BBA 45506

FACTORS AFFECTING THE PATHWAY OF GLUTAMATE OXIDATION IN RAT-LIVER MITOCHONDRIA

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(Received July 14th, 1966)

SUMMARY

1. The pathway of glutamate oxidation in isolated rat-liver mitochondria in the presence of phosphate and phosphate acceptor has been studied.

2. In freshly prepared mitochondria, in experiments extending over 6–40 min at 25°, an average of 90% of the glutamate oxidized was converted to aspartate and 10% to ammonia, independent of the initial concentration of glutamate. Most of the ammonia was formed during the first 10 min.

3. When malonate is present as well as glutamate, the transamination pathway is suppressed and the formation of ammonia is stimulated.

4. Ageing of mitochondria leads to inhibition of the transamination pathway and stimulation of the deamination of glutamate. In aged mitochondria the contribution of the deamination pathway increases as the initial concentration of glutamate is lowered.

5. Uncoupling agents inhibit the transamination pathway of glutamate oxidation. This inhibition is localized in the reactions leading from malate to aspartate.

6. Uncoupling agents stimulate the deamination of glutamate. This is correlated with an increase in the oxidation level of NADP.

INTRODUCTION

Glutamate is rapidly oxidized by mitochondria from a variety of tissues. In 1960–1961, a number of investigators[1–5] showed that it is oxidized not only *via* glutamate dehydrogenase, but also by a transamination pathway, first described by MÜLLER AND LEUTHARDT[6], in which glutamate is converted to aspartate. In muscle mitochondria, which contain little or no glutamate dehydrogenase, the transamination pathway is obligatory (see ref. 7). The mechanism of glutamate oxidation in isolated liver mitochondria, which do contain an active glutamate dehydrogenase, has been the subject of some controversy. According to BORST[3,7], the contribution of the transamination pathway to glutamate disappearance in isolated rat-liver mitochondria is 90%. Other investigators[1,8–10] have found lower values, ranging from 80% to 18–20%. HIRD AND MARGINSON[10] measured ammonia production from

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glutamate in rat-liver mitochondria and calculated that at a substrate concentration of 10 mM, 21–27 % of the glutamate utilized was converted to ammonia; this conversion was increased at lower substrate concentrations and amounted to 63–85 % at 0.125 M glutamate. QUAGLIARIELLO *et al.*[11] and TAGER AND DE HAAN[12] have shown that the small amount of ammonia that is formed from glutamate is produced mainly in the first few minutes of the incubation. As PAPA, PALMIERI AND QUAGLIARIELLO[13] have stressed, the contribution of the deamination pathway will therefore tend to be higher if short incubation times are used. However, this does not explain the differences in experimental results obtained in different laboratories where similar incubation times have been used.

In view of these discrepancies, we have re-examined this problem, and our results are presented in this paper. The importance of the structural integrity of the mitochondria in determining the pathway of glutamate oxidation is stressed and the effect of uncouplers and of substrate concentration is shown. Some of the results have been presented in a preliminary form[12,14,15]. In the accompanying papers[16,17], the control of glutamate dehydrogenase activity during glutamate oxidation, and the problem of citrulline synthesis in rat-liver mitochondria are discussed.

METHODS

Preparation of mitochondria

Rat-liver mitochondria were prepared by the method of HOGEBOOM[18] exactly as described by MYERS AND SLATER[19].

Aged mitochondria

Mitochondria were aged by incubating the mitochondrial suspension (in 0.25 M sucrose) at room temperature (about 20°) for various periods with occasional shaking. When an aged preparation was used in a reaction, the zero-time control was prepared with a preparation aged for the same length of time. This was done to prevent errors due to changes in the content of certain metabolites during ageing of the mitochondria. In particular, the free ammonia content rose during ageing.

Sonicated mitochondria

Mitochondria were subjected to ultrasonic vibrations for two periods of 1 min in a M.S.E. sonic disintegrator with an output of 20 kHz. The mitochondrial suspension was kept cold during the treatment.

Reaction conditions

Unless otherwise stated, the standard reaction mixture (1 ml) used in the experiments contained 15 mM KCl, 5 mM $MgCl_2$, 2 mM EDTA, 50 mM Tris–HCl buffer, 0.1–0.5 mM ADP, 20–30 mM potassium phosphate buffer, 20–30 mM glucose, 150 Cori units (5 μmoles substrate per min) hexokinase (EC 2.7.1.1) and 25 mM sucrose (derived from the mitochondrial suspension). The final pH was 7.5. The reaction temperature was 25° unless otherwise stated. The reaction was carried out in Warburg flasks (gas volume, 6–7 ml) in most experiments. O_2 uptake was measured with differential manometers with a small capillary. The reaction was started by the addition of mitochondria.

The reaction was stopped by the addition of 0.1 ml 35 % $HClO_4$, except when

NAD(P)H was to be measured. After removal of the protein by centrifugation, $HClO_4$ was removed in the cold as $KClO_4$ (see ref. 16).

For the measurement of NAD(P)H, the reaction was stopped by the addition of 0.5 ml 1 M KOH in ethanol. Neutralization of the extract was carried out as described in the accompanying paper[16].

Analytical procedures

Glutamate was determined in early experiments with glutamate decarboxylase (EC 4.1.1.15) by the method of GALE[20]. Later, the more accurate spectrophotometric method of BERNT AND BERGMEYER[21] using glutamate dehydrogenase (EC 1.4.1.3) was employed.

Malate was determined with malate dehydrogenase (EC 1.1.1.37) according to HOHORST[22].

