized by a mechanism in which reducing equivalents can be provided by α-oxoglutarate or by the products of succinate oxidation. The inhibition of NADH oxidation brought about by the rapid oxidation of succinate might possibly play a contributory role as suggested by KREBS. Thus, in the presence of dinitrophenol, glutamate is synthesized by a mechanism essentially that proposed by KREBS. However, malate is a relatively ineffective donor under these conditions, since energy is necessary for the maximal reduction of α-oxoglutarate + NH_3 by malate and could not be available in the presence of dinitrophenol.

In our opinion, our results show unequivocally that, although conditions can be found in which the mechanism proposed by KREBS satisfactorily explains the synthesis of glutamate (*cf.* ERNSTER[51]), under other conditions the type of mechanism first proposed by CHANCE AND HOLLUNGER[38] operates.

In a preliminary report of this investigation[18], we suggested that even in the presence of dinitrophenol glutamate was synthesized by reversal of the respiratory chain, the energy being provided by the dinitrophenol-resistant[52,53] substrate-linked phosphorylation step associated with α-oxoglutarate oxidation (*cf.* ERNSTER[44,45]). This explanation had been made attractive by the suggestion of AZZONE AND ERNSTER[54] that the ATP formed in this reaction was not accessible to the dinitrophenol-induced ATPase. Further study has, however, brought the following evidence against our earlier interpretation:

1. Since the succinate-linked reduction of NAD^+ is insensitive to oligomycin[21,34,45,55], an energy-rich intermediate of oxidative phosphorylation that is formed before the oligomycin block must react in reversed electron transfer. Oligomycin should, therefore, completely inhibit reversed electron transfer when ATP is the energy source, regardless of whether ATP is added exogenously (*cf.* ref. 56) or is formed in the substrate-linked phosphorylation step. In the experiment shown in Table II, oligomycin has a relatively slight effect on glutamate synthesis and this can be explained by the inhibition by oligomycin of the dinitrophenol-induced ATPase, necessary to supply the ADP for Reaction 5.

2. Our own studies[57] on the mechanism of oxidation of glutamate in the presence of dinitrophenol and absence of phosphate did not support AZZONE AND ERNSTER's conclusion that the ATP formed in the substrate-linked phosphorylation step is inaccessible to the dinitrophenol-induced ATPase. It is unlikely, then, that this ATP will be available for reversed electron transport in the presence of dinitrophenol.

3. It is unlikely that, in the presence of the high concentrations of inorganic phosphate used in this work, ATP formed in the substrate-linked phosphorylation step would be able to provide sufficient energy to promote Reaction 10 (*cf.* ref. 36).

Studies reported in this and in subsequent papers on the mechanism of glutamate synthesis with different hydrogen donors have revealed important differences in principle. Reduction of α-oxoglutarate ($+NH_3$) by succinate is an endergonic reaction and requires the provision of high-energy compounds (either intermediates of oxidative phosphorylation or ATP) in stoicheiometric amounts. Reduction by malate also requires energy[22,40,41], but in less than stoicheiometric amounts, since one high-energy bond is able to promote the formation of more than one molecule of aspartate[37]. It is noteworthy that Reaction 9, the sum reaction when malate is the hydrogen donor, is easily reversible. That reduction of α-oxoglutarate + NH_3 by isocitrate, an exergonic reaction, does not require energy is shown by the fact that Amytal or antimycin, which block the aerobic oxidation of isocitrate and therefore the supply of high-energy compounds, do not inhibit this reaction, which was also the only one not stimulated by oligomycin.

It is noteworthy that amino acid synthesis is not inhibited by addition of phosphate acceptor when succinate (Table V) or isocitrate (Table X) is the hydrogen donor, and is only slightly inhibited when malate is the donor (Table IX). Since the addition of phosphate acceptor promotes the oxidation of NADH, it appears that in all cases glutamate dehydrogenase can compete readily with the respiratory chain for the NADH. These findings are in striking contrast with the results obtained by ERNSTER[45] in similar experiments in which acetoacetate is the hydrogen acceptor. ERNSTER found that the reduction of acetoacetate by succinate was completely abolished by addition of phosphate and phosphate acceptor.

A possible explanation is that the nicotinamide nucleotide which reacts with glutamate dehydrogenase in intact mitochondria is NADPH, rather than NADH, as assumed in this paper until now. Although the isolated dehydrogenase reacts with both NAD and NADP, several lines of evidence led KLINGENBERG and coworkers[8,40,43] to suggest that the enzyme in the intact mitochondria is specific for NADP. Since KLINGENBERG AND SLENCZKA[43] have also shown that NADPH responds much more slowly than NADH to addition of phosphate acceptor, this suggestion would be in line with the insensitivity of glutamate synthesis to the addition of phosphate acceptor. It would also be in agreement with the well-established role of NADPH in various synthetic reactions (*cf.* KREBS[58,59]).

The insensitivity of glutamate synthesis to the addition of phosphate acceptor when succinate is hydrogen donor raises an additional difficulty, which is related to the large stimulation by oligomycin, both in the absence and presence of phosphate acceptor. This will be discussed in a later paper[36]. The stimulation of aspartate synthesis by oligomycin (Tables II and V) is probably secondary to this increased glutamate synthesis, which leads to increased formation of fumarate and therefore of malate available for aspartate synthesis.

Inorganic phosphate or arsenate was required for the maximum synthesis of amino acids when either succinate, malate or isocitrate was the hydrogen donor, although the effect was most marked with succinate. The fact that phosphate is required with all three hydrogen donors, whether or not energy is required for the reduction of α-oxoglutarate ($+NH_3$), suggests that this requirement is unrelated to the energy requirement. Since neither the reduction of mitochondrial NAD^+ by succinate[34] nor the reduction of acetoacetate by succinate[45] requires phosphate, it appears that this requirement is localized in the glutamate dehydrogenase reaction. Its exact nature, however, remains obscure.

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