Glutamate Synthesis and the Control of Reactions Linked with the Nicotinamide-Adenine Dinucleotide Coenzymes in Mitochondria

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Jones & Gutfreund (1961) showed that the oxidation of glutamate by guinea-pig-liver and mammary-gland mitochondria proceeds largely, and perhaps entirely, via transamination and the oxidation of α -oxoglutarate to oxaloacetate, resulting in the overall reaction proposed by Krebs & Bellamy (1960):

Borst & Slater (1960) and Jones & Gutfreund (1961) studied rat-heart sarcosomes and guinea-pig-mammary-gland mitochondria respectively, and found that the above is the only possible pathway for glutamate oxidation, since no glutamate-dehydrogenase activity can be detected in these tissues. In liver mitochondria we found, however, a very high concentration of glutamate dehydrogenase and yet this enzyme accounts for less than one-quarter of the oxygen uptake due to glutamate oxidation. We extended our studies of the reactions of mitochondrial glutamate dehydrogenase to investigate the reverse reaction, the synthesis of glutamate from ammonia and α -oxoglutarate, by various mitochondrial preparations under a variety of conditions. The reductive amination of α -oxoglutarate catalysed by glutamate dehydrogenase is much more rapid than glutamate oxidation and is considerably favoured thermodynamically. Either NADPH or NADH can serve as hydrogen donor. Krebs, Eggleston & Hems (1948) carried out extensive studies on glutamate synthesis in rat-liver homogenates and discussed previous work in this field. Studies of glutamate synthesis by various mitochondrial preparations can present a very sensitive test for the presence of glutamate dehydrogenase in systems deficient in this enzyme, and can prove that the enzyme is at least partially in an active form in intact mitochondria. Further, in liver mitochondria, which have very high glutamate-dehydrogenase activity, one can adjust conditions so as to oxidize NADH and NADPH at various rates. It is thus possible to study various steps of the citric acid cycle in the steady state in the absence of the complex kinetic control of respiration by replacing this pathway for the oxidation of NADH and NADPH by a relatively-simple easily-controlled single-enzyme reaction.

The situations involving the two different pathways for the oxidation of NADH and NADPH are not necessarily identical in their structural and kinetic relations to the enzyme systems involved in the nucleotide reduction. 'Compartmentation' may well play an important role in the control of mitochondrial metabolism, and the respiratory chain and the glutamate-dehydrogenase system could have different relationships with various 'compartments' containing the nicotinamide-adenine dinucleotides.

EXPERIMENTAL

Mitochondria were prepared from guinea-pig liver or mammary gland by the methods of Jones & Gutfreund (1961) and were used within 5 hr. of preparation for experiments involving intact mitochondria. For experiments with broken mitochondria we used material which had been frozen at -20° and thawed out just before the experiment. In some specified experiments, the freezing and thawing was repeated several times.

The standard incubation medium (pH 7.4) for all experiments with intact mitochondria contained tris (20.0 mM), potassium phosphate (16 mM) and MgSO_4 (8 mM). Other additions are specified for each experiment.

Respiration was measured with a Clark oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, Ohio, U.S.A.); a potential of -0.6 V was applied to the platinum electrode. The current flow between the silver and platinum electrodes in air-saturated solutions was approximately $1\text{ }\mu\text{A}$ greater than the small residual current in anaerobic solutions. This current was recorded on a 0–100 μV scale by connexion of a W. G. Pye and Co. Ltd. d.c. amplifier (11370) across a 100 Ω resistance. The output of the amplifier was connected to a 1 mA, 1500 Ω recorder (Record Electrical Co. Ltd., Altrincham, Cheshire), and a 10 k Ω variable resistance was connected in series with the recorder for fine adjustment of sensitivity. A submersible magnetic stirrer (Rank Bros., Bottisham, Cambridge) was mounted in a thermostatically-controlled water bath and the reaction vessel was fixed to it.

Respiratory experiments involving subsequent analysis of incubation mixtures for glutamate and citrate were carried out by the conventional Warburg technique. All kinetic measurements were carried out at 25° .