Ammonia was determined with glutamate dehydrogenase (ammonia-free) by a modification of the method of KIRSTEN, GEREZ AND KIRSTEN[23]. The reaction mixture (1.25–1.45 ml) contained 80–120 mM Tris–HCl buffer (pH 7.5), 15 mM α-oxoglutarate, 80 μM NADH (free of ammonia; see below), ammonia-free glutamate dehydrogenase (0.3 mg) and a sample of the neutralized $HClO_4$ extract (containing not more than 110 nmoles NH_3). The reaction was started by the addition of either enzyme or sample. The reaction was completed in 20–30 min. No precautions were taken to exclude diffusion from the air into the reaction mixture, but it was found that no further decrease in absorbance occurred after 20–30 min. Furthermore, the results presented later in Table II indicate that added ammonia is recovered almost quantitatively. The small amount of ammonia found in the zero-time control was probably derived from the hexokinase preparation used.

α-Oxoglutarate was determined with glutamate dehydrogenase as described by SLATER AND HOLTON[24].

Aspartate was determined with malate dehydrogenase and aspartate transaminase (EC 2.6.1.1) by the method of PFLEIDERER, GRÜBER AND WIELAND[25].

Protein was determined by the biuret method as described by CLELAND AND SLATER[26].

$NAD(P)^+$ and $NAD(P)H$ were determined by the methods of KLINGENBERG[27] as modified by VAN DAM[28].

Source of enzymes

Hexokinase was prepared from yeast by the method of DARROW AND COLOWICK[29], omitting the final crystallization step.

Aspartate transaminase was prepared by the method of BORST AND PEETERS[30] as modified by TAGER AND SLATER[31].

The sources of the other enzymes were: glutamate decarboxylase, Sigma Chemical Co.; glutamate dehydrogenase, malate dehydrogenase, alcohol dehydrogenase (EC 1.1.1.1), glucose-6-phosphate dehydrogenase (EC 1.1.1.49) and lactate dehydrogenase (EC 1.1.1.27), Boehringer und Soehne, Mannheim.

Special reagents

The sources of the special reagents were: ADP, ATP, oxaloacetate, NAD^+, Tris and L-malate, Sigma Chemical Co.; glutamate and aspartate, British Drug Houses; NADH, Mycofarm, Delft; dicoumarol, Amsterdamsche Chinine Fabriek.

Ammonia-free NADH was prepared by a method suggested by Dr. W. C. HÜLSMANN. 50 mg NADH in a minimum volume of 10 mM Tris–HCl (pH 7.5) were introduced onto a DEAE-cellulose column (diameter 1 cm, and height 8 cm), previously equilibrated with the same buffer. The column was then washed with 10–15 ml of the buffer. The NADH was eluted from the column with 1 % K_2HPO_4.

RESULTS

Pathway of glutamate oxidation in fresh rat-liver mitochondria

In Table I, the results of a series of 42 experiments are shown in which the stoicheiometry of glutamate oxidation by rat-liver mitochondria in State 3 (ref. 32) in the absence of malonate was measured. These experiments were carried out at 25°, and the reaction time was 6–40 min. In agreement with the results of BORST[3,7], it was found that 90 % of the glutamate that disappears under these conditions is recovered as aspartate. Table I shows that the remaining 10 % is converted to ammonia. Very little α-oxoglutarate accumulates (see also BORST[7]).

TABLE I

STOICHEIOMETRY OF GLUTAMATE OXIDATION IN FRESH RAT-LIVER MITOCHONDRIA

Experimental conditions described under METHODS. Reaction mixture contained the standard components *plus* 5–10 mM glutamate, 2.3–8.2 mg mitochondrial protein and (where present) 20 mM malonate. Reaction time, 6–40 min.

	Malonate absent			*Malonate present*		
	natoms or nmoles/min per mg protein		*Number of measurements*	*natoms or nmoles/min per mg protein*		*Number of measurements*
	Mean	*Range*		*Mean*	*Range*	
$-\Delta$ O	94.0	33.8–140.6	42	32.5	17.6–48.3	16
$-\Delta$ Glutamate	30.2	10.8– 53.0	42	17.1	7.3–27.2	15
Δ Aspartate	27.3	10.0– 44.9	41	1.1	0 – 3.2	16
Δ NH_3	3.2	0.7– 6.5	37	16.5	9.8–22.8	13
Δ α-Oxoglutarate	1.1	0 – 3.2	15	1.5	0.7– 2.8	6

Table I also shows the stoicheiometry of glutamate oxidation in the presence of malonate. The transamination pathway is suppressed (*cf.* refs. 1, 2, 5, 7, 10–12) and concomitantly, the deamination of glutamate is greatly stimulated. As COPENHAVER AND LARDY[33] and BORST[7] found, very little α-oxoglutarate accumulates in the presence of malonate.

The time course of glutamate oxidation is shown in Fig. 1. During the first 10 min of the reaction, a small amount of ammonia was formed. In this experiment, as in most others carried out by us under these experimental conditions, we could detect no further increase after 10 min in the ammonia found. In the experiments of QUAGLIARIELLO *et al.*[11], ammonia production continued for longer than 10 min, particularly when the reaction was carried out at 30° instead of 25°.

