Control of Glutamate Oxidation in Brain and Liver Mitochondrial Systems

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(Received 4 November 1964)

1. Glutamate oxidation in brain and liver mitochondrial systems proceeds mainly through transamination with oxaloacetate followed by oxidation of the α -oxoglutarate formed. Both in the presence and absence of dinitrophenol in liver mitochondria this pathway accounted for almost 80% of the uptake of glutamate. In brain preparations the transamination pathway accounted for about 90% of the glutamate uptake. 2. The oxidation of [1- 14 C]- and [5- 14 C]-glutamate in brain preparations is compatible with utilization through the tricarboxylic acid cycle, either after the formation of α -oxoglutarate or after decarboxylation to form γ -aminobutyrate. There is no indication of γ -decarboxylation of glutamate. 3. The high respiratory control ratio obtained with glutamate as substrate in brain mitochondrial preparations is due to the low respiration rate in the absence of ADP: this results from the low rate of formation of oxaloacetate under these conditions. When oxaloacetate is made available by the addition of malate or of NAD^+ , the respiration rate is increased to the level obtained with other substrates. 4. When the transamination pathway of glutamate oxidation was blocked with malonate, the uptake of glutamate was inhibited in the presence of ADP or ADP plus dinitrophenol by about 70 and 80% respectively in brain mitochondrial systems, whereas the inhibition was only about 50% in dinitrophenol-stimulated liver preparations. In unstimulated liver mitochondria in the presence of malonate there was a sixfold increase in the oxidation of glutamate by the glutamate-dehydrogenase pathway. Thus the operating activity of glutamate dehydrogenase is much less than the 'free' (non-latent) activity. 5. The following explanation is put forward for the control of glutamate metabolism in liver and brain mitochondrial preparations. The oxidation of glutamate by either pathway yields α -oxoglutarate, which is further metabolized. Since aspartate aminotransferase is present in great excess compared with the respiration rate, the oxaloacetate formed is continuously removed by the transamination reaction. Thus α -oxoglutarate is formed independently of glutamate dehydrogenation, and the question is how the dehydrogenation of glutamate is influenced by the continuous formation of α -oxoglutarate. The results indicate that a competition takes place between the α -oxoglutarate-dehydrogenase complex and glutamate dehydrogenase, probably for NAD^+ , resulting in preferential oxidation of α -oxoglutarate.

Several alternative pathways are available for the oxidation of glutamate in mammalian tissues. Until recently it has been assumed that glutamate is mainly oxidized by a pathway involving glutamate dehydrogenase (reaction 1):

However, the activity of glutamate dehydrogenase is very low in some tissues that nevertheless oxidize glutamate at a high rate (Jones & Gutfreund, 1961;

Borst, 1962). Recent experiments *in vitro* from different Laboratories have indicated that in most tissues glutamate oxidation proceeds mainly through transamination with oxaloacetate followed by oxidation of the α -oxoglutarate formed (reaction 2) (cf. Krebs & Bellamy, 1960; Jones & Gutfreund, 1961; Borst, 1962; Haslam & Krebs, 1963b; Chappell, 1964b):

Besides these routes glutamate can also be oxidized through transamination with other α -oxo acids, and in brain there is a by-pass of the tricarboxylic acid cycle, the γ -aminobutyrate shunt, which involves glutamate as an intermediate (Roberts, 1956). There are a few quantitative data published for the contribution of these different pathways of glutamate oxidation, but the data are not comparable owing to the different experimental conditions used (e.g. Krebs & Bellamy, 1960; Borst, 1962). In the present work the different routes of glutamate oxidation were measured in brain and liver mitochondrial preparations in the resting and active states of mitochondrial respiration, and the effect of pyruvate on the oxidation of glutamate has also been investigated (Balázs, 1965). Factors that might control the relative contribution of the different pathways were also studied. A part of this investigation has already been reported in a preliminary form (Balázs, 1963).

EXPERIMENTAL

Mitochondrial preparations. Rats lightly narcotized with ether were decapitated. The two hemispheres of the brain were homogenized in 0.3M-sucrose containing EDTA (sodium salt), pH 7.2 (0.5 mM), with an Aldridge homogenizer (Aldridge, Emery & Street, 1960), and a 10% suspension was prepared. The nuclei and cell debris were separated at 1000g for 10 min. in a Spinco model L centrifuge. The low-speed fraction was resuspended in the sucrose solution and sedimented at the same speed and time. The combined supernatants were again spun at 1000g for 10 min. The mitochondrial fraction was separated by centrifugation at 18000g for 10 min. The fluffy layer was removed as far as possible by gentle swirling and this treatment was repeated at the following two washings. Finally the mitochondrial fraction was suspended in the sucrose solution (about 1g. wet wt./ml.).

Liver mitochondrial fractions were prepared by the method of Aldridge (1958).

Manometric techniques and incubations. Conventional Warburg technique was used (Umbreit, Burris & Stauffer,

Table 1. *Effect of different concentrations of mitochondria on the oxidation of glutamate*

The incubation mixture contained basic medium (see the Experimental section), ADP (2 mM) and 2,4-dinitrophenol (40 μ M). The concentration of glutamate was 9.6 mM for liver mitochondria and 9.1 mM for brain mitochondria. The incubation volume was 3.0 ml. Metabolic changes are given in μ moles/hr./vessel.

	Vessel no....	Liver mitochondria			Brain mitochondria		
		1	2	3	1	2	3
Mitochondrial protein (mg.)...		1.28	2.56	5.12	1.52	3.05	6.10
Metabolic changes:							
O ₂		-5.30	-12.3	-25.7	-5.88	-13.1	-24.9
Glutamate		-3.52	-8.20	-17.3	-4.40	-8.57	-16.1
Aspartate		+2.68	+7.09	+14.0	+3.56	+7.61	+13.1
Conversion of glutamate into aspartate:							
Aspartate							
Glutamate							
$\times 100$		76	86	81	81	89	81

Table 2. *Effect of glutamate concentration on the utilization of glutamate*

The incubation mixture contained basic medium (see the Experimental section), ADP (2 mM) and dinitrophenol (40 μ M). Liver and brain mitochondrial fractions contained 2.56 and 3.05 mg. of protein respectively. The incubation volume was 3 ml. except in vessels 4 (6 ml.). Metabolic changes are given in μ moles/hr./vessel.