The oxidation and reduction of NAD and NADP in intact mitochondria were observed by a fluorimetric technique. The direct-reading fluorimeter (Electronic Instruments Ltd., Richmond, Surrey) Model 27A was used as follows. The filter combination was a Chance OX1

filter mounted in front of a mercury lamp to select the 3655 Å band for excitation of fluorescence, and a Chance OY 13 with a Wratten H filter mounted between fluorescing sample and the photomultiplier. This gives a wide transmission band between 440 and 530 m μ . The output of the fluorimeter was recorded with the same equipment used for recording that of the oxygen electrode. No absolute concentrations of mitochondrial reduced and oxidized NAD and NADP were recorded: the technique was only employed to study relative rates and amounts of their reduction under various conditions.

The concentrations of NADH and NADPH were estimated spectrophotometrically with a Unicam SP. 500 assuming 6.2 (1 cm. optical path) to be the extinction change at 340 m μ on reduction of a mM solution of either NAD⁺ or NADP⁺. Some comparative kinetic measurements were carried out on a 'home-made' single-beam recording spectrophotometer.

Protein concentrations were determined by the micro-Kjeldahl technique, the factor 6.25 being used to convert nitrogen concentration into protein concentration. Citrate was estimated by the method of McArdle (1955). Glutamic acid and aspartic acid were separated from other ninhydrin-positive material by electrophoresis on Whatman no. 30 paper in 2% (w/v) sodium borate at 4 v/cm. for 3 hr. The strips were dried in air at room temperature and dipped in a mixture of 100 ml. of 0.3% (w/v) ninhydrin in 95% (v/v) ethanol, 6.5 ml. of 0.02% EDTA and 4 ml. of acetic acid. The strips were then heated to 100° until the bands were clearly visible; the bands were then eluted and glutamic acid plus aspartic acid was estimated by the method of Krebs & Bellamy (1960). Two glutamic acid standards were included in each batch of five strips. The decarboxylase method (Krebs & Bellamy, 1960) was used to determine separately the glutamic acid and aspartic acid synthesized.

RESULTS

Respiration and glutamate synthesis by intact mitochondria in the presence of ammonia and citric acid-cycle substrates

Table 1 shows that the rate of oxygen uptake by suspensions of guinea-pig-liver mitochondria provided with different substrates is inhibited on addition of ammonia. (The exception found on addition of succinate is discussed below.) All the substrates yield α -oxoglutarate and reduced nicotinamide-adenine dinucleotides. In the absence of ammonia the reduced nicotinamide-adenine dinucleotides are available for reoxidation via the respiratory chain, but in the presence of ammonia some will be reoxidized by glutamate dehydrogenase during synthesis of glutamate.

Gutfreund, Ebner & Mendiola (1961) showed that the glutamate dehydrogenase of liver mitochondria can utilize NADH and NADPH for glutamate synthesis at a rate comparable with that of their production by mitochondrial dehydrogenases. No glutamate-dehydrogenase activity could be detected in guinea-pig-mammary-gland mitochondria (Jones & Gutfreund, 1961; Gutfreund *et al.* 1961)

and this is confirmed by the absence of any effect on the rate of oxidation of α -oxoglutarate in the presence of ADP on addition of ammonia at concentrations up to 10 mM. Also no glutamate could be detected after incubation of mammary-gland mitochondria with various citric acid-cycle intermediates and ammonia. In an experiment carried out under optimum conditions for glutamate synthesis, mammary-gland mitochondria (3.32 mg. of protein/ml.) were incubated in the presence of excess of hexokinase, glucose (8 mM), ATP (0.4 mM), α -oxoglutarate (4 mM), succinate (4 mM) and ammonium chloride (8 mM). Glutamate analyses showed that less than 1 μ m-mole of glutamate was formed/min./mg. of mitochondrial protein. This is less than $\frac{1}{10}$ th of the rate of glutamate synthesis by liver mitochondria under these conditions and is the limit of the accuracy of our analytical procedure. One unexplained anomaly is the inhibition by ammonia (6, 13 and 20 mM-ammonia cause 17, 58 and 83% inhibition respectively) of citrate and isocitrate oxidation by mammary-gland mitochondria. The isocitrate-dehydrogenase activity of broken mitochondria was not inhibited by the addition of ammonia.