The possibility was considered that the deamination reaction takes place to furnish intermediates of the transamination pathway and that, as soon as a certain steady-state level of the intermediates is reached, the deamination stops. However, no

lag in aspartate formation, corresponding to the decline in deamination and extending over 10 min, could be detected in this experiment (Fig. 1; see also refs. 13, 16). It is possible to detect a very short lag in aspartate formation when the first 2 min of the reaction are studied, but as shown in the following paper[16], this lag lasts for a few seconds only, after which the rate of formation of aspartate is perfectly linear.

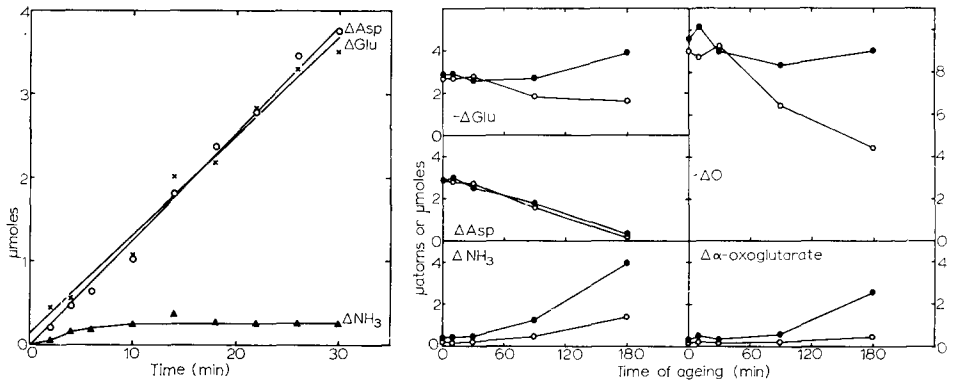


Fig. 1. Time course of glutamate oxidation in rat-liver mitochondria. Experimental conditions as in METHODS. Reaction mixture contained the standard components *plus* 8 mM glutamate and 4.6 mg mitochondrial protein.

Fig. 2. Effect of ageing of rat-liver mitochondria on the pathway of glutamate oxidation. Experimental conditions as described under METHODS. Reaction mixture contained the standard components *plus* 9.3 mM glutamate, 4.4 mg mitochondrial protein and (where present) 0.45 mM NAD^+. Reaction temperature, 25°. Reaction time, 30 min. ○, without NAD^+; ●, with NAD^+.

TABLE II

EFFECT OF ADDED AMMONIA ON THE DEAMINATION OF GLUTAMATE

Experimental conditions described under METHODS. Reaction mixture contained the standard components *plus* 10 mM glutamate, the concentrations of NH_4Cl indicated, and 3.8 mg mitochondrial protein. Reaction time, 20 min.

NH_4Cl added (*μmoles*)	*NH_3 found* (*μmoles*)		*Δ NH_3* (*μmole*)
	at zero time	*after 20 min*	
0	0.3	0.5	0.2
0.2	0.5	0.7	0.2
1.0	1.2	1.6	0.4
2.0	2.2	2.5	0.3

Another possible explanation of the results is that the accumulation of ammonia inhibits further deamination of glutamate, but this is ruled out by the experiment reported in Table II, which shows that the amount of ammonia formed from glutamate is independent of an initial ammonia concentration of up to 2.2 mM. Similar results were obtained when the mitochondria were preincubated for 8 min with NH_4Cl, in order to overcome a possible permeability barrier, before adding glutamate. Furthermore, as shown in the accompanying paper[17], removal of ammonia for citrulline

synthesis does not lead to any further deamination of glutamate. The factors controlling the activity of glutamate dehydrogenase during glutamate oxidation are discussed further in the following paper[16].

Glutamate oxidation in aged rat-liver mitochondria

When fresh rat-liver mitochondria are disrupted by ultrasonic vibration or by treatment with a detergent, only oxidative deamination of glutamate takes place (not shown), although transamination can occur if oxaloacetate or one of its precursors is added as well as glutamate (see Fig. 7). These results suggested that the pathway of glutamate oxidation may be a function of the structural integrity of the mitochondria. In order to examine this further, the experiment of Fig. 2 was carried out. Mitochondria were aged by leaving the preparation at room temperature (20°) with occasional shaking. At the times indicated in Fig. 2, samples of mitochondria were taken and the stoicheiometry of glutamate oxidation was determined in the absence and in the presence of added NAD^+. When the reaction was studied in the absence of added NAD^+, ageing caused a gradual decline in oxygen uptake, a decline in glutamate disappearance, a marked decline in aspartate formation and a stimulation of ammonia formation. The addition of NAD^+ to aged mitochondria restored the O_2 uptake, restored or even enhanced glutamate utilization, had no effect on aspartate formation and markedly stimulated ammonia formation. In fresh mitochondria, added NAD^+ had very little effect on O_2 uptake or glutamate disappearance, and increased the amount of ammonia that was found from 0.1 to 0.2 μmole. Fig. 2 also shows that ageing brought about an increase in α-oxoglutarate accumulation, especially when NAD^+ was present.

The effect of substrate concentration on the pathway of glutamate oxidation

HIRD AND MARGINSON[10] have reported that when glutamate is oxidized by rat-liver mitochondria, the percentage of glutamate that is oxidatively deaminated is dependent on the concentration of the substrate, being higher at low concentrations of substrate. As the substrate concentration was increased, the percentage deamination gradually decreased, but even when the initial glutamate concentration was 10 mM, appreciable deamination still occurred. We have not been able to confirm this with freshly prepared rat-liver mitochondria under our experimental conditions (Fig. 3).