	Vessel no....	Liver				Brain			
		1	2	3	4	1	2	3	4
Glutamate concn. (mM):									
Initial...9.6		7.7	5.8	3.8		9.1	7.2	5.4	3.6
Final...6.9		5.1	3.3	2.7		6.2	4.8	3.5	2.7
Metabolic changes:									
O ₂		-12.3	-11.4	-11.3	-10.6	-13.1	-11.7	-10.0	-9.64
Glutamate		-8.20	-7.89	-7.52	-6.70	-8.57	-7.28	-5.62	-5.50
Aspartate		+7.09	+6.70	+6.54	+5.28	+7.61	+5.70	+4.85	+3.92
Conversion of glutamate into aspartate:									
Aspartate									
Glutamate									
$\times 100$		86	85	87	79	89	78	86	71

1945). The basic medium contained MgCl_2 (6mm), potassium phosphate buffer, pH 7.2 (15mm), tris-HCl buffer, pH 7.2 (20mm), EDTA (sodium salt), pH 7.2 (2mm), sucrose (20–50mm) introduced with the mitochondrial suspension, and KCl to give an osmolality of 0.3 after the addition of all constituents. The pH of substrates was adjusted to 7.2 and the substrates were used in 7–10mm final concentration. When yeast hexokinase was added (type III, Sigma Chemical Co., St Louis, Mo., U.S.A., or Aldrich Chemical Co., Milwaukee, Wis., U.S.A.) the inorganic phosphate concentration was increased to 40mm. The centre well of the flasks contained 0.2ml. of 2N-NaOH. The incubation was carried out at 37.5° in an O_2 atmosphere for 40–60min., and readings of O_2 uptake were taken after a temperature equilibration of 7–10min. The reaction was stopped by tipping 2N-HCl from the side arm (final concn. 0.4N). The samples were kept in the deep-freeze until analysed, except when oxaloacetate was estimated, a procedure which was carried out without delay.

Since the rate of glutamate oxidation is much lower in the absence than in the presence of ADP the experimental conditions had to be adjusted in a way to enable the measurement of metabolic changes under the different experimental conditions. Unless otherwise stated, the vessels contained in the absence of ADP 4–10mg. of mitochondrial protein and the incubation volume was 1.5ml. In the presence of ADP (2mm) plus 2,4-dinitrophenol (40–50 μM) the incubation volume was 3ml. and less mitochondrial suspension was added (1.5–4mg. of protein). When only ADP was included in the medium the volume was 2–3ml. and 3–8mg. of mitochondrial protein was added. The initial concentration of glutamate was the same in all cases and the procedure described made it possible to keep the final concentration of substrate within the same range under the different experimental conditions. This procedure was justified by the observations given below. The concentration of mitochondria within the limits investigated in Table 1 had only a small effect on the metabolism of glutamate. Further, the conversion of glutamate into aspartate was only affected when the concentration of glutamate was lower than about 3.5mm (Table 2). In the results given in Tables 3 and 7, only those experiments were included in which the final concentration of substrate was higher than 3.5mm. The time-course of glutamate oxidation was also investigated and did not indicate any marked change in the pattern of glutamate metabolism during 1 hr. incubation (Fig. 1). de Haan, Tager & Slater (1964) observed a small increase in the amount of glutamate utilized through the glutamate-dehydrogenase pathway in the second 20min. of incubation at 37°; this was not apparent in the present experiments.

Analytical methods. Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951) and the protein content of the mitochondrial fraction was expressed as mg. of albumin. Aspartate and glutamate plus glutamine were determined by the method of Krebs & Bellamy (1960) by using *Nocardia globulara* and *Clostridium welchii* respectively (kindly supplied by Mr A. Tuffrey, Laboratory Animal Centre, Carshalton, Surrey) for the decarboxylation of the amino acids. As glutamate and glutamine were never assayed individually the values for glutamate removal may include endogenous glutamine and refer to glutamate metabolized by pathways other than glutamine synthesis. The following enzymic determinations were carried out by spectrophotometric methods with

Fig. 1. Time-course of glutamate oxidation in mitochondrial systems of liver (a) and brain (b). The incubation was carried out in a conical flask in a Dubnoff metabolic shaking incubator at 37.5° in O_2 atmosphere. The total volume was 8.0ml. containing the basic medium (see the Experimental section), ADP (2mm), dinitrophenol (40 μM) and L-glutamate (10mm). The reaction was started by the addition of 0.8ml. of cold mitochondrial suspension (20 and 24mg. of protein for liver and brain mitochondria respectively). Then 1.2ml. portions of the medium were withdrawn at the time-intervals indicated and were acidified and analysed. The glutamate and aspartate contents found in the samples after the first 10min. incubations served as the control. ○, Glutamate uptake; ●, aspartate formation.

neutralized portions of the samples (Bergmeyer, 1962). α -Oxoglutarate was measured by recording the oxidation of NADH in the presence of glutamate dehydrogenase and ammonia. Pyruvate and oxaloacetate were determined by measuring the oxidation of NADH on the addition of lactate dehydrogenase and malate dehydrogenase respectively. Malate was determined by the reduction of NAD^+ on the addition of malate dehydrogenase. The extinction change due to NADH was determined with a Unicam SP.700

recording spectrophotometer at 340 m μ by using micro-cells containing 0.4 ml. total volume and having a light-path of 1 cm.

Chromatography of amino acids. A portion of the HCl extract of the incubated samples was evaporated in a desiccator over NaOH. The dry residue was taken up in 75% (v/v) ethanol and centrifuged. Two-dimensional chromatography was done by the method of Gaitonde (1961). Alternatively, in a few experiments the amino acids were determined from neutralized perchloric acid filtrates by the modified technique of Gaitonde (1964).