Citrate and glutamate analyses of three reaction mixtures after incubation of liver mitochondria with citrate (4 mM) and ammonia (4 mM) indicated that 45, 56 and 38% respectively of the citrate utilized was converted into glutamate.

The addition of succinate (4 mM) to a mitochondrial suspension oxidizing citrate increased the rate of respiration by 12% and decreased the rate at which citrate disappeared by 31%. When

Table 1. *Effect of ammonia concentration on the rate of respiration of guinea-pig-liver mitochondria in the presence of different substrates*

The concentration of the added substrate was 4 mM. Measurements were made at 25° with the oxygen electrode.

Substrate	Concn. of ammonia (mM)	Inhibition of rate of O ₂ uptake (%)
α -Oxoglutarate	7.4	27
	13.8	42
	19.3	55
Citrate	3.8	45
	7.4	72
	13.8	88
Malate	7.4	41
	13.8	52
	19.3	68
Succinate	7.4	-10*
	13.8	-19*

* Signifies percentage increase in oxygen uptake.

ammonia and succinate were added to a mitochondrial suspension oxidizing citrate, the rate of citrate disappearance was 22 % greater than when citrate alone was being oxidized in the absence of ammonia. Ammonia increased the rate of citrate disappearance in the presence of succinate twofold. In the incubation mixtures containing succinate as well as citrate and ammonia, the disappearance of citrate corresponded almost quantitatively to the amount of glutamate formed, which was 5-6 times that synthesized in the absence of succinate (see also Table 2). The interpretation of the role of succinate is discussed below, together with other effects of succinate on mitochondrial reactions.

We also investigated the effect on glutamate synthesis of a number of reagents which alter the ratio of oxidized to reduced nicotinamide-adenine dinucleotides. Our mitochondrial preparations all exhibited good respiratory control (Lardy, 1956), i.e. in the absence of ADP as phosphate acceptor the intramitochondrial NAD and NADP were in the reduced form (giving maximum fluorescence) and respiration was only about 10 % of that observed during coupled phosphorylation of ADP. Table 2 gives data for the relative rates of glutamate synthesis by guinea-pig-liver mitochondria in the presence and absence of phosphate acceptor, as well as during conditions of uncoupled respiration in the presence of DNP and under conditions of inhibited respiration in the presence of antimycin. The effect of the presence of succinate with and without the various reagents affecting respiration is also shown in Table 2. The interpretation of these data depends on further information, presented below, on the effects of the different components on the rate of respiration and on the ratio of oxidized to reduced nicotinamide-adenine dinucleotides.

As stated in the Experimental section, the routine estimation of glutamate by paper electrophoresis and ninhydrin assay gave values for glutamate plus aspartate, and all values quoted for glutamate include any aspartate formed. In view of the high transaminase activity of the mitochondria (Gutfreund *et al.* 1961) the aspartate concentration of some incubation mixtures was estimated: it was found to be less than 5 % of the combined glutamate and aspartate under all the conditions used, except when malate or succinate was added, when it could be as much as 30 % of the total.

Fluorimetric studies of the oxidation and reduction of intramitochondrial nicotinamide-adenine dinucleotides

Our studies of relative changes of fluorescence were intended to supplement previous investigations on the effects of various substrates and inhibitors on the state of oxidation of mitochondrial NAD and NADP (Chance & Williams, 1956; Chance, 1957). Fig. 1 (*a*) shows the changes in fluorescence of mitochondrial suspensions observed on oxidation and reduction of nicotinamide-adenine dinucleotides. We assume that any increase in fluorescence under the conditions of our experiments can be directly correlated with increased concentration of reduced nicotinamide-adenine dinucleotides. The reaction mixtures contained approximately 3 mg. of mitochondrial protein/ml. in our standard incubation medium. The proportion of the total mitochondrial nicotinamide-adenine dinucleotides which was in the reduced state under various conditions was estimated by certain calibration experiments, in each series of experiments, carried out with the same concentration of one mitochondrial preparation.

Table 2. *Rate of glutamate synthesis by guinea-pig-liver mitochondria in the presence of ammonia and of different substrates*

The concentration of ammonia and of the added substrates was 4 mM. Incubation was at 25°. The effects of the presence of excess ADP or absence of phosphate acceptor as well as the effects of antimycin and DNP are recorded.