A low steady-state concentration of glutamate can be maintained by using

TABLE III

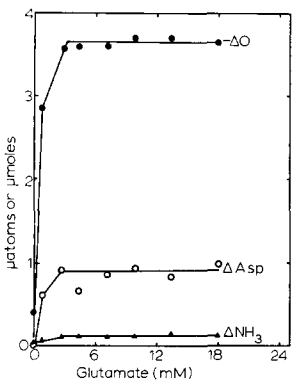
PRODUCTS OF GLUTAMINE AND PROLINE OXIDATION IN RAT-LIVER MITOCHONDRIA

Experimental conditions described under METHODS. Reaction mixture contained the standard components *plus* 6.7 mg (Expt. 1), 4.2 mg (Expt. 2) or 6.0 mg (Expt. 3) mitochondrial protein and either 10 mM glutamine or 10 mM L-proline (added as DL-proline). Reaction time, 20 min. In Expts. 1 and 2, which were carried out in collaboration with Mr. H. F. TABAK, each value is the mean of 3 separate incubations.

Expt.	*Substrate*	*Δ Glutamate (μmole)*	*Δ Aspartate (μmoles)*	*ΔNH_3 (μmoles)*
1	Glutamine	0.47	1.35	1.87
2	Glutamine	0.51	0.92	1.67
3	Proline	0.43	1.15	0.02

glutamine and proline as respiratory substrates. Glutamate is formed from glutamine by the action of mitochondrial glutaminase (EC 3.5.1.2) and from proline by oxidation. The results of experiments using these substrates are presented in Table III. In the two experiments with glutamine the glutamate concentration could never have exceeded the value for ΔNH_3 (1.9 and 1.7 mM, respectively). In the experiment with proline the glutamate concentration was measured during the course of the incubation and was found to be less than 0.5 mM at all times. Calculations based on the data of Table III show that the contribution of the deamination pathway to the oxidation of glutamate was 2 %, 12 % and 2 % in Expts. 1, 2 and 3, respectively.

We did obtain results somewhat similar to those of HIRD AND MARGINSON[10] when we used aged mitochondria (Fig. 4). A possible reason for the difference between our results and those of HIRD AND MARGINSON[10] with freshly prepared rat-liver mitochondria may lie in the experimental conditions. We performed our experiments



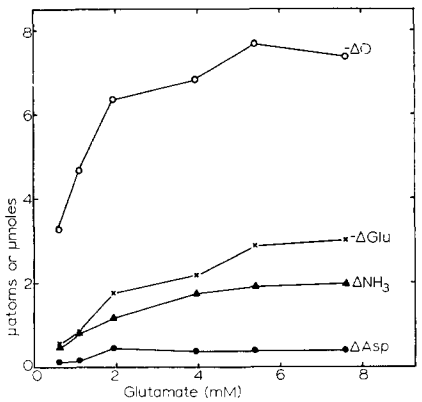


Fig. 3. Effect of substrate concentration on the pathway of glutamate oxidation in fresh rat-liver mitochondria. Experimental conditions described under METHODS. Reaction mixture contained the standard components *plus* 3.8 mg mitochondrial protein and the concentrations of glutamate indicated. Reaction time, 16 min.

Fig. 4. Effect of substrate concentration on the pathway of glutamate oxidation in aged rat-liver mitochondria. Experimental conditions described under METHODS. Mitochondria were aged at room temperature (about 20°) for 90 min. Reaction mixture contained the standard components *plus* 0.45 mM NAD^+, 4.4 mg mitochondrial protein and the concentrations of glutamate indicated in the figure. Reaction temperature, 25°. Reaction time, 28 min.

at 25°, and the incubation time was kept short in order to prevent the substrate being exhausted. HIRD AND MARGINSON[10] carried out their experiments at 38° for 40–45 min, and we have confirmed[12] that the deamination pathway is quantitatively important under these experimental conditions. However, we have also shown[12] that the mitochondria lose their respiratory control under these conditions. We believe that the loss of structural integrity that this implies is responsible for the enhanced contribution of the deamination pathway to glutamate oxidation.

Effect of uncouplers on the pathway of glutamate oxidation

As reported earlier[15,35], the addition of uncouplers such as 2,4-dinitrophenol or dicoumarol to a system in which glutamate is being oxidized by rat-liver mitochondria in the presence of phosphate and phosphate acceptor brings about an inhibition of

aspartate formation and a stimulation of the deamination of glutamate. The magnitude of these effects depends on the concentration of uncoupler. This is shown in Fig. 5 for 2,4-dinitrophenol (see also ref. 34). On increasing the concentration of uncoupler, the inhibition of aspartate formation increases. Ammonia formation is progressively stimulated until an optimum concentration of uncoupler is reached that varies in different experiments. When the optimum concentration is exceeded, there is a slight decline in ammonia formation. In the absence of uncoupler, no malate is found. With increasing concentrations of 2,4-dinitrophenol, increasing amounts of malate accumulate. (In the presence of malonate, which itself stimulates deamination, there is only a slight further stimulation by uncoupler of ammonia production (see Fig. 6, Curve 1).)

In order to determine at which point in the transamination pathway (Reaction 6) the inhibition by uncouplers takes place, the effect of 2,4-dinitrophenol on the individual steps (Reactions 1–5) of this pathway was studied.