Radioactive tracer techniques. The general techniques used were those described by Haslam & Krebs (1963*a,b*). The respiratory $^{14}\text{CO}_2$ was absorbed in 0.2 ml. of 2*N*-NaOH placed in the centre well of the Warburg flasks. The flasks were incubated for approx. 1 hr. after the medium had been acidified. The $^{14}\text{CO}_2$ trapped in the alkali was converted into BaCO_3 and plated on filter-paper disks (3 cm. diam.). The planchets were assayed for radioactivity with a Nuclear-Chicago gas-flow counter. The counts were corrected for background, dead-time and self-absorption. The specific activities of [^{14}C]glutamate and [^{14}C]aspartate were determined by assay of the $^{14}\text{CO}_2$ liberated by decarboxylation with *C. welchii* and *N. globetula* respectively. The specific activity of [^{14}C]glutamate, kindly given by Dr R. J. Haslam, was determined by direct plating and counting at infinite thinness, and by measuring the amount of glutamate by decarboxylation with *C. welchii*. Values for the relative specific activity of residual substrates are defined as:

$$\frac{\text{Sp. activity of residual } [^{14}\text{C}]\text{substrate}}{\text{Sp. activity of added } [^{14}\text{C}]\text{substrate}}$$

Enzymic assays. Aspartate aminotransferase (EC 2.6.1.1) was assayed by the method of Gutfreund, Ebner & Mendiola (1961). The reaction mixture contained L-glutamate (40 mM), oxaloacetate (1 mM), potassium phosphate buffer, pH 7.66 (0.1 M), NADPH (0.17 mM), NH_4Cl (0.15 M) and 100 μg . of crystalline glutamate dehydrogenase. The rate of the reaction increased in the first 5–6 min. after the addition of oxaloacetate, but remained constant for another 5 min. The enzyme was also assayed in the reverse direction by the method of Karmen (1955) as modified by Boyd (1961). The observed rate was higher in the latter direction, mainly in liver (Table 10), than in the other direction under the conditions of the assays.

Glutamate dehydrogenase (EC 1.4.1.3) was assayed in both directions by the method of Olson & Anfinsen (1952) in the presence of 1 mM-ADP (Frieden, 1959; Mildvan & Greville, 1962). When the activity was measured in the direction of glutamate + NAD^+ + H_2O to α -oxoglutarate + NH_3 + NADH + H^+ the reaction mixture contained potassium phosphate buffer, pH 7.66 (0.1 M), L-glutamate (13.3 mM), NAD^+ (0.77 mM) and ADP (1 mM). The composition of the medium for the reverse direction was potassium phosphate buffer, pH 7.66 (0.1 M), α -oxoglutarate (10 mM), NH_4Cl (0.15 M), NADH (0.17 mM) and ADP (1 mM).

The mitochondrial preparations were diluted in Triton X-100 (0.1% final concn.; Charles Lenning and Co. Ltd., London, W.C.1) before the assay. The change of extinction given by NADH was followed at 340 m μ with a Unicam SP.700 recording spectrophotometer.

Chemicals. DL-[^{14}C]Glutamate (obtained from The Radiochemical Centre, Amersham, Bucks.) was dissolved in unlabelled L-glutamate solution to decrease the proportion

of D-glutamate to a negligible value (about 1%). The final specific activity of the L-glutamate was 10 $\mu\text{C}/\text{m-mole}$. 2,4-Dinitrophenol was recrystallized before use. The following chemicals were obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany: ADP (sodium salt), NAD^+ , NADH, malate dehydrogenase, lactate dehydrogenase and glutamate dehydrogenase. The other chemicals were commercially available analytical-grade preparations.

Ordinary glass-distilled water was passed through an Elgastat deionizer.

RESULTS

Glutamate oxidation in brain mitochondrial systems. The fate of glutamate in brain mitochondrial systems under different experimental conditions is shown in Table 3. Glutamate uptake and $^{14}\text{CO}_2$ formation were comparable, indicating that the dilution by endogenous glutamate was small. The glutamate content of ten mitochondrial preparations was only $7.7 \pm 1.62 \mu\text{moles}/100\text{mg.}$ of protein. Further, glutamate was not formed in appreciable amounts during the incubations, as shown by the observation that the relative specific activity of the glutamate remaining after incubation was near to 1.0 (0.94–1.01 in five experiments). Aspartate formation accounted for a large fraction of the glutamate removed (last line of Table 3). Paper chromatography confirmed that the amino acid product of glutamate oxidation was almost exclusively aspartate. Alanine was not detected and in a few experiments only trace amounts of γ -aminobutyrate were found. Since the accumulation of α -oxoglutarate was very small (0.04–0.1 mM final concn.) the glutamate removed was apparently oxidized beyond this α -oxo acid. Therefore the contribution of the transamination pathway to the oxidation of glutamate can be calculated on the basis of reaction (2), and under the conditions tested the percentage value of the conversion of glutamate into aspartate can be used for assessing the magnitude of this pathway.

If all the glutamate consumed were oxidized through the transamination pathway the expected ratio of oxygen consumed to glutamate removed would be 1.5 (reaction 2). In fact this ratio was much higher in the absence of ADP (1.93) and the values were also greater in the active state (last two columns of Table 3). This finding is explicable by the observation that a portion of the disappeared glutamate was not converted into aspartate and thus was probably oxidized by glutamate dehydrogenase. Since oxaloacetate accumulation was very low under all conditions tested (less than 0.01 mM final concn.) it is probable that a part of oxaloacetate which did not react by transamination was further metabolized. This was shown to occur in experiments in which the mitochondrial suspension was incubated with [^{14}C]glutamate as substrate (Table 4). The oxaloacetate derived from [^{14}C]glutamate will be

Table 3. *Glutamate oxidation in rat-brain mitochondrial systems*

Rat-brain mitochondrial preparations were incubated in the basic medium (see the Experimental section) with L-glutamate (7–10 mM) (in some experiments [$1\text{-}^{14}\text{C}$]glutamate, $10\text{ }\mu\text{C/m-mole}$, was used), and also ADP (2 mM), 2,4-dinitrophenol ($50\text{ }\mu\text{M}$) and 0.5 mg. of yeast hexokinase (Sigma type III) plus glucose (40 mM) when added. When the hexokinase–glucose trapping system was used the concentration of inorganic phosphate was 40 mM. Incubation was for 40–60 min. at 37.5° in O_2 atmosphere. The metabolic changes are given in $\mu\text{moles/hr./100 mg. of protein}$ ($\pm \text{S.E.M.}$; no. of experiments in parentheses). The mean of the ratio of O_2 uptake to glutamate uptake and the mean of the percentage conversion of glutamate into aspartate are given with the range in square brackets.