Substrates added	Glutamate synthesized (μ m-moles/min./mg. of protein)	
	With phosphate acceptor	Without phosphate acceptor
Citrate	5, 6, 7, 6	3
α -Oxoglutarate	14, 15, 12	6
Citrate + α -oxoglutarate	28	17.3
Citrate + succinate	45, 32, 36, 27, 30	4, 5
Citrate + malate	25	11
α -Oxoglutarate + succinate	32, 36	—
Citrate + antimycin (20 μ M)	12, 15	—
Citrate + succinate + antimycin (20 μ M)	22	11
Citrate + DNP (mM)	4, 5	—
Citrate + succinate + DNP (mM)	30, 25	30

First the fully oxidized state was observed by addition of ADP under aerobic conditions in the absence of substrate, and secondly the fully reduced state was observed anaerobically in the presence of succinate (Chance & Williams, 1956).

The rate of reoxidation of intramitochondrial NAD and NADP by glutamate dehydrogenase on addition of ammonium chloride was studied in the

following reaction mixtures with intact guinea-pig-liver mitochondria. First the effect of different NH_4^+ -ion concentrations on the reoxidation reaction in the presence of citrate or α -oxoglutarate was investigated. The results are illustrated in Figs. 1 (b) and 1 (c). Such experiments could only be carried out in the absence of ADP, and therefore without respiration, since under conditions of active oxidative phosphorylation the coenzymes of guinea-pig-liver mitochondria are largely oxidized. Fig. 1 (d) shows the oxidation of the coenzymes in the presence of citrate on addition of a small amount of ADP, and their reduction when the ADP has been phosphorylated to ATP. Intramitochondrial nicotinamide-adenine dinucleotides which have been through an ADP cycle of oxidation and reduction are oxidized very rapidly on addition of ammonia, as can be seen by comparison of Figs. 1 (b) and 1 (d). To investigate further this rapid reoxidation by ammonia after pretreatment with ADP, experiments were carried out with mitochondria and citrate and the addition of an amount of ATP equivalent to that formed from ADP in the previous experiment: no effect on the rate of reoxidation of mitochondrial NADH and NADPH was obtained on subsequent addition of ammonia.

Experiments with guinea-pig-mammary-gland mitochondria, which had the same respiratory and fluorescence responses to citrate, ADP and succinate as described for liver mitochondria, showed that no reoxidation of NADH and NADPH occurred in this system on addition of ammonia. This was so even for mammary-gland mitochondria pretreated with ADP, and is further evidence for the connexion between ammonia-stimulated reoxidation and the utilization of NADH and NADPH by glutamate dehydrogenase for glutamate synthesis.

Experiments with broken mitochondria

Recent studies of the enzyme activities in mitochondria disrupted by freezing and thawing indicated that the NAD- and NADP-linked enzymes of guinea-pig-liver mitochondria could, under optimum conditions, reduce coenzymes much more rapidly than they were oxidized by the respiratory chain (Gutfreund *et al.* 1961). In the present investigation we wished to compare the maximum rate of glutamate synthesis by intact and by broken liver mitochondria. The maximum rate of glutamate synthesis in intact mitochondria was $0.045 \mu\text{mole/min./mg. of protein}$. Broken mitochondria will synthesize glutamate on incubation with isocitrate, NADP^+ , ammonia and traces of Mn^{2+} ion. Aconitase activity was very low and citrate was not utilized in this system. When mitochondria were frozen and thawed four times and

Fig. 1 (a-d). Records of fluorescence changes during the oxidation and reduction of nicotinamide-adenine dinucleotides in suspensions of guinea-pig-liver mitochondria containing approx. 3 mg. of mitochondrial protein/ml. Additions were made at the points indicated. The concentrations given for the reagents added are mM for the reaction mixture at the time of addition. The broken line in Fig. 1 (b) refers to an experiment in which the substrate was 4 mM- α -oxoglutarate instead of 4 mM-citrate.