Glutamate + oxaloacetate	$\rightleftarrows$ aspartate + α-oxoglutarate	(1)
α-Oxoglutarate + O	$\rightarrow$ succinate + CO_2	(2)
Succinate + O	$\rightarrow$ fumarate	(3)
Fumarate	$\rightleftarrows$ malate	(4)
Malate + O	$\rightarrow$ oxaloacetate	(5)
Sum: Glutamate + 3 O	$\rightarrow$ aspartate + CO_2	(6)

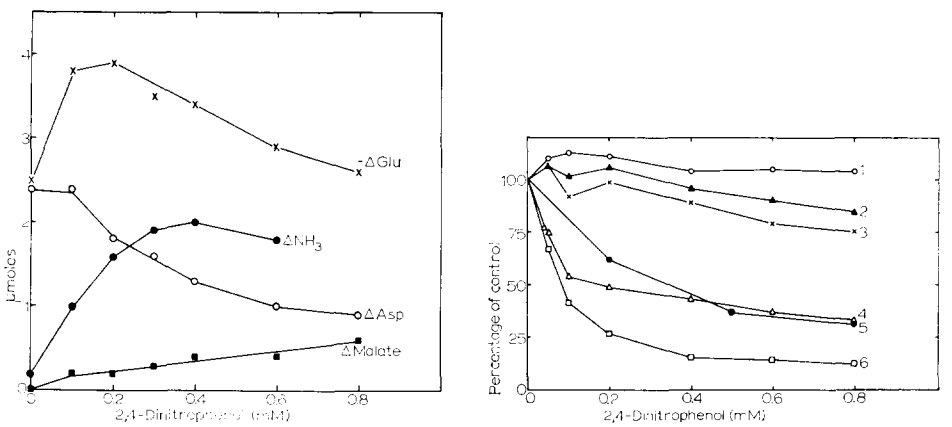


Fig. 5. Effect of concentration of 2,4-dinitrophenol on the pathway of glutamate oxidation in fresh rat-liver mitochondria. Experimental conditions as described under METHODS. Reaction mixture contained the standard components *plus* 10 mM glutamate, 2.5 mg mitochondrial protein and 2,4-dinitrophenol as indicated in the figure. Reaction time, 30 min.

Fig. 6. Effect of concentration of 2,4-dinitrophenol on the oxidation of various substrates by fresh rat-liver mitochondria. This figure combines the results of several experiments in which different substrates were tested. Experimental conditions as described under METHODS. The reaction mixture contained the standard components *plus* 2.4–7.3 mg mitochondrial protein. In the experiments of Curve 4, P_i was omitted from the reaction mixture and the mitochondria were preincubated at 25° with 8 mM malate *plus* 8 mM pyruvate in order to deplete them of endogenous P_i and thus to prevent the oxidation of any α-oxoglutarate subsequently formed. After 14 min, either 30 mM P_i (control) or 2,4-dinitrophenol (at the concentration indicated in the figure) was tipped into the reaction mixture from the sidearm of the Warburg flask. Further additions were as follows: Curve 1, 10 mM glutamate *plus* 25 mM malonate (NH_3 formation was measured); Curve 2, 10 mM α-oxoglutarate *plus* 10 mM malonate (O_2 uptake was measured); Curve 3, 40 mM succinate *plus* 2 mM Amytal (O_2 uptake was measured); Curve 4, 8 mM malate *plus* 8 mM pyruvate (O_2 uptake was measured); Curve 5, 15 mM glutamate (aspartate formation was measured); Curve 6, 10 mM glutamate *plus* 10 mM malate *plus* 1 mM arsenite (aspartate formation was measured). The reaction time was 20 min. The values are expressed as % of the control without 2,4-dinitrophenol.

The results of these experiments are shown in Fig. 6. The following points emerge:

(i) The transamination pathway (Reaction 6) became progressively more inhibited as the concentration of 2,4-dinitrophenol was increased to approx. 0.5 mM (Curve 5). Little further change occurred between 0.5 and 0.8 mM.

(ii) The oxidation of α-oxoglutarate to succinate (Reaction 2) was studied in the presence of malonate. The oxidation of succinate to fumarate *plus* malate (Reactions 3 and 4) was measured in the presence of Amytal. These reactions were very little affected by the uncoupler even at a concentration of 0.8 mM (Curves 2 and 3, respectively).

(iii) Reaction 5 was coupled with Reaction 1 by using malate *plus* glutamate as substrates in the presence of arsenite. The net result of these 2 reactions is the conversion of malate to aspartate; this is very markedly inhibited by the uncoupler (Curve 6).

These results demonstrate that the locus of the inhibition of the transamination pathway is Reaction 1 and/or Reaction 5. The finding that malate accumulates during the oxidation of glutamate in the presence of uncoupler (Fig. 5) is in agreement with this.

The aspartate transaminase reaction (1), in which oxaloacetate is one of the metabolites, is inhibited by dicoumarol in intact mitochondria, but not in sonicated mitochondria (Fig. 7). In other experiments (not shown) we have found that uncouplers have no effect on isolated aspartate transaminase (contrast ref. 36).

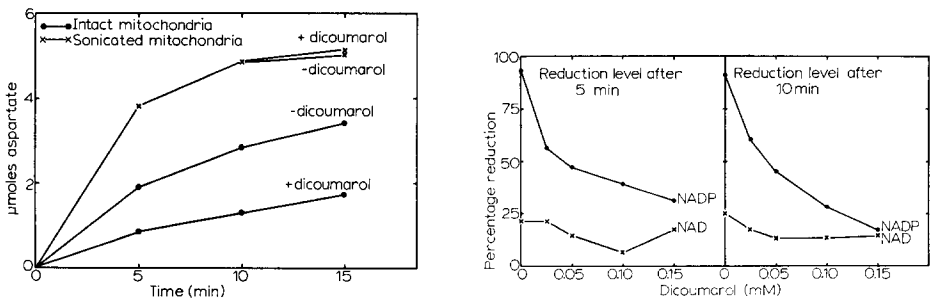


Fig. 7. Effect of dicoumarol on the transamination between oxaloacetate and glutamate in intact and in sonicated rat-liver mitochondria. Reaction mixture contained the standard components *plus* 1 mM arsenite, 8 mM glutamate, 8 mM oxaloacetate and 5.0 mg mitochondrial protein. The reaction was carried out in open centrifuge tubes at 25° in a Dubnoff metabolic shaker. Each point represents a separate incubation.