Metabolic changes:	Additions... None	ADP	ADP + dinitrophenol	ADP + hexokinase
O_2	-45.8 ± 1.50 (5)	-207 ± 11.1 (8)	-474 ± 19.7 (7)	-474 ± 19.9 (3)
$^{14}\text{CO}_2$	$+23.6 \pm 1.14$ (5)	$+117 \pm 20.6$ (6)	$+278 \pm 12.2$ (5)	$+271$ (2)
Glutamate	-24.5 ± 1.16 (4)	-139 ± 8.5 (8)	-283 ± 9.2 (7)	-272 ± 16.9 (3)
Aspartate	$+19.0 \pm 2.26$ (4)	$+121 \pm 6.3$ (8)	$+249 \pm 8.9$ (7)	$+258 \pm 20.9$ (3)
$\alpha\text{-Oxoglutarate}$	$+0.31 \pm 0.04$ (4)	3.29 ± 0.49 (7)	3.18 ± 0.79 (6)	5.09 (2)
$\Delta \text{O}_2/\Delta \text{glutamate}$:	1.93	1.49	1.68	1.73
	[1.58–2.09]	[1.33–1.65]	[1.53–1.92]	[1.64–1.80]
Conversion of glutamate into aspartate:				
$\frac{\Delta \text{Aspartate}}{\Delta \text{Glutamate}} \times 100$	78 (4)	87 (8)	88 (7)	94 (3)
	[62–89]	[77–100]	[82–97]	[89–99]

Table 4. *Oxidation of [$5\text{-}^{14}\text{C}$]glutamate in brain mitochondrial systems*

The incubation mixture contained basic medium (see the Experimental section) and [$5\text{-}^{14}\text{C}$]glutamate (9.7 mM ; $40\text{ }\mu\text{C/m-mole}$), and also ADP (2 mM) when added. The mitochondrial fraction contained 10.9 mg. of protein per vessel. The incubation volume was 1.5 ml. in the absence of ADP and 3.0 ml. in the presence of ADP. Metabolic changes are given in $\mu\text{moles/hr./vessel}$.

	Vessel no. ... 1	2
Additions...	None	ADP
Metabolic changes:		
O_2	-5.50	-23.4
$^{14}\text{CO}_2$	$+0.48$	$+1.46$
Glutamate	-3.00	-14.9
Aspartate	$+2.15$	$+11.0$
$\alpha\text{-Oxoglutarate}$	$+0.92$	$+0.12$
Rel. sp. activity of residual aspartate:	—	0.48

labelled equally in C-1 and C-4 due to randomization of ^{14}C . The $^{14}\text{CO}_2$ will be produced from the labelled oxaloacetate if further metabolized, either by decarboxylation to pyruvate or by entering the tricarboxylic acid cycle and yielding $^{14}\text{CO}_2$ at the steps of oxidation of isocitrate and $\alpha\text{-oxoglutarate}$. The formation of $^{14}\text{CO}_2$ in the absence of ADP was 16% of the glutamate removed (vessel 1) and less than 10% in the presence of ADP (vessel 2). If the oxygen consumption calculated on the basis of glutamate removal through the transamination and dehydrogenase pathways is corrected for the further metabolism of oxaloacetate according to the results of Table 4, the oxygen uptake can be almost com-

pletely accounted for by the oxidation of glutamate even in the absence of ADP, e.g. the calculated value of oxygen uptake in vessel 1 of Table 2 is 5.3 and 5.6 depending on whether pyruvate was assumed to be formed from oxaloacetate or from endogenous sources.

In a few experiments the activity of C-4 of aspartate formed during the metabolism of [$1\text{-}^{14}\text{C}$]glutamate was measured, but the counts were not significantly above the background, confirming the observations of Haslam & Krebs (1963b) that a γ -decarboxylation of glutamate, proposed by Cohen, Simon, Berry & Chain (1962), did not occur. Moreover, when [$5\text{-}^{14}\text{C}$]glutamate was the substrate the relative specific activity of residual aspartate was near to 0.5 (vessel 2 in Table 4), which is the theoretical value, when glutamate is not oxidized through γ -decarboxylation.

These results indicated that both in the resting and in the active state of mitochondrial respiration the major pathway of glutamate oxidation proceeds through transamination. This was confirmed by the effect of malonate on glutamate oxidation. Malonate inhibits succinate oxidation and thus should block the operation of the pathway of glutamate utilization through transamination with oxaloacetate. In the presence of malonate the conversion of glutamate into aspartate was negligible under the experimental conditions (Table 5). Oxygen and glutamate uptake in the presence of ADP or ADP plus 2,4-dinitrophenol was only a small fraction of that obtained in the uninhibited systems. The inhibition was the smallest in the absence of ADP, when oxygen uptake was about half of that obtained

Table 5. *Effect of malonate on glutamate oxidation in brain mitochondrial systems*

The incubation mixture contained basic medium (see the Experimental section) and L-glutamate (9.4 mM), and also ADP (2 mM), dinitrophenol (50 μ M) and malonate (20 mM) when added. The incubation volume was 1.5 ml. in the absence of ADP and 3.0 ml. in the presence of ADP. Incubation was for 1 hr. in O₂ atmosphere. Changes in metabolites are given in μ moles/hr./vessel.

	Normal			Malonate		
Vessel no. ...	1	2	3	4	5	6
Additions...	None	ADP	ADP+ dinitrophenol	None	ADP	ADP+ dinitrophenol
Mitochondrial protein added (mg.)...	8.2	4.1	4.1	8.2	8.2	8.2
Metabolic changes:						
O ₂	-4.81	-10.3	-19.5	-2.13	-3.62	-4.09
Glutamate	-2.25	-6.67	-11.1	-2.08	-3.54	-3.74
Aspartate	+1.75	+6.29	+9.21	0*	0*	0*
α-Oxoglutarate	+0.03	+0.16	+0.08	+0.01	+0.02	+0.02
ΔO ₂ /Δglutamate:	2.14	1.54	1.76	1.02	1.02	1.09
Rates in the presence of malonate as % of control:						
O ₂				44	18	10
Glutamate				93	27	17

* Not measurable.