then incubated in the presence of isocitrate (4 mM), ammonia (4 mM), NADP^+ (0.1 mM) and manganous sulphate ($33 \mu\text{M}$), they synthesized $0.17 \mu\text{mole}$ of glutamate/min./mg. of protein. Spectroscopic observation of absorption at $340 \text{ m}\mu$ showed that during the steady state the NADP was fully reduced and consequently the steady-state concentration of α -oxoglutarate was 0.1 mM, since such pretreated mitochondria did not oxidize α -oxoglutarate and the conversion of isocitrate into glutamate was quantitative. When four-times-frozen mitochondria were incubated with α -oxoglutarate (2 mM), ammonia (4 mM) and NADH they synthesized glutamate at the rate of $0.31 \mu\text{mole/min.}$ /mg. of protein.

Glutamate dehydrogenase can use either NADH or NADPH , although at different rates, as hydrogen donor for the synthesis of glutamate; therefore any final comparison of the activity of the enzymes in intact and disrupted mitochondria has to await the answer to the question which of the two coenzymes is more readily available for glutamate synthesis in the intact particles.

Bendall & de Duve (1960) reported that glutamate-dehydrogenase activity of intact rat-liver mitochondria is only about 5% of that found in mitochondria treated with detergent or repeated freezing and thawing. Our comparisons indicate that the maximum activity for the synthesis of glutamate by intact guinea-pig-liver mitochondria is about 26 and 14% of that by broken mitochondria in the presence of NADPH and NADH respectively.

DISCUSSION

The experiments with mammary-gland mitochondria described above served both to establish the absence of glutamate-dehydrogenase activity in these respiratory particles and as useful controls for some of the experiments with liver mitochondria. Comparative experiments proved that in spite of some latency glutamate dehydrogenase is at least partially in an active form in intact liver mitochondria, though there is no detectable activity of this enzyme in mammary-gland mitochondria. Intact liver mitochondria converted citrate into glutamate in the presence of ammonia, and broken liver mitochondria converted isocitrate into glutamate in the presence of NADP^+ and ammonia. Neither intact nor broken mammary-gland mitochondria could be stimulated to convert citrate or isocitrate into glutamate, though isocitrate oxidation proceeded at a similar rate in mitochondria from the two tissues (Jones & Gutfreund, 1961; Gutfreund *et al.* 1961). Parallel experiments, with mammary-gland and liver mitochondria, to study each effect of the addition of ammonia on oxidation and reduction phenomena,

indicated the contributions due to reversal of the glutamate-dehydrogenase reaction. For instance, the addition of ammonia to mammary-gland mitochondria up to a concentration of 10 mM had no effect on the oxidation of α -oxoglutarate, showing that the lowering of the rate of oxygen uptake on addition of ammonia to liver mitochondria oxidizing α -oxoglutarate is due to oxidation of a proportion of the reduced coenzymes by glutamate dehydrogenase instead of by the respiratory chain. This proportion depends on the ammonia concentration, which controls the rate of glutamate synthesis under the conditions of the experiments reported; the K_m of glutamate dehydrogenase for ammonia is 57 mM (Olson & Anfinsen, 1953), so that at ammonia concentrations up to 10 mM the rate of glutamate synthesis is to a first approximation directly proportional to the ammonia concentration.

The decrease in respiration on addition of ammonia to mammary-gland mitochondria oxidizing citrate or isocitrate cannot be explained at present; we could not find any single enzymic reaction involved in this system that is inhibited at the concentrations of ammonia used. It will, however, be necessary to consider this inhibition when attempting to interpret quantitatively the kinetics of the utilization of reduced nicotinamide-adenine dinucleotides by the alternative pathways of the respiratory chain and glutamate synthesis.

The potential activity of the dehydrogenases of disrupted mitochondria is much greater than the rate of respiration/mg. of mitochondrial protein (Gutfreund *et al.* 1961). However, the only dehydrogenase of the citric acid cycle which appears to be in a fully free and active form in intact mitochondria is NADP -linked isocitrate dehydrogenase (no NAD -linked oxidation of isocitrate could be detected in our mitochondrial preparations). No detailed information is available about the rate of hydrogen transfer between the two coenzymes. If the activities of the intramitochondrial enzymes are only about 10% of their potentialities as free enzymes, then the rates of oxidation and reduction of NAD and NADP are closely balanced and could easily account for the predominance of the oxidized form in the respiratory steady state [Fig. 1 (a)].