Fig. 8. Effect of the concentration of dicoumarol on the reduction level of NAD(P) during glutamate oxidation in rat-liver mitochondria. Reaction mixture contained the standard components *plus* 10 mM glutamate, 2.7 mg mitochondrial protein and the concentrations of dicoumarol indicated in the figure. After 5 and 10 min, the reaction was stopped with $HClO_4$ or ethanolic KOH and $NAD(P)^+$ and NAD(P)H were measured in the neutralized acid and alkali extracts, respectively, as described under METHODS.

Since oxaloacetate takes part in both Reaction 1 and Reaction 5, the effect of 2,4-dinitrophenol on another system involving this metabolite was studied, namely, the oxidation of pyruvate *plus* malate (Reactions 7, 5, 8 and 9). This system is also markedly inhibited by the uncoupler (Curve 4 of Fig. 6).

The effect of different concentrations of dicoumarol on the oxidoreduction level

Pyruvate + CoA + O	→ acetyl-CoA + CO_2	(7)
Malate + O	→ oxaloacetate	(5)
Acetyl-CoA + oxaloacetate	→ citrate + CoA	(8)
Sum: Pyruvate + malate + 2 O	→ citrate + CO_2	(9)

of NAD(P) was also studied. Fig. 8 shows that as the concentration of dicoumarol was increased, the reduction level of NADP progressively decreased. There is also a progressively greater stimulation of the deamination of glutamate with increasing concentrations of uncoupler (Fig. 5). The reduction level of NAD was low even in the absence of dicoumarol, and remained relatively unaffected by addition of the uncoupler.

DISCUSSION

Rat-liver mitochondria contain high levels of the enzymes involved in both the deamination and transamination pathways of glutamate oxidation. Yet the extensive investigations in this laboratory initiated by BORST[2] have shown that freshly prepared, isolated rat-liver mitochondria oxidize glutamate preferentially by the transaminase pathway. In confirmation of the initial observations of BORST[2,3,7], we have found that the contribution of the transamination pathway to glutamate disappearance in isolated rat-liver mitochondria incubated at 25° is at least 90 %. We have also shown that only 10 % of the glutamate that disappears is deaminated (contrast refs. 10, 37).

In most of our experiments, we have found that the small amount of ammonia that is found is produced in the first 10 min of incubation. QUAGLIARIELLO and co-workers[11,13,34] have also shown that deamination is more extensive during the initial stages of the incubation than subsequently. However, in their experiments, particularly those carried out at 30°, deamination continued for longer than 10 min. Although the relative contribution of the two pathways of glutamate oxidation depends in part on the incubation time, as QUAGLIARIELLO and co-workers have stressed[11,13,34], the marked differences in experimental results obtained in different laboratories (see also, *e.g.*, refs. 1, 8 and 10) suggest that other factors must also be involved.

HIRD and co-workers[10,37] have reported that the contribution of the deamination pathway is increased by lowering the concentration of glutamate. We have been unable to detect an effect of substrate concentration on the relative contribution of the two pathways of glutamate oxidation in freshly prepared mitochondria.

We have shown[12] that the contribution of the deamination pathway can be increased by raising the incubation temperature, particularly if the incubation time is lengthened as well. However, we also found that mitochondria lose their respiratory control after prolonged incubation at higher temperatures. This suggested that an uncoupled state of the mitochondria might be responsible for the enhanced contribution of the deamination pathway. Indeed, we have found that when the structural integrity of the mitochondria is impaired by ageing, so that they become uncoupled, the contribution of the transamination pathway decreases and that of the deamination pathway increases (Fig. 2). Furthermore, this can also be achieved by the addition of uncoupling agents (Fig. 5).

The inhibition by uncouplers of the transamination pathway has been localized in the steps in which malate is converted to aspartate (Reactions 1 and 5; see Fig. 6).

It has also been found[38-41] that energy is required for the transfer of reducing equivalents from malate to α-oxoglutarate *plus* ammonia (Reactions 10, 11, 1 and 12).

Malate	⇆ oxaloacetate + 2 H	(10)
α-Oxoglutarate + NH_3 + 2 H	→ glutamate	(11)
Glutamate + oxaloacetate	⇆ aspartate + α-oxoglutarate	(1)
Sum: Malate + NH_3	→ aspartate	(12)

Two possible explanations for this were brought forward by TAGER[39]. The first was that energy is required for the transfer of NADH from the NAD-specific malate dehydrogenase to a 'compartment' where it can react with glutamate dehydrogenase or into a form which reacts with this enzyme. The second was that energy is necessary for the removal of oxaloacetate from malate dehydrogenase, which it strongly inhibits, so that it can react with aspartate transaminase. Subsequent studies[35,42] showed that the primary energy requirement is not for the removal of NADH from malate dehydrogenase, and focussed attention on the second explanation. KLINGENBERG[40,41] advanced a different explanation, namely, that in the absence of energy, aspartate accumulates within the mitochondrion and inhibits further transamination. An inhibition by uncoupler of the aspartate transaminase reaction can, indeed, be demonstrated in intact mitochondria (Fig. 7). However, uncouplers also inhibit the oxidation of pyruvate *plus* malate (Fig. 6) in which aspartate does not play a role. It seems more likely that energy is involved in the intramitochondrial compartmentation of oxaloacetate. CHAPPELL[43] has shown that added oxaloacetate does not inhibit succinate oxidation unless the mitochondria are preincubated with uncoupler, and KUNZ[44] and TAGER[45] have demonstrated that added oxaloacetate can even stimulate the oxidation of succinate in State 4 by acting as a sink for reducing equivalents in the energy-linked reversal of the respiratory chain.