* Not measurable.

without malonate, and glutamate uptake was only slightly affected. The ratio of oxygen uptake to glutamate removal was about 1 in the presence of malonate, which is the theoretical value for the oxidation of glutamate to succinate through the glutamate-dehydrogenase route (reaction 1). It may be noted that in the presence of malonate the stimulation of glutamate oxidation by dinitrophenol is very small, whereas this uncoupler approximately doubles the uptake of glutamate in the absence of malonate. Since α -oxoglutarate accumulation was negligible in the presence of malonate the rate-limiting step of glutamate oxidation under these conditions in intact mitochondria is apparently the glutamate-dehydrogenase reaction.

Respiratory control with glutamate as substrate. It has been reported (Balázs, Biesold & Magyar, 1963) that with brain mitochondrial preparations the highest respiratory control ratio, measured by comparing the respiration rate in the presence of an excess of a phosphate-acceptor system with the rate in the absence of added ADP, can be obtained with glutamate as substrate. When pyruvate plus malate or α -oxoglutarate were the substrates the respiratory control ratio was 5–6 under these conditions, whereas it was 10–17 with glutamate. The high control ratio was mainly due to the low respiration rate with glutamate as substrate in the absence of added ADP. The rate was only half of that obtained with α -oxoglutarate (compare vessel 1 with vessel 7 in Table 6), whereas the oxygen uptakes in the stimulated state were comparable with the two substrates (Balázs *et al.* 1963).

Since a large fraction of the glutamate utilized yields aspartate (Table 3) it is possible that the rate of glutamate oxidation is controlled by the rate of oxaloacetate formation in the absence of ADP. This assumption was tested by supplementing the mitochondrial system with malate, which approximately doubled the rates of glutamate removal and of aspartate formation (vessel 2 in Table 6). In the presence of malate the oxidation of glutamate proceeded completely through the transamination pathway, whereas in the control (vessel 1) only about 70% was attributed to this route. Almost all the α -oxoglutarate formed was oxidized, since the amount accumulating, although increased significantly, was very low. The concentration of oxaloacetate was very low, although it was increased on the addition of malate. These experiments indicate that the low glutamate oxidation in the absence of ADP is related to a limitation in the supply of oxaloacetate.

Since in the resting state the mitochondrial nicotinamide nucleotides are mainly in the reduced form, it is possible that the oxidation of NAD⁺-linked substrates was decreased by an unfavourable NAD⁺/NADH ratio. The effect of added NAD⁺ was therefore tested. The mitochondria became apparently permeable to NAD⁺ in the absence of added ADP, so it is probable that they were damaged under these conditions. In contrast with mitochondrial systems containing ADP, when added NAD⁺ had no effect on the respiration rate, 0.1 mM-NAD⁺ more than doubled the rate of glutamate oxidation in the unfortified system (vessel 3). The

Table 6. *Effect of malate and NAD⁺ on glutamate oxidation in the absence of ADP*

The incubation mixture contained basic medium (see the Experimental section) and [1-¹⁴C]glutamate (8.4 mm), and also α -oxoglutarate (10 mm), L-malate (7.3 mm) and NAD⁺ (0.1 mm) plus nicotinamide (25 mm) when added. The brain mitochondrial preparation contained 10.8 mg. of protein/vessel. Changes in metabolites are given as μ moles/50 min./vessel.

Vessel no. ...	1	2	3	4	5	6	7
Additions.....	Glutamate	Glutamate + malate	Glutamate + NAD ⁺	Glutamate + malate + NAD ⁺	Malate	Malate + NAD ⁺	α -Oxoglutarate
Metabolic changes:							
O ₂	-4.0	-8.6	-10.0	-14.4	-4.6	-11.6	-8.3
¹⁴ CO ₂	+1.94	+3.71	+5.18	+4.73			
Glutamate	-2.1	-3.7	-5.4	-4.9			
Aspartate	+1.4	+3.6	+4.2	+4.1			
α -Oxoglutarate	+0.02	+0.23	+0.08	+1.38	+0.02	+0.10	-8.36
Malate	—	-5.56	—	-10.43	-4.59	-8.82	+3.63
Oxaloacetate	+0.008	+0.019	+0.016	+0.005	+0.018	+0.093	0
Pyruvate	+0.008	+0.015	+0.027	+0.078	+0.009	+0.051	+0.04

Table 7. *Glutamate oxidation in liver mitochondrial systems*

Rat-liver mitochondrial preparations were incubated in the basic medium (see the Experimental section) with L-glutamate (8–10 mm) (in some experiments [1-¹⁴C]glutamate, 10 μ C/m-mole, was used), and also ADP (2 mm) and dinitrophenol (40 μ M) when added. Incubation was for 40–60 min. at 37.5° in O₂ atmosphere. Metabolic changes are given in μ moles/hr./100 mg. of protein ($\pm$ S.E.M.; no. of experiments in parentheses). The mean of the ratio of O₂ uptake to glutamate removal and the mean percentage conversion of glutamate into aspartate are given with the range in square brackets.

Metabolic changes:	Additions... None	ADP	ADP + dinitrophenol
O ₂	-84.1 (2)	-139 $\pm$ 10.4 (6)	-495 $\pm$ 15.3 (7)
¹⁴ CO ₂	+48.7 (2)	+87.1 $\pm$ 9.20 (4)	+327 $\pm$ 22.5 (5)
Glutamate	-61.9 (2)	-96.0 $\pm$ 3.91 (9)	-357 $\pm$ 10.1 (9)
Aspartate	+41.0 (2)	+74.9 $\pm$ 4.09 (9)	+292 $\pm$ 11.7 (9)
α -Oxoglutarate	+1.55 (2)	+8.02 $\pm$ 1.41 (4)	+8.9 $\pm$ 1.88 (4)
Δ O ₂ / Δ glutamate:	1.35 (2)	1.46 (6) [1.28–1.62]	1.41 (7) [1.30–1.62]
Conversion of glutamate into aspartate:			
$\frac{\Delta \text{Aspartate}}{\Delta \text{Glutamate}} \times 100$	66 (2)	78 [70–88]	82 [70–94]

further addition of malate did not increase the uptake of glutamate in the presence of added NAD⁺ (vessel 4).