Both the amounts and the rates of reoxidation of intramitochondrial coenzymes on addition of ammonia pose a number of interesting questions. Under the conditions of the experiments recorded in Fig. 1 (b), mitochondria incubated with citrate and ammonia (3.5 mM) in the absence of respiration, the rate of glutamate synthesis is only about $5 \mu\text{mole/min.}$ /mg. of protein. In such a system the rate of reoxidation of nicotinamide-adenine dinucleotides is only about 10–20% of that of the

respiratory chain (Gutfreund *et al.* 1961). It is therefore surprising that glutamate synthesis can shift the steady state of NAD and NADP to half-oxidized, against the rate of reduction by the citric acid-cycle dehydrogenases. The rate at which the new steady-state rate is established on addition of ammonia is also puzzling. Approximate calculations based on the rate of respiration and various estimates of the NAD and NADP content of mitochondria suggest that the oxidation-reduction turnover of the coenzymes is less than 10 sec. and perhaps as little as 2 sec., and yet a new steady state is approached with a half-time of about 1 min. The almost instantaneous oxidation of the coenzymes on addition of ammonia after pretreatment with ADP would be more consistent with the kinetic picture. At this stage one can only suggest that the availability of NAD and NADP in the right form is a predominant factor in the phenomena observed. This availability could be controlled by 'compartmentation' with variable permeability or by association or dissociation of the coenzyme with some components of the system. Other features and possible causes of latency are discussed by Gutfreund *et al.* (1961).

The various conditions affecting the rate of glutamate synthesis from citrate suggest that, in the absence of respiration, NADP is almost fully reduced and α -oxoglutarate formation is inhibited. The optimum conditions of glutamate synthesis in the presence of citrate and succinate are such that there is simultaneous rapid reduction and reoxidation of the coenzymes. Though succinate oxidation is not directly linked to the nicotinamide-adenine dinucleotides, the addition of succinate does cause their rapid reduction [see Figs. 1 (a) and 1 (b), and also Krebs (1961) and Chance & Hollunger (1960)].

The effect of ammonia on succinate oxidation can be caused by the formation of glutamate and transamination to form aspartate from oxaloacetate. This would remove a powerful inhibitor of succinate oxidation. Preliminary studies with $[2,3-^{14}\text{C}_2]$ succinate indicate that incubation of succinate with citrate under our conditions stimulated glutamate synthesis, there being very little succinate converted into citrate and an almost quantitative conversion of citrate into glutamate. The partial inhibition by succinate of the oxidation of NADH (Krebs, 1961) could account for both the observed accumulation of malate and the decrease in the competition between glutamate synthesis and respiration for the reduced coenzymes.

SUMMARY

1. The rate of glutamate synthesis from citrate or α -oxoglutarate and ammonia by intact respiring guinea-pig-liver mitochondria was studied under various conditions affecting the relative proportions of reduced and oxidized intramitochondrial NAD and NADP. Conditions of rapid oxidation and reduction of NAD and NADP by respiration favour a high rate of glutamate synthesis.

2. The rate of reoxidation of intramitochondrial NADH and NADPH on addition of ammonia was studied fluorimetrically in the absence of respiration and was found to be much more rapid when α -oxoglutarate was the substrate than when citrate was being oxidized.

3. The activities of isocitrate dehydrogenase and glutamate dehydrogenase were studied in broken liver mitochondria, and the rate of glutamate synthesis by such systems in the presence of isocitrate, ammonia and NADPH was compared with glutamate synthesis by intact mitochondria.

4. Guinea-pig-mammary-gland mitochondria do not contain any detectable glutamate dehydrogenase; neither in the presence of citrate nor of α -oxoglutarate did the addition of ammonia stimulate the oxidation of NADH and NADPH or the synthesis of glutamate.

5. The kinetics of the various phenomena studied can be interpreted in terms of delays in availability of NAD and NADP both to enzymes which reduce them and to systems which oxidize them, such as the respiratory chain or a special synthetic couple like glutamate dehydrogenase.

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