Our explanation for the inhibition by ageing or uncoupling agents of the transamination pathway of glutamate oxidation, then, is that, in the absence of energy, oxaloacetate is not sufficiently rapidly removed from the malate dehydrogenase compartment to aspartate transaminase, so that malate dehydrogenase is inhibited and malate accumulates (see Fig. 5).

In the experiments with aged mitochondria and with uncoupling agents, an inhibition of the transamination pathway was always accompanied by a stimulation of the deamination of glutamate. The factors controlling the activity of glutamate dehydrogenase during glutamate oxidation in rat-liver mitochondria are discussed more fully in an accompanying paper[16]. It is shown[16] that glutamate dehydrogenase in isolated rat-liver mitochondria reacts preferentially with NADP, as originally postulated by KLINGENBERG AND SLENCZKA[46], and that the main factor controlling the activity of glutamate dehydrogenase is the oxidoreduction state of NADP. The rate of deamination of glutamate is dependent on the extent of oxidation of NADP, and an increase in $NADP^+$ is always accompanied by increased deamination. The same correlation is observed when uncoupler is present. When increasing concentrations of uncoupler are added, there is a progressive increase in the level of $NADP^+$ (Fig. 8) and also a progressive increase in the rate of deamination up to an optimum concentration of uncoupler (Fig. 5).

In the systems discussed above, glutamate deamination is stimulated under

conditions where the transamination pathway is inhibited. However, the deamination of glutamate can also be stimulated without influencing the transamination pathway. As shown in an accompanying paper[16] this can be done by adding 2-methyl-1,4-naphthoquinone, which oxidizes NADPH and stimulates the deamination of glutamate, but has no effect on the transamination pathway.

ACKNOWLEDGEMENTS

The authors wish to thank Dr. P. BORST for many helpful discussions and Miss B. KELDER and Mr. H. HORN for their technical assistance. This investigation was supported in part by a grant from the Life Insurance Medical Research Fund.

NOTE ADDED IN PROOF (Received November 11th, 1966)

Since the above paper was submitted, HIRD AND MARGINSON[47] have reported an increased ammonia production from glutamate in rat-liver mitochondria at 38° on the addition of ADP. Since our measurements (*e.g.* Table I above) were carried out in the presence of ADP, hexokinase and glucose, this appears to be in disagreement with the results of our experiments carried out at 25°.

We have carried out two experiments exactly as described by HIRD AND MARGINSON[47]. In one of them, ammonia was produced, after a slight lag, at a steady state comparable with that reported by HIRD AND MARGINSON[47]. An extensive oxidation of NADP was also observed in this experiment. In a second experiment, which lasted only 10 min, little ammonia was formed, and NADP was completely reduced. Thus, the correlation between the degree of reduction of NADP and the deamination of glutamate is confirmed by these experiments.

We interpret these results in the following manner. In tightly coupled mitochondria, the NADPH/$NADP^+$ ratio is very high even in the presence of ADP (*cf.* ref. 16), presumably because the energy-linked nicotinamide nucleotide transhydrogenase competes very effectively with ADP for the high-energy intermediate of oxidative phosphorylation. Incubation at 38°, however, can, with some preparations, apparently cause some loosening of the coupling, with the result that added ADP can cause oxidation of NADPH.

REFERENCES

1 H. A. KREBS AND D. BELLAMY, *Biochem. J.*, 75 (1960) 523.
2 P. BORST AND E. C. SLATER, *Biochim. Biophys. Acta*, 41 (1960) 170.
3 P. BORST, *Een biochemisch onderzoek over mitochondriën geïsoleerd uit een ascitescel tumor*, M.D. Thesis, Jacob van Campen, Amsterdam, 1961.
4 J. B. CHAPPELL AND G. D. GREVILLE, *Nature*, 190 (1961) 502.
5 E. A. JONES AND H. GUTFREUND, *Biochem. J.*, 79 (1961) 608.
6 A. F. MÜLLER AND F. LEUTHARDT, *Helv. Chim. Acta*, 33 (1950) 268.
7 P. BORST, *Biochim. Biophys. Acta*, 57 (1962) 256.
8 R. BALÁZS, *Biochem. J.*, 95 (1965) 497.
9 S. PAPA, C. SACCONE, F. PALMIERI, A. FRANCAVILLA AND E. QUAGLIARIELLO, *Boll. Soc. Ital. Sper.*, 39 (1963) 617.
10 F. J. R. HIRD AND M. A. MARGINSON, *Nature*, 201 (1964) 1224.
11 E. QUAGLIARIELLO, S. PAPA, C. SACCONE, F. PALMIERI AND A. FRANCAVILLA, *Biochem. J.*, 95 (1965) 742.

12 J. M. TAGER AND E. J. DE HAAN, in E. QUAGLIARIELLO, *Atti del Seminario di Studi Biologici*, Vol. 1, Cressati, Bari, 1964, p. 307.
13 S. PAPA, F. PALMIERI AND E. QUAGLIARIELLO, in J. M. TAGER, S. PAPA, E. QUAGLIARIELLO AND E. C. SLATER, *Regulation of Metabolic Processes in Mitochondria*, BBA Library, Vol. 7, Elsevier, Amsterdam, 1966, p. 153.
14 E. J. DE HAAN, J. M. TAGER AND E. C. SLATER, *Biochim. Biophys. Acta*, 89 (1964) 375.
15 E. J. DE HAAN, *Abstr. 2nd Meeting Fed. European Biochem. Soc., Vienna, 1965*, p. 165.
16 S. PAPA, J. M. TAGER, A. FRANCAVILLA, E. J. DE HAAN AND E. QUAGLIARIELLO, *Biochim. Biophys. Acta*, 131 (1967) 14.
17 R. CHARLES, J. M. TAGER AND E. C. SLATER, *Biochim. Biophys. Acta*, 131 (1967) 29.
18 G. H. HOGEBOOM, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. 1, Academic Press, New York, 1962, p. 16.
19 D. K. MYERS AND E. C. SLATER, *Biochem. J.*, 67 (1957) 558.
20 E. F. GALE, *Biochem. J.*, 39 (1945) 46.
21 E. BERNT AND H. U. BERGMEYER, in H. U. BERGMEYER, *Methods of Enzymatic Analysis*, Academic Press, New York, 1963, p. 384.
22 H. J. HOHORST, in H. U. BERGMEYER, *Methods of Enzymatic Analysis*, Academic Press, New York, 1963, p. 328.
23 E. KIRSTEN, C. GEREZ AND R. KIRSTEN, *Biochem. Z.*, 337 (1963) 312.
24 E. C. SLATER AND F. A. HOLTON, *Biochem. J.*, 55 (1953) 530.
25 G. PFLEIDERER, W. GRÜBER AND TH. WIELAND, *Biochem. Z.*, 326 (1955) 446.
26 K. W. CLELAND AND E. C. SLATER, *Biochem. J.*, 53 (1953) 547.
27 M. KLINGENBERG, in H. U. BERGMEYER, *Methods of Enzymatic Analysis*, Academic Press, New York, 1963, pp. 528, 531, 535, 537.
28 K. VAN DAM, *Nicotinamide–adenine dinucleotide en de ademhalingsketenfosforylering*, Ph.D. Thesis, Jacob van Campen, Amsterdam, 1966.
29 R. A. DARROW AND S. P. COLOWICK, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. 5, Academic Press, New York, 1962, p. 226.
30 P. BORST AND E. M. PEETERS, *Biochim. Biophys. Acta*, 54 (1961) 188.
31 J. M. TAGER AND E. C. SLATER, *Biochim. Biophys. Acta*, 77 (1963) 227.
32 B. CHANCE AND G. R. WILLIAMS, *Advan. Enzymol.*, 17 (1956) 65.
33 J. H. COPENHAVER AND H. A. LARDY, *J. Biol. Chem.*, 195 (1952) 225.
34 E. QUAGLIARIELLO AND S. PAPA, in E. QUAGLIARIELLO, *Atti del Seminario di Studi Biologici*, Vol. 1, Cressati, Bari, 1964, p. 351.
35 J. M. TAGER AND E. J. DE HAAN, in E. QUAGLIARIELLO, *Atti del Seminario di Studi Biologici*, Vol. 1, Cressati, Bari, 1964, p. 339.
36 E. QUAGLIARIELLO, F. PALMIERI, M. A. ELICIO AND S. PAPA, *Boll. Soc. Ital. Biol. Sper.*, 40 (1964) 1455.
37 F. J. R. HIRD AND D. J. MORTON, *Biochim. Biophys. Acta*, 85 (1964) 353.
38 J. M. TAGER, *Biochem. J.*, 84 (1962) 64P.
39 J. M. TAGER, *Biochim. Biophys. Acta*, 77 (1963) 258.
40 M. KLINGENBERG, in *Symp. über Redoxfunktionen cytoplasmatischer Strukturen*, Gemeinsame Tagung der Deutschen Gesellschaft für physiologische Chemie und der Österreichischen biochemischen Gesellschaft, Wien, 1962, p. 163.
41 M. KLINGENBERG, in B. CHANCE, *Energy-linked Functions of Mitochondria*, Academic Press, New York, 1963, p. 121.
42 J. M. TAGER, *Abstr. 6th Intern. Congr. Biochem., New York, 1964*, Vol. X, p. 789.
43 J. B. CHAPPELL, *Proc. 1st IUB/IUBS Symp. Biological Structure and Function, Stockholm, 1960*, Vol. 2, Academic Press, New York, 1961, p. 71.
44 W. KUNZ, *Z. Physiol. Chem.*, 334 (1963) 128.
45 J. M. TAGER, in J. M. TAGER, S. PAPA, E. QUAGLIARIELLO AND E. C. SLATER, *Regulation of Metabolic Processes in Mitochondria*, BBA Library, Vol. 7, Elsevier, Amsterdam, 1966, p. 202.
46 M. KLINGENBERG AND W. SLENCZKA, *Biochem. Z.*, 331 (1959) 486.
47 F. J. R. HIRD AND M. A. MARGINSON, *Arch. Biochem. Biophys.*, 115 (1966) 247.