It is known that the oxidation of malate by mitochondrial preparations is very slow (cf. Chappell, 1964a), as is also shown in Table 6 (vessel 5). The equilibrium constant of the malate-dehydrogenase reaction is very strongly in favour of malate formation, so that the oxidation of malate will depend on the rate of removal of oxaloacetate. Another means of increasing the rate of malate oxidation is by increasing the concentration of NAD⁺. When NAD⁺ was added to a mitochondrial system containing malate the uptake of malate and oxygen was indeed

doubled (vessel 6). Thus the low rates of glutamate and malate oxidation in the absence of added ADP are probably due to the same limiting factor, and only after the removal of this rate-limiting step by added NAD⁺ do the coupling reactions become the pace-makers as a result of a relative lack of phosphate acceptor. When these precautions are met the respiratory control ratio with glutamate as substrate is comparable with that obtained with α -oxoglutarate.

Oxidation of glutamate by liver mitochondrial preparations. Contradicting values have been reported from different Laboratories for the contribution of the transamination pathway to glutamate oxidation

Table 8. *Effect of malonate on glutamate oxidation in liver mitochondrial systems*

The incubation mixture contained basic medium (see the Experimental section) and [1-¹⁴C]glutamate (10.4 mM), and also ADP (2 mM), dinitrophenol (40 μ M) and malonate (20 mM) when added. The incubation volume was 1.5 ml. in vessels 1 and 4 and 3.0 ml. in the other vessels. Incubation was for 50 min. Metabolic changes are given in μ moles/50 min./vessel.

Vessel no. ... Additions...	Normal			Malonate		
	1 None	2 ADP	3 ADP + dinitrophenol	4 None	5 ADP	6 ADP + dinitrophenol
Mitochondrial protein added (mg.)...	9.1	9.1	4.55	9.1	9.1	9.1
Metabolic changes:						
O ₂	-5.99	-13.6	-21.3	-1.68	-11.3	-12.6
¹⁴ CO ₂	+3.71	+8.36	+12.2	+1.88	+9.65	+11.6
Glutamate	-4.48	-8.47	-13.7	-2.24	-11.7	-12.7
Aspartate	+2.48	+6.37	+10.9	+0.11	+0.32	+0.26
α -Oxoglutarate	+0.22	+0.57	+0.45	+0.37	+0.22	+0.25
Rates in the presence of malonate as % of control:						
O ₂				28	83	30
Glutamate				50	138	46
Δ O ₂ /Δ glutamate:	1.34	1.61	1.55	0.75	0.97	0.99

by liver mitochondrial preparations (e.g. Krebs & Bellamy, 1960; Borst, 1962), but owing to the differences in the experimental conditions the results are not comparable. In the following experiments the effect of the state of mitochondrial respiration, which was one of the major differences in the conditions used by Krebs & Bellamy (1960) and Borst (1962), on the relative contribution of the different pathways to glutamate oxidation was studied (Table 7). The major pathway of glutamate oxidation was through transamination whether the mitochondria were incubated in the presence or absence of dinitrophenol, since about 80% of the glutamate uptake was accounted for as aspartate formed. As in brain mitochondria α -oxoglutarate accumulation was very small. Respiratory ¹⁴CO₂ formation was comparable with the removal of [1-¹⁴C]glutamate (cf. Table 8), although in a few experiments significantly less ¹⁴CO₂ was formed relative to glutamate uptake. The addition of malonate blocked almost completely the transamination of glutamate, since aspartate formation was negligible (vessels 4-6 in Table 8). In the presence of malonate the ratio of oxygen uptake to glutamate consumption was near to 1. This value is to be expected when the oxidation of glutamate to succinate proceeds through the glutamate-dehydrogenase pathway (reaction 1). With mitochondrial preparations incubated with added ADP (vessel 5) the inhibition of respiration was small, whereas glutamate uptake was stimulated by 38%. Thus under these conditions the oxidation of glutamate proceeded exclusively through the glutamate-dehydrogenase pathway, which compensated almost completely for the oxygen uptake.

In the absence of ADP the oxidation of glutamate differed in some respects from that described above. The respiration rate was very low, and less glutamate was oxidized through the transamination pathway than in the presence of ADP (vessel 1). Malonate inhibited substantially both oxygen and glutamate uptake (vessel 4), whereas in the presence of ADP (vessel 5) this did not occur. Malonate had a similar, although less marked, effect on α -oxoglutarate oxidation in the absence of ADP (vessel 4 in Table 8), so that it is probable that the mitochondrial preparations were damaged under these conditions.

In the presence of dinitrophenol malonate inhibited oxygen uptake by 70% and the removal of glutamate by 54% (vessel 6 in Table 8). The rate-limiting reaction under these conditions must be that catalysed by the glutamate dehydrogenase, because α -oxoglutarate accumulation was very small. Further, when the oxidation of α -oxoglutarate was measured under comparable conditions (vessel 6 in Table 9), it was observed that the rate of α -oxoglutarate uptake was almost four times that of glutamate. When the oxidation of glutamate and α -oxoglutarate are compared in liver mitochondrial systems by calculating the ratios of oxygen uptake to substrate removal, the results indicate that a larger section of the tricarboxylic acid cycle is involved in glutamate oxidation (α -oxoglutarate to oxaloacetate) than in the oxidation of α -oxoglutarate (α -oxoglutarate to malate) (cf. Chappell, 1964b). Similar results were obtained with brain mitochondrial preparations.

'NADP-specificity' of glutamate dehydrogenase as

Table 9. *Oxidation of α -oxoglutarate in liver mitochondrial systems*

The incubation mixture contained basic medium (see the Experimental section) and α -oxoglutarate (20.1 μ moles to vessels 1 and 4, 30.1 μ moles to the other vessels), and also ADP (2 mM), dinitrophenol (40 μ M) and malonate (20 mM) when added. The incubation volume was 2.0 ml. The same mitochondrial preparation as that in Table 6 was used. Incubation was for 50 min. Metabolic changes are given in μ moles/50 min./vessel.

	Normal			Malonate		
Vessel no. ...	1	2	3	4	5	6
Additions...	None	ADP	ADP+ dinitrophenol	None	ADP	ADP+ dinitrophenol
Mitochondrial protein added (mg.)...	9.1	9.1	4.55	9.1	9.1	4.55
Metabolic changes:						
O ₂	-10.5	-14.0	-23.3	-5.5	-12.7	-12.0
α-Oxoglutarate	-11.1	-14.8	-25.4	-8.3	-24.5	-23.5
Rates in the presence of malonate as % of control:						
O ₂				52	91	51
α-Oxoglutarate				75	165	92
Δ O ₂ /Δ α-oxoglutarate:	0.95	0.95	0.92	0.66	0.52	0.51

Table 10. *Aspartate-aminotransferase and glutamate-dehydrogenase activities in liver and brain mitochondrial fractions*

The O₂ uptake was calculated from the mean values given in Tables 3 and 7 for the dinitrophenol-containing systems and is expressed as μ g. atom/min./mg. of protein at 37.5°. The enzyme assays were as described in the Experimental section. The total activities were measured in mitochondrial preparations that had been diluted 20–40-fold in Triton X-100 (final concn. 0.1%) before the assay. The enzyme activities are given in μ moles/min./mg. of protein at 25°.

	Liver	Brain
O ₂ uptake:	0.165	0.158
Aspartate-aminotransferase activity:		
(a) Glutamate + oxaloacetate $\rightarrow$ α -oxoglutarate + aspartate	0.891, 0.877, 1.429, 1.28, 1.0 (mean 1.10)	0.798, 1.085, 1.01 (mean 0.964)
(b) α -Oxoglutarate + aspartate $\rightarrow$ glutamate + oxaloacetate	3.16, 2.15, 2.10 (mean 2.65)	1.48, 1.53, 1.38, 0.91, 1.61 (mean 1.33)
Glutamate-dehydrogenase activity:		
(a) Glutamate + NAD ⁺ + H ₂ O $\rightarrow$ α -oxoglutarate + NH ₃ + NADH + H ⁺	0.251, 0.229, 0.234, 0.22 (mean 0.233)	0.046, 0.030, 0.0295 (mean 0.035)
(b) α -Oxoglutarate + NH ₃ + NADH + H ⁺ $\rightarrow$ glutamate + NAD ⁺ + H ₂ O	2.19, 2.86, 3.10 (mean 2.71)	0.33, 0.273, 0.252, 0.382 (mean 0.309)

a factor in the control of the glutamate-dehydrogenase pathway. The operating activity of glutamate dehydrogenase both in the active and the resting state was much higher in the presence than in the absence of malonate (Table 8). The total activity of this enzyme as measured in disrupted liver mitochondrial preparations is even higher than the respiration rate (Table 10) (cf. Jones & Gutfreund, 1961; Borst, 1962; Chappell, 1964b), but there is no reason to believe that malonate has 'unmasked' the glutamate-dehydrogenase activity. Thus these results suggest that the glutamate-dehydrogenase pathway is subject to some control mechanism. Borst (1962) suggested that the control may be explicable by the assumption of Klingenberg & Slenczka (1959) as to

the NADP specificity of glutamate dehydrogenase in mitochondria (cf. the Discussion section). In the next experiments this assumption was tested (Table 11). The 'free' activity of glutamate dehydrogenase was assayed in dinitrophenol-stimulated systems in the presence of malonate by measuring oxygen and glutamate uptake, and the effect of added nicotinamide nucleotides on the enzymic activity was determined. The permeability of the mitochondrial membrane for nicotinamide nucleotides was increased by pretreatments that produced only slight or no 'unmasking', like a single freezing and thawing or hypo-osmotic conditions (0.1 M-sucrose; Bendall & de Duve, 1960). Whichever nicotinamide nucleotide is the cofactor of the glutamate dehydrogenase

Table 11. *Effect of mild structural damage of liver mitochondria on the glutamate-dehydrogenase activity in the presence and absence of NAD⁺ and NADP⁺*

The incubation mixture contained basic medium (see the Experimental section), L-glutamate (9.5 mM), ADP (2 mM), dinitrophenol (40 μ M) and malonate (20 mM). A portion of the mitochondrial preparation was frozen and thawed once. Another portion was diluted to 0.1 M-sucrose concentration and kept for 10 min. in an ice-water bath before incubation started. Untreated mitochondrial fraction was used in the control (vessel 1). When nicotinamide nucleotides (2 mM) were added nicotinamide (25 mM) was also included in the medium. The osmolarity of all media was adjusted with KCl to 0.3. The mitochondrial protein added was 10.7 mg. The incubation volume was 2 ml. except for mitochondrial preparations pretreated in 0.1 M-sucrose (3 ml.). Metabolic changes are given in μ moles/40 min./vessel.

Vessel no.	...	...	1	2	3	4	5	6	7
Pretreatment...	None			Freezing and thawing			Pretreatment in 0.1 M-sucrose		
Additions	...	...	None	None	NAD ⁺	NADP ⁺	None	NAD ⁺	NADP ⁺
Metabolic changes:									
O ₂			-11.3	-10.5	-13.6	-7.25	-10.3	-13.6	-7.80
Glutamate			-9.58	-8.60	-11.9	-6.02	-10.6	-13.3	-5.88
Aspartate			+0.40	+0.36	+0.14	+0.23	+0.10	0.0	+0.35
α -Oxoglutarate			+0.32	+0.31	+0.49	+0.16	+0.72	+0.93	+0.56

the rate of glutamate removal should be stimulated or unaffected by the addition of the cofactor, depending on whether or not the enzyme is saturated with the coenzyme. The results in Table 11 show that after both treatments the rate of glutamate uptake, which under the present conditions is by the glutamate-dehydrogenase pathway, was only slightly affected, unless nicotinamide nucleotides were added (compare vessel 1 with vessels 2 and 5). On the addition of NAD⁺ the rate of glutamate uptake was increased by 38% (vessel 3) and by 26% (vessel 6). On the addition of NADP⁺ the rate of glutamate removal was substantially decreased (vessels 4 and 7), a finding that is at variance with the alleged role of NADP⁺ in glutamate oxidation.

DISCUSSION

The observations reported in the present paper agree with the findings of Krebs & Bellamy (1960) in showing that transamination is the main pathway of glutamate oxidation in brain mitochondrial preparations. However, in contrast with their results, this was found to be the major route of glutamate oxidation also in liver mitochondrial preparations; this confirms the observations of other investigators (Jones & Gutfreund, 1961; Borst, 1962; Papa, Saccone, Palmieri, Francavilla & Quagliariello, 1963; Chappell, 1964b; Hird & Marginson, 1964; de Haan *et al.* 1964). The oxidation of glutamate by pathways other than transamination was estimated from the difference between glutamate removal and aspartate formation and also by blocking the transamination reaction by malonate. It was shown that the activity of the pathways other than trans-

amination is much lower in brain than in liver mitochondrial preparations. The mean rates accounted for by these pathways in the active state in the presence and absence of malonate were in brain mitochondria 45 and 33 and in liver mitochondria 161 and 70 μ moles/hr./100 mg. of protein. Dinitrophenol increased the rate of oxidation of glutamate both in brain and in liver mitochondria without changing the relative proportions oxidized by transamination and by other routes (Tables 3 and 7). The ratio of these two pathways remained approximately constant during incubation for 1 hr. at 37.5° (Fig. 1) and it was not affected by changing the glutamate concentration within the range 3.5–10 mM. The glutamate that was not oxidized through the transamination pathways was oxidized in liver mitochondria by the glutamate dehydrogenase; this was also indicated by measurements of the formation of ammonia during the oxidation of glutamate (Papa *et al.* 1963; Hird & Marginson, 1964). In brain mitochondrial preparations the activity of glutamate dehydrogenase was much lower than in liver mitochondria (Table 10).

In brain preparations glutamate can also be metabolized by the glutamate decarboxylase. Quantitative amino acid analysis by paper chromatography was carried out in a few experiments, but only trace amounts of γ -aminobutyric acid were found at the end of the incubation. This would not exclude the possibility that this compound was formed and metabolized further by transamination with α -oxoglutarate (Bessman, Rossen & Layne, 1953). However, the contribution of this pathway must be small, since in previous experiments it was found that the respiration rate with or without

added α -oxoglutarate was only slightly increased by high concentrations of γ -aminobutyric acid (Balázs *et al.* 1963); also, the activity of the glutamate decarboxylase in these Triton-treated mitochondrial preparations was low ($19.5 \mu\text{moles/hr./100 mg.}$ of protein; compare with rates given in Table 10).

In liver mitochondrial preparations in the presence of ADP only a fraction of the total glutamate uptake ($28 \mu\text{moles/hr./100 mg.}$ of protein; Table 8) could be accounted for by the glutamate-dehydrogenase pathway, whereas when transamination was blocked with malonate the oxidation of glutamate by this route was increased about sixfold ($154 \mu\text{moles/hr./100 mg.}$ of protein). Thus the available (i.e. not latent) activity of glutamate dehydrogenase was much higher than the operating activity under these conditions.

It is suggested that the control of glutamate-dehydrogenase activity is attributable to a competition for NAD^+ between the enzymes oxidizing glutamate and α -oxoglutarate. The oxidation of glutamate yields α -oxoglutarate, which is further metabolized in the tricarboxylic acid cycle, as indicated by the low concentration (0.1 mM) of this metabolite during incubation. The aspartate-aminotransferase activity of the mitochondria is in great excess compared with the rate of respiration or with the activity of glutamate dehydrogenase (Table 10). Therefore oxaloacetate is continuously removed by the transamination reaction. Thus after the reactions are started α -oxoglutarate is formed independently of the dehydrogenation of glutamate. The main question is therefore how the glutamate-dehydrogenase activity is influenced by the presence of α -oxoglutarate. A direct inhibition of the glutamate-dehydrogenase reaction by α -oxoglutarate does not seem to be a major effect, since the final concentrations of α -oxoglutarate were of the same order of magnitude in the presence and absence of malonate; nevertheless, the oxidation of glutamate by the dehydrogenase was increased several-fold in the presence of malonate (vessels 2 and 5 in Table 8). The results indicate that competition takes place between the enzymes oxidizing the two substrates, resulting in preferential oxidation of α -oxoglutarate (Tables 3 and 7). This would agree with the general considerations of Krebs (1959). In view of the very complex nature of the two enzyme systems (α -oxoglutarate-dehydrogenase complex and glutamate dehydrogenase) and of our limited knowledge of their behaviour in the mitochondria, the detailed mechanism of the competition has yet to be elucidated.

The preferential oxidation of α -oxoglutarate is probably related to the observation that the apparent K_m for NAD^+ of the purified α -oxoglutarate-dehydrogenase complex is much lower ($4.5 \times 10^{-6} \text{ M}$; Sanadi, 1963) than that of the glutamate dehydro-

genase ($9 \times 10^{-5} \text{ M}$; Frieden, 1959). It was also found that at high substrate concentrations the rate of α -oxoglutarate oxidation by 'intact' liver mitochondria was about four times as high as that of the oxidation of glutamate by the glutamate dehydrogenase in the presence of malonate and dinitrophenol (Tables 8 and 9). The relative contribution of the two pathways of glutamate oxidation will depend on the relative rates of substrate oxidation by the α -oxoglutarate dehydrogenase and glutamate dehydrogenase under any particular conditions. A further consideration is that some oxaloacetate will escape transamination by being further metabolized; this is indicated by the formation of $^{14}\text{CO}_2$ from $[5\text{-}^{14}\text{C}]\text{glutamate}$ under these conditions (Table 4).

Klingenberg & Slenczka (1959) and Klingenberg & Pette (1962) gave circumstantial evidence as to the NADP -specificity of mitochondrial glutamate dehydrogenase. It was suggested by Borst (1962) that the control of the pathways of glutamate oxidation can be explained by the NADP -specificity of this enzyme, