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CONTROL OF GLUTAMATE DEHYDROGENASE ACTIVITY DURING GLUTAMATE OXIDATION IN ISOLATED RAT-LIVER MITOCHONDRIA

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SUMMARY

1. The kinetics of the reaction of glutamate dehydrogenase with the intramitochondrial nicotinamide nucleotides has been followed in isolated rat-liver mitochondria preincubated with phosphate and phosphate acceptor in order largely to oxidize intramitochondrial nicotinamide nucleotides. In the presence of rotenone and arsenite, the oxidation of glutamate to α-oxoglutarate is accompanied by extensive reduction of $NADP^+$ and very little reduction of NAD^+, and ceases when NADP is maximally reduced, even though the level of NAD^+ is still quite high.

2. In mitochondria preincubated in the presence of ATP, oligomycin or rotenone in order largely to reduce intramitochondrial nicotinamide nucleotides, NADPH was oxidized much more rapidly than NADH on the addition of ammonia (α-oxoglutarate was already present).

3. On addition of glutamate to mitochondria preincubated as in 1, a small amount of ammonia is formed and some α-oxoglutarate accumulates in the first few minutes of incubation, during which $NADP^+$ is rapidly reduced and NAD^+ slowly reduced. Aspartate is not formed in the first few seconds. α-Oxoglutarate formation, on the other hand, is greater during the first few seconds than in the subsequent 2 min.

4. When malonate is added together with glutamate, there is a marked deamination of glutamate and only a partial reduction of $NADP^+$.

5. 2-Methyl-1,4-naphthoquinone stimulates the deamination of glutamate markedly both in the absence and presence of malonate, and brings about an oxidation of NADPH.

6. It is concluded that the most important factor controlling the activity of glutamate dehydrogenase during glutamate oxidation in isolated rat-liver mitochondria is the oxidoreduction state of NADP.

INTRODUCTION

The oxidation of glutamate in freshly prepared rat-liver mitochondria proceeds primarily through the transamination pathway of MÜLLER AND LEUTHARDT[1]. Although glutamate dehydrogenase (EC 1.4.1.3) is present in high concentration in

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liver mitochondria, it contributes very little to the oxidation of this substrate in the isolated mitochondria (*cf.* refs. 2–7, but contrast refs. 8–10). The deamination pathway of glutamate oxidation can be stimulated by damaging the mitochondria[5,6], by the addition of uncouplers[4,5,7], or by the addition of malonate[2,4–7] or arsenite[4,7,11].

The question arises of why the transamination pathway is able to compete so successfully with the deamination pathway for the oxidation of glutamate in isolated liver mitochondria. KLINGENBERG and co-workers[12,13] postulated that glutamate dehydrogenase in intact mitochondria is specific for NADP, in contrast to the isolated enzyme, which reacts with both NAD and NADP. BORST[2] invoked this postulate in his suggestion that the low contribution of glutamate dehydrogenase to the oxidation of glutamate may be due to an accumulation of NADPH. QUAGLIARIELLO and co-workers[4,7] followed the time course of glutamate oxidation in isolated liver mitochondria and found that some deamination occurs during the first few minutes of incubation, but that the rate of deamination decreases, at first rapidly and then more slowly. Similar observations were made by TAGER AND DE HAAN[5]. QUAGLIARIELLO and co-workers[4,7] observed that the initial, rapid decline in glutamate deamination was accompanied by a sharp increase in the extent of reduction of the nicotinamide nucleotides. They assumed this to reflect an almost complete reduction of NADP (*cf.* refs. 12, 14, 15) and proposed that the accumulation of NADPH inhibits glutamate deamination.

In this paper, the results of a detailed examination of the kinetics of glutamate oxidation in rat-liver mitochondria under various conditions are given and some features of the control of the pathway of glutamate oxidation in intact, isolated mitochondria are discussed. In particular, the role of the oxidoreduction state of NADP is considered. Some of the results have been presented in a preliminary form[16,17]. Studies of some of the conditions under which glutamate deamination may be stimulated in isolated mitochondria, and of the problem of citrulline synthesis in rat-liver mitochondria, are reported in the accompanying papers[18,19].

METHODS

Rat-liver mitochondria

Rat-liver mitochondria were prepared by the method of HOGEBOOM[20], exactly as described by MYERS AND SLATER[21].

Experimental conditions

The standard reaction mixture contained 15 mM KCl, 5 mM $MgCl_2$, 50 mM Tris–HCl buffer, 10, 20 or 25 mM potassium phosphate buffer and 25 mM sucrose (derived from the mitochondrial suspension). Other additions are indicated in the legends to the figures. The final pH was 7.5 and the reaction temperature was 25° unless otherwise indicated. The incubation was carried out in Warburg flasks, except for the short-time experiments. The final volume of the reaction mixture in the Warburg flasks was 1 or 3 ml, and the centre well contained 0.1 or 0.3 ml 2 M KOH, respectively, and a fluted filter paper. The reaction was stopped with 0.1 or 0.3 ml 35 % $HClO_4$, respectively.

For the short-time experiments, a technique developed by VAN DAM[22] was used, in which the reaction was carried out in 5-ml beakers with magnetic stirring.

The temperature was kept at 25° by means of a jacket through which water from a constant-temperature bath was circulated. The volume of the reaction mixture was 1 ml. Rapid additions to the reaction mixture were made by blowing out concentrated solutions of the reagents from pipettes. Each point on a curve represents a separate incubation. The reaction was stopped by rapidly blowing 0.1 ml 35 % $HClO_4$, or 0.5 ml 1 M KOH in ethanol, from a pipette into the reaction mixture.

Neutralization of the $HClO_4$ extracts

Several methods were employed. In the first, the protein was removed by centrifugation and a volume of the supernatant was neutralized in an ice-water bath by the addition of 0.5 vol. of 0.25 M Tris–HCl (pH 7.4) and the dropwise addition of the required amount of 3 M KOH (determined previously); magnetic stirring was employed. The mixture was frozen, thawed, and kept in the cold for approx. 30 min, after which the $KClO_4$ was removed by centrifugation. In the second method, the $HClO_4$ extracts were neutralized directly without prior removal of the protein. Again, after approx. 30 min in the cold, $KClO_4$ and protein were removed together by centrifugation. This method was used only in some preliminary experiments for the determination of $NAD(P)^+$. In some short-time experiments, and in all the Warburg experiments, Tris–HCl buffer was not added after the $HClO_4$, and after removal of protein, neutralization was effected by the dropwise addition of 1 M KOH. This led to a more complete precipitation of $KClO_4$ and facilitated the microestimation of $NAD(P)^+$ and α-oxoglutarate.

Neutralization of the alkali extracts

The alkali extracts were neutralized by the dropwise addition, with magnetic stirring, of 0.5 ml of a solution containing 0.5 M triethanolamine, 0.5 M HCl, 0.4 M KH_2PO_4 and 0.1 M K_2HPO_4.

Determination of nicotinamide nucleotides

NAD^+ and $NADP^+$ were determined in the neutralized acid extracts, and NADH and NADPH in the neutralized alkali extracts, by the methods of KLINGENBERG[23] as modified by VAN DAM[22], using the Aminco-Chance double-beam spectrophotometer (wavelength pair 340–374 mμ or 350–375 mμ).

Determination of α-oxoglutarate

α-Oxoglutarate was determined in the neutralized acid extracts with NH_4Cl, NADH and glutamate dehydrogenase, using the Aminco-Chance double-beam spectrophotometer (wavelength pair 340–374 mμ or 350–375 mμ). The reaction mixture (3.0 ml) contained 125 mM Tris–HCl buffer (pH 7.4), 33 mM NH_4Cl, 70 μM NADH and an aliquot of the neutralized reaction mixture (containing a maximum of 120 nmoles α-oxoglutarate); the reaction was started by the addition of 0.6 mg glutamate dehydrogenase.

Determination of ammonia

Ammonia was determined in the neutralized acid extracts by a modification[18] of the method of KIRSTEN, GEREZ AND KIRSTEN[24].

Determination of aspartate

Aspartate was determined by the method of PFLEIDERER, GRÜBER AND WIELAND[25]. For the determination of small amounts (up to 100 nmoles), the Aminco-Chance double-beam spectrophotometer (wavelength pair 350–375 mμ) was used. The reaction mixture (3.0 ml) contained 125 mM Tris–HCl buffer (pH 7.4), 3.3 mM α-oxoglutarate, 70 μM NADH, 10 μg malate dehydrogenase (EC 1.1.1.37) and an aliquot of the neutralized reaction mixture; the reaction was started by the addition of 0.1 mg aspartate transaminase (EC 2.6.1.1). Since a drift in NADH oxidation occurs, the value for the final absorbance was obtained by extrapolation to the time of addition of aspartate transaminase. The cause of the drift is unknown.

Determination of glutamate

Glutamate was determined with NAD^+ and glutamate dehydrogenase (ammonia-free) as described by BERNT AND BERGMEYER[26].

Determination of protein

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

Preparation of hexokinase

Hexokinase (EC 2.7.1.1) was prepared as described by DARROW AND COLOWICK[28], omitting the final crystallization step. In some experiments, hexokinase obtained from Sigma Chemical Co. was used (Type III or IV, dialyzed against 1 % glucose to remove NH_4^+).

Preparation of aspartate transaminase

Mitochondrial aspartate transaminase was prepared by the method of BORST AND PEETERS[29], as modified by TAGER AND SLATER[30], using either horse- or ox-heart muscle as the starting material. For assays with the Aminco-Chance double-beam spectrophotometer, aspartate transaminase obtained from Boehringer und Soehne, Mannheim, was used.

Special chemicals and enzymes

Special chemicals and enzymes were obtained from the following sources: ADP, NAD^+, α-oxoglutarate, sodium pyruvate, glucose 6-phosphate, malate dehydrogenase, lactate dehydrogenase (EC 1.1.1.27), glutamate dehydrogenase (in $(NH_4)_2SO_4$ for α-oxoglutarate determination and in glycerol for glutamate and ammonia determination), alcohol dehydrogenase (EC 1.1.1.1), aspartate transaminase and glucose-6-phosphate dehydrogenase (EC 1.1.1.49), Boehringer und Soehne; 2-methyl-1,4-naphthoquinone, hexokinase (Type III), Sigma Chemical Co.; glutamate and malonate, British Drug Houses.

RESULTS

Nicotinamide nucleotide specificity of glutamate dehydrogenase in rat-liver mitochondria

In Fig. 1, the results of an experiment are shown in which the kinetics were studied of the reduction of intramitochondrial NAD^+ and $NADP^+$ and of the

formation of α-oxoglutarate and aspartate after the addition of glutamate to mitochondria. The mitochondria were preincubated aerobically for 4 min in the presence of arsenite, ADP and P_i; glutamate was then added and, 5 sec later, rotenone. The preincubation served to oxidize NAD(P)H and was carried out for 4 min in order to

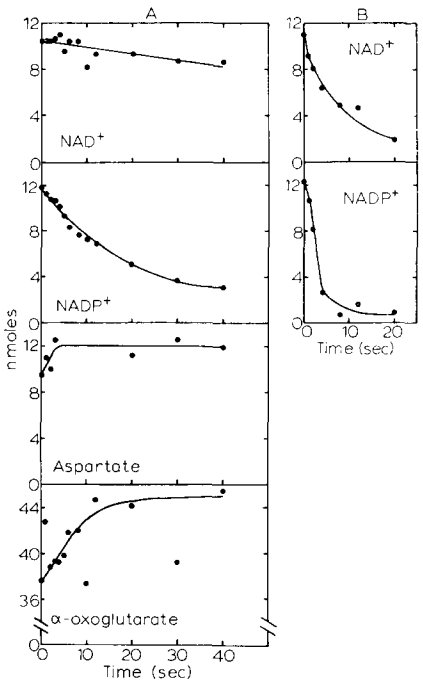


Fig. 1. Reduction of NAD^+ and $NADP^+$ by glutamate and β-hydroxybutyrate, and formation of α-oxoglutarate and aspartate from glutamate in rat-liver mitochondria. The reaction mixture contained the standard components *plus* 5 mM ADP, 1 mM arsenite and 2.7 mg mitochondrial protein. After a preincubation of 4 min in order to oxidize NAD(P)H, 10 mM glutamate was added in A, followed 5 sec later by 2 μg rotenone. The time of addition of rotenone was taken as zero time. In B, rotenone was added immediately after the 4-min preincubation, followed 5 sec later by 10 mM β-hydroxybutyrate. The time of addition of β-hydroxybutyrate was taken as zero time. The reaction was stopped with $HClO_4$ at the times indicated.

allow sufficient time for arsenite inhibition to set in (*cf.* ref. 30). The reason for the 5-sec interval between the addition of glutamate and the addition of rotenone was to ensure that most of the precursors of oxaloacetate, as well as oxaloacetate itself, that might be present would be converted to aspartate by transamination with glutamate. By blocking the oxidation of NADH *via* the respiratory chain with rotenone, by inhibiting the oxidation of α-oxoglutarate with arsenite, by converting oxaloacetate and its precursors to aspartate, and by measuring α-oxoglutarate and aspartate formation and $NAD(P)^+$ reduction, the kinetics and stoicheiometry of the glutamate dehydrogenase reaction (1) could be studied unequivocally*.

$$\text{Glutamate} + \text{NAD(P)}^+ + \text{H}_2\text{O} \rightleftharpoons \alpha\text{-oxoglutarate} + \text{NAD(P)H} + \text{H}^+ + \text{NH}_3 \qquad (1)$$

* In preliminary studies (see Fig. 1 of ref. 16 and Fig. 4 of ref. 31) not all of these precautions were taken, so that the results obtained were equivocal; in particular, the α-oxoglutarate that was found could have been formed not only in the glutamate dehydrogenase reaction but also by transamination.

Fig. 1A shows that at zero time, when rotenone was added, 9.5 nmoles aspartate and 37.6 nmoles α-oxoglutarate were present. Furthermore, some aspartate formation took place in the first 3 sec after the addition of rotenone and then ceased. On the other hand, α-oxoglutarate formation continued at a relatively rapid rate for approx. 10 sec and the rate then gradually declined. Thus the α-oxoglutarate formed after the first 3 sec must have been synthesized according to Eqn. 1. The rapid phase of α-oxoglutarate formation was correlated with a rapid reduction of $NADP^+$, and the decline in its synthesis occurred when NADP had become almost maximally reduced, even though at this stage the level of NAD^+ was still high. The rate of NAD^+ reduction was slow throughout the experimental period of 40 sec. Between 3 and 20 sec, 5 nmoles α-oxoglutarate were synthesized, and 4.5 nmoles $NADP^+$ and 0.5 nmole NAD^+ were reduced.

As a control, the reduction of $NAD(P)^+$ by β-hydroxybutyrate was studied in the same experiment (Fig. 1B). In this case, rotenone was added before the β-hydroxybutyrate. Some important differences in the behaviour of this substrate as compared with glutamate emerge.

(i) With β-hydroxybutyrate, the initial rate of reduction of NAD^+ was more rapid than that of $NADP^+$. However, after 1 sec the rate of reduction of $NADP^+$ exceeded that of NAD^+ because of the functioning of the energy-linked transhydrogenase (*cf.* ref. 32).

(ii) After 4 sec, the level of $NADP^+$ had decreased from 12.5 to 2.5 nmoles, and that of NAD^+ from 11 to 6.5 nmoles. With glutamate, the level of $NADP^+$ decreased to 3 nmoles after 40 sec, and at this stage the level of NAD^+ had declined only from 10.3 to 8.3 nmoles.

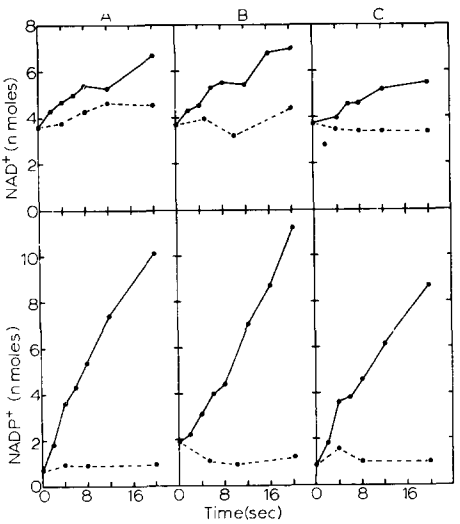


Fig. 2. Oxidation of NADH and NADPH by α-oxoglutarate (*plus* ammonia) in rat-liver mitochondria. Reaction mixture (1 ml) contained the standard components (with the exception of P_i) *plus* 10 mM α-oxoglutarate, 1 mM arsenite, 0.1 mM ATP and 2.3 mg (A and C) or 2.8 mg (B) mitochondrial protein. In addition, 2.5 μg oligomycin was present in Expt. B and 1 μg rotenone in Expt. C. After a preincubation of 30 sec (Expts. A and C) or 60 sec (Expt. B) at 0°, 10 mM NH_4Cl *plus* 10 mM P_i were added and the incubation was continued at 0°. In the controls, only P_i was added. The reaction mixture was kept in ice for 20–30 min before adding the mitochondria, in order to ensure that the temperature was 0° during the preincubation and incubation. The reaction was stopped at the times indicated with $HClO_4$. ●—●, NH_4Cl + P_i; ●---●, P_i.

(iii) The rate of reduction of $NAD(P)^+$ was much greater in the presence of β-hydroxybutyrate than of glutamate.

Fig. 2 shows the results of an experiment in which the reaction was studied from the opposite direction. Mitochondria were preincubated at 0° for 30 or 60 sec with α-oxoglutarate and arsenite, in State 4 as defined by CHANCE AND WILLIAMS[33] (Fig. 2A), in the presence of oligomycin (Fig. 2B) or in the presence of rotenone (Fig. 2C). After 30 or 60 sec, ammonia and P_i were added (or, in control experiments, only P_i), and the incubation was continued at 0°. Since VAN DAM[22] has shown that the transhydrogenase reaction occurs at a negligible rate at 0°, NADH and NADPH could only be oxidized by α-oxoglutarate (*plus* ammonia) in these experiments. In all 3 experiments, NADPH became oxidized more rapidly than NADH, indicating that glutamate dehydrogenase reacts preferentially with NADPH.

Time course of glutamate oxidation in rat-liver mitochondria

In Fig. 3 the results of an experiment are shown in which the kinetics of ammonia production, α-oxoglutarate accumulation and $NAD(P)^+$ reduction were followed during the course of glutamate oxidation by rat-liver mitochondria over a period of 30 min. Since in freshly prepared mitochondria it was found that NADP is almost completely reduced and NAD to the extent of 50–70 %, the mitochondria

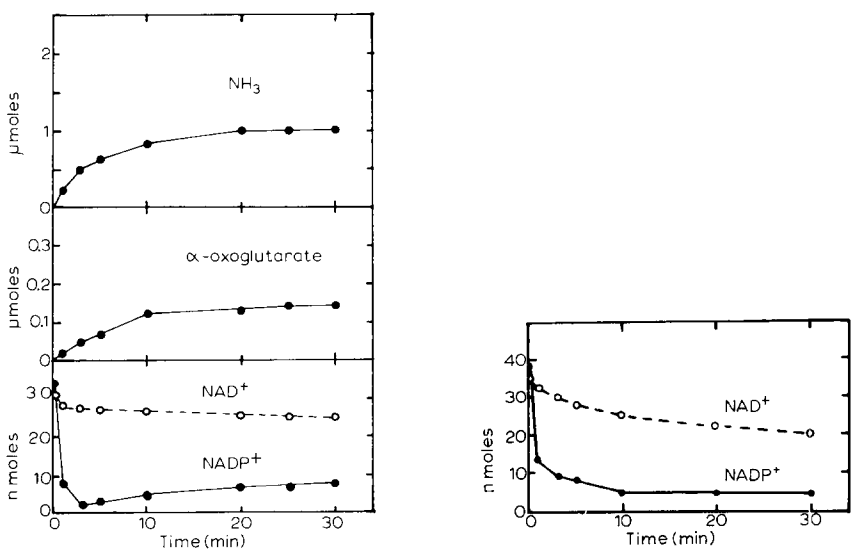
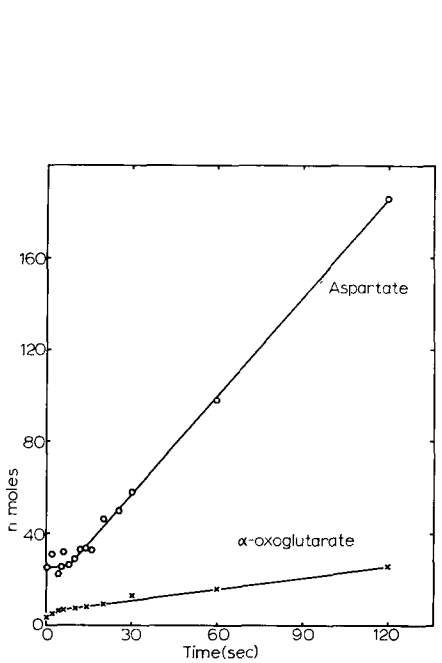


Fig. 3. Kinetics of ammonia formation, α-oxoglutarate accumulation and $NAD(P)^+$ reduction during glutamate oxidation in rat-liver mitochondria, preincubated with ADP. Reaction mixture (3 ml) contained the standard components *plus* 0.3 mM ADP, 30 mM glucose, 3 mg hexokinase (Sigma Type III) and 16 mg mitochondrial protein. After a preincubation of 4 min, 10 mM glutamate was added from the side-arm of the Warburg flask. The reaction was stopped at the times indicated with $HClO_4$. In 2 parallel samples, the reaction was stopped with ethanolic KOH immediately after the 4-min preincubation in order to determine NAD(P)H. The results of these analyses were: NADH, 0.25 nmole; NADPH, 7 nmoles.

Fig. 4. Kinetics of $NAD(P)^+$ reduction during the oxidation of pyruvate *plus* malate by rat-liver mitochondria, preincubated with ADP. Conditions as in Fig. 3, except that 17.5 mg mitochondrial protein were present and 10 mM malate and 10 mM pyruvate were added after the 4-min preincubation. 0.4 nmole NADH and 9.5 nmoles NADPH were found after the preincubation.

were preincubated aerobically with P_i and phosphate acceptor for 4 min in order to oxidize the nicotinamide nucleotides. By this treatment NAD was completely oxidized, while NADP remained 20 % reduced. On the addition of glutamate, there was a rapid formation of ammonia during the first 3 min. Between 3 and 20 min the rate of ammonia formation declined steadily. No ammonia formation occurred during the 20-30 min period (*cf.* refs. 4, 5 and 7). There was an accumulation of α-oxoglutarate during the first 10 min, after which there was little further change. NADP became almost completely reduced (95 %) in the first 2–3 min, after which it began to be slowly oxidized. After 30 min, NADP was 81 % reduced. NAD was slowly reduced during the entire experimental period, and after 30 min, the extent of reduction of NAD was 25 %.

For comparison, the time course of the reduction of nicotinamide nucleotides when pyruvate (*plus* malate) was used as respiratory substrate instead of glutamate is shown in Fig. 4. Mitochondria were preincubated for 4 min in State 2. On the addition of pyruvate *plus* malate, $NADP^+$ became rapidly reduced in the first minute and then more slowly between 1 and 10 min (*cf.* Fig. 3), after which there was no further



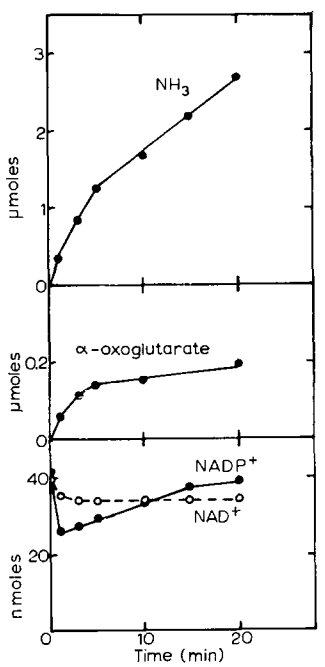


Fig. 5. Kinetics of aspartate formation and α-oxoglutarate accumulation during the first 2 min of glutamate oxidation by rat-liver mitochondria, preincubated with ADP. Reaction mixture contained the standard components *plus* 30 mM glucose, 5 units (μmoles substrate per min) hexokinase, 0.5 mM ADP and 2.4 mg mitochondrial protein. After a 2-min preincubation, 10 mM glutamate was added. The reaction was stopped at the times indicated with $HClO_4$.

Fig. 6. Kinetics of ammonia formation, α-oxoglutarate accumulation and $NAD(P)^+$ reduction during the oxidation of glutamate, in the presence of malonate, by rat-liver mitochondria, preincubated with ADP. Conditions as in Fig. 3, except that 18 mg mitochondrial protein were present and 10 mM glutamate *plus* 20 mM malonate were added after the 4-min preincubation. At the times indicated, the reaction was stopped with $HClO_4$.

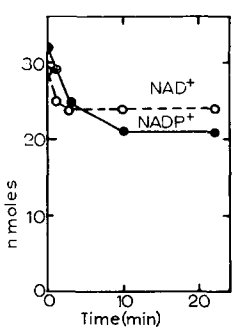
change. In contrast to the situation with glutamate (Fig. 3), no secondary reoxidation of NADPH occurred when pyruvate (*plus* malate) was the substrate (Fig. 4).

The initial rates of aspartate formation and α-oxoglutarate accumulation when glutamate is oxidized by rat-liver mitochondria were compared in the experiment shown in Fig. 5. Mitochondria were preincubated in State 2 for 2 min before glutamate was added. After the addition of glutamate, there was a definite lag of about 4–5 sec in aspartate formation, after which the rate of production of this product of the transamination pathway of glutamate oxidation became constant. This lag in aspartate formation was accompanied by a more rapid rate of α-oxoglutarate accumulation in the first 2–3 sec than in the 3–120-sec period.

Effect of malonate on glutamate oxidation

As will be discussed below, another factor besides direct reduction by glutamate dehydrogenase can contribute to the accumulation of NADPH during glutamate oxidation, namely, the operation of the dehydrogenases involved in the transaminase pathway and the energy-linked transhydrogenase reaction.

Malonate blocks the transamination pathway of glutamate oxidation almost completely[2,4,5,7,8,34]. In the experiment shown in Fig. 6, glutamate and malonate were added to mitochondria which had been preincubated for 4 min in State 2. It can be seen that, in the presence of malonate, a considerable accumulation of ammonia occurred (*cf.* Fig. 3). Ammonia formation and α-oxoglutarate accumulation were more rapid in the first 5 min than in the 5–20-min period. Indeed, the amount of α-oxoglutarate found increased only very slightly in the 5–20-min period, in agreement with earlier observations[2,6,35] that very little α-oxoglutarate accumulates during glutamate oxidation even in the presence of malonate. The reduction of NAD^+ showed a similar pattern in the presence of glutamate *plus* malonate as in the presence of



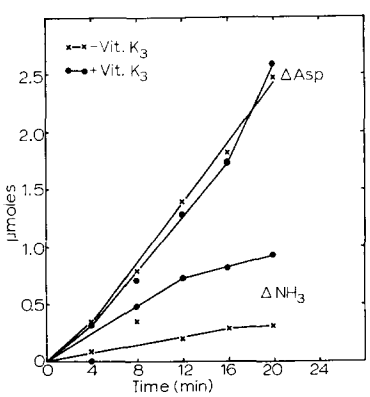
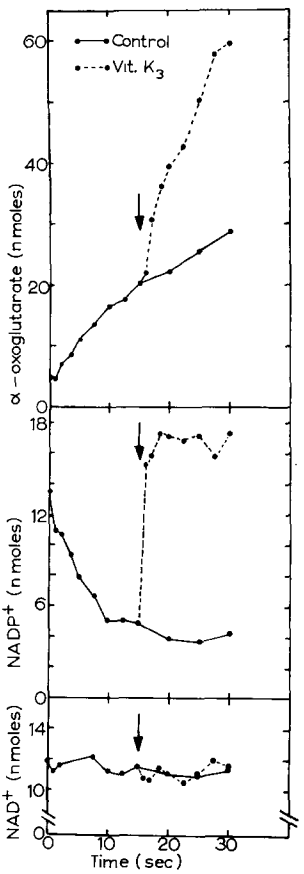


Fig. 7. Kinetics of $NAD(P)^+$ reduction during the oxidation of α-oxoglutarate in the presence of malonate by rat-liver mitochondria, preincubated with ADP. Conditions as in Fig. 3, except that 15 mg mitochondrial protein were present and 10 mM α-oxoglutarate and 20 mM malonate were added after the 4-min preincubation.

Fig. 8. Effect of 2-methyl-1,4-naphthoquinone on aspartate and ammonia formation from glutamate in rat-liver mitochondria. Reaction mixture (1 ml) contained the standard components *plus* 30 mM glucose, 0.5 mM ADP, 5 units yeast hexokinase, 10 mM glutamate and 2.6 mg mitochondrial protein. The reaction was stopped with $HClO_4$ at the times indicated. Where present, 2-methyl-1,4-naphthoquinone was used at a concn. of 10^{-5} M. Abbreviation: vit. K_3, 2-methyl-1,4-naphthoquinone.

glutamate alone (Figs. 3 and 6). However, malonate caused a marked difference in the response of NADP. In the presence of glutamate *plus* malonate, NADP reached a maximum extent of reduction of 50 % in 1 min, after which it became slowly reoxidized and was only 23 % reduced after 20 min.

In a control experiment, α-oxoglutarate *plus* malonate were added instead of glutamate *plus* malonate. The results are shown in Fig. 7. NAD^+ became partly reduced in the first 3 min, and then showed no further change. $NADP^+$ also became partly reduced in the first 3 min, and this was followed by a slight further reduction.



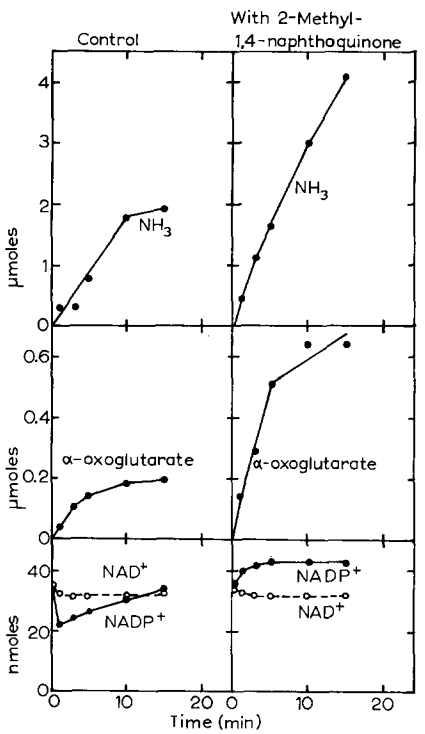


Fig. 9. Kinetics of glutamate oxidation in rat-liver mitochondria (preincubated with ADP) during the first 30 sec after addition of glutamate, and the effect of 2-methyl-1,4-naphthoquinone. Reaction mixture (1 ml) contained the standard components *plus* 5 mM ADP, and 3.1 mg mitochondrial protein. After a 90-sec preincubation, 10 mM glutamate was added. After a further 15 sec, 10^{-5} M 2-methyl-1,4-naphthoquinone and 2 % ethanol (or, in the controls, only ethanol) were added. The reaction was stopped at the times indicated with $HClO_4$.

Fig. 10. Effect of 2-methyl-1,4-naphthoquinone on the kinetics of ammonia formation, α-oxoglutarate accumulation and $NAD(P)^+$ reduction during the oxidation of glutamate, in the presence of malonate, by rat-liver mitochondria, preincubated with ADP. Conditions as in Fig. 3, except that 10 mM glutamate and 20 mM malonate were added after the 4-min preincubation. In the right-hand figure, 10^{-5} M 2-methyl-1,4-naphthoquinone was added together with the glutamate *plus* malonate. The reaction was stopped with $HClO_4$ at the times indicated.

In contrast to the situation when glutamate *plus* malonate were added, no secondary reoxidation of NADPH occurred in the presence of α-oxoglutarate *plus* malonate.

Effect of 2-methyl-1,4-naphthoquinone

2-Methyl-1,4-naphthoquinone accepts hydrogens from both NADH and NADPH[36,37]. In the presence of this carrier, intramitochondrial nicotinamide nucleotides become oxidized *via* NAD(P)H dehydrogenase (EC 1.6.99.2) and the cytochrome system, and the oxidation of NADPH becomes independent of the transhydrogenase reaction[38].

The effect of 2-methyl-1,4-naphthoquinone on the oxidation of glutamate, both in the absence and in the presence of malonate, was studied. As shown in Fig. 8, in the absence of malonate, 2-methyl-1,4-naphthoquinone had no effect on the formation of aspartate from glutamate, but stimulated the deamination of glutamate markedly. This effect of 2-methyl-1,4- naphthoquinone was examined more closely in an experiment in which the kinetics of the reaction were studied in the first 30 sec after adding glutamate to rat-liver mitochondria preincubated in State 2 for 2 min. The results are shown in Fig. 9. In the absence of 2-methyl-1,4-naphthoquinone, the rate of α-oxoglutarate formation was more rapid in the first 10 sec, the period during which $NADP^+$ was being rapidly reduced, than in the 10–20-sec period, when the $NADP^+$ level was low. The NAD^+ level remained the same throughout the experimental period. When 2-methyl-1,4-naphthoquinone was added after 15 sec, there was an abrupt rise in the level of $NADP^+$, which reached a maximum value within 3 sec, and a very marked stimulation of α-oxoglutarate formation. 2-Methyl-1,4-naphthoquinone had no effect on the level of NAD^+.

The effect of 2-methyl-1,4-naphthoquinone on the oxidation of glutamate in the presence of malonate is shown in Fig. 10. 2-Methyl-1,4-naphthoquinone stimulated ammonia formation and α-oxoglutarate accumulation, brought about an oxidation of NADPH and had no effect on the level of NAD^+. The increase in the level of $NADP^+$ that occurred in the presence of 2-methyl-1,4-naphthoquinone was not preceded by a decrease, as was the case when only glutamate and malonate were present (see also Fig. 6).

DISCUSSION

When freshly isolated rat-liver mitochondria oxidize glutamate in the presence of phosphate and phosphate acceptor, the main product of the reaction is aspartate. In confirmation of the results of BORST[2], DE HAAN, TAGER AND SLATER[6,18] have found that in experiments of 6–40 min at 25°, at least 90% of the glutamate that disappears is recovered as aspartate and little as ammonia. However, PAPA, PALMIERI AND QUAGLIARIELLO[17] showed that relatively more ammonia appears during the first few minutes of incubation. This was confirmed by DE HAAN and co-workers [5,18] and by Fig. 3 of this paper in which the initial production of ammonia was increased by preincubating the mitochondria with phosphate and ADP. Even under these conditions, little ammonia production took place after 10 min.

The reason for the dominance of the transamination pathway and the inhibition of the deamination pathway of glutamate oxidation in isolated liver mitochondria has been discussed by BORST[2], QUAGLIARIELLO and co-workers[3,4,7] and TAGER AND

DE HAAN[5]. The possible role of an accumulation of the products of the reaction in inhibiting glutamate deamination has been considered by BORST[2]. He suggested that the inhibition is unlikely to be due to an accumulation of α-oxoglutarate, since this product is not found in the reaction mixture in high concentrations, and proposed that the accumulation of reduced nicotinamide nucleotides brings about the inhibition (see also refs. 4, 7). During the oxidation of glutamate in State 3, NADP is almost completely reduced (*cf.* refs. 12, 14, 15, and this paper). If glutamate dehydrogenase in liver mitochondria is an NADP-linked enzyme (*cf.* refs. 12, 13), the factor controlling the rate of oxidation of glutamate by this enzyme may be the rate of reoxidation of NADPH.

The results presented in this paper provide the following direct evidence that the deamination of glutamate by glutamate dehydrogenase and, consequently, the contribution of the deamination pathway to glutamate oxidation, are controlled by the rate of reoxidation of NADPH and the availability of $NADP^+$.

(1) In the presence of rotenone and arsenite, the oxidation of glutamate to α-oxoglutarate is accompanied by an extensive reduction of $NADP^+$ and very little reduction of NAD^+ (Fig. 1). The oxidation ceases when NADP is maximally reduced even though the level of NAD^+ is still high (Fig. 1). Also in the absence of these inhibitors, a close correspondence is found between the level of $NADP^+$ and the rate of deamination of glutamate (Figs. 3, 6, 9 and 10). Furthermore, glutamate dehydrogenase in the mitochondria reacts more rapidly with NADPH than with NADH (Fig. 2) under conditions where the transhydrogenase reaction is negligible[22]. These results suggest that glutamate dehydrogenase in rat-liver mitochondria reacts preferentially with NADP (*cf.* ref. 12).

(2) As mentioned above, a close correspondence is always found between the rate of deamination of glutamate and the level of $NADP^+$. In the absence of malonate, the initial decline in ammonia production is accompanied by a very rapid disappearance of $NADP^+$ (Figs. 3 and 9). The constant supply of reducing equivalents to NAD^+ by the dehydrogenases involved in the transamination pathway and the operation of the energy-linked transhydrogenase favour the reduction of $NADP^+$. The oxidation of NADPH by NAD^+ is rendered impossible by the unfavourable equilibrium of the energy-linked transhydrogenase under conditions where there is a constant supply of reducing equivalents to NAD^+. Because of the lack of $NADP^+$ under these conditions, glutamate deamination declines. In the presence of 2-methyl-1,4-naphthoquinone, there is a bypass of the transhydrogenase reaction, NADPH is oxidized directly, and as a result there is a marked stimulation of the deamination of glutamate (Figs. 8 and 9).

(3) The deamination of glutamate is also stimulated by malonate. Malonate has two effects. In the first place, by inhibiting succinate dehydrogenase (EC 1.3.99.1), it prevents the regeneration of oxaloacetate, so that the transaminase pathway cannot function. In the second place, the extent of reduction of NADP is decreased in the presence of malonate and the increased availability of $NADP^+$ is correlated with a greatly increased rate of deamination of glutamate (Fig. 6). The reason for the decreased reduction of NADP is of interest (see ref. 17). A decreased extent of reduction of NADP is brought about by malonate not only in the presence of glutamate (Fig. 6) but in the presence of α-oxoglutarate as well (Fig. 7), indicating the importance of succinate dehydrogenase and/or malate dehydrogenase in supplying

reducing equivalents for the reduction of $NADP^+$. However, even in the presence of malonate, glutamate deamination does not occur at a maximal rate and may be stimulated by the addition of 2-methyl-1,4-naphthoquinone (Fig. 10). This stimulation of ammonia production is accompanied by an increase in the level of $NADP^+$ (Fig. 10). Our interpretation of these results is that in the presence of malonate, the rate of reoxidation of NADPH by NAD^+ still limits the rate of deamination. Addition of 2-methyl-1,4-naphthoquinone allows NADPH oxidation *via* NAD(P)H dehydrogenase[36–38] thus bypassing the transhydrogenase reaction. The result is that deamination proceeds at an increased rate.

(4) As discussed in the accompanying paper[18] and elsewhere[4,5,7], uncouplers stimulate the deamination of glutamate. This effect may be due in part at least to an inhibition of the energy-linked transhydrogenase. Indeed, it has been found that increasing concentrations of uncoupler bring about a progressive increase both in the oxidation state of NADP and in the extent of deamination of glutamate[18].

The question remains of the role of an accumulation of ammonia and α-oxoglutarate in the inhibition of the deamination of glutamate. As shown in an accompanying paper[19], the small amounts of ammonia that are formed during glutamate oxidation, in the absence of inhibitors, can be utilized for citrulline synthesis, and removal of this ammonia does not lead to any increase in deamination. Thus, it is unlikely that the rate of deamination of glutamate under normal conditions is limited by the fact that the reaction catalysed by glutamate dehydrogenase approaches equilibrium. In the presence of arsenite and absence of other inhibitors, however, α-oxoglutarate accumulates and the rate of glutamate oxidation under these conditions is limited by the rate of efflux of α-oxoglutarate from the mitochondria[39]. This is also the case in the presence of arsenite and rotenone (S. PAPA AND A. FRANCAVILLA, unpublished observations).

The experiments described in this paper provide a striking demonstration of the effectiveness of the energy-linked transhydrogenase in reducing mitochondrial $NADP^+$. For example, only 4 sec after adding β-hydroxybutyrate to mitochondria, in the presence of rotenone, twice as much $NADP^+$ is reduced as NAD^+ (Fig. 1B), despite the fact that β-hydroxybutyrate dehydrogenase is NAD-specific. Similarly, pyruvate *plus* malate were much more effective in reducing $NADP^+$ than NAD^+ (Fig. 4).

During the oxidation of glutamate by rat-liver mitochondria, either in the absence or in the presence of malonate, a slow oxidation of NADPH takes place during the latter stages of the incubation (Figs. 3 and 6). Possible reasons for this have been discussed by PAPA *et al.*[17]. No oxidation of NADPH occurs when the substrate is malate *plus* pyruvate (Fig. 4) or α-oxoglutarate *plus* malonate (Fig. 6).

In the experiment shown in Fig. 5, a small lag in aspartate formation was observed. This lag lasted for a few seconds, and during this time the rate of α-oxoglutarate accumulation was more rapid than subsequently. It is possible that the lag is caused by a shortage of oxaloacetate and its precursors and that they are provided by the oxidation of glutamate *via* glutamate dehydrogenase. Once these precursors are present, the oxidation of glutamate *via* the transamination pathway occurs with great efficiency, in spite of the fact that the steady-state concentration of intermediates of the transamination pathway, such as oxaloacetate and malate*, is rel-

* E. J. DE HAAN, unpublished observations.

atively low. This indicates that there must be little or no loss of these intermediates from the mitochondrion.

In conclusion, these results raise the question of what the source of ammonia is for citrulline synthesis in the intact liver (see refs. 40 and 41). This is discussed in the accompanying paper[19].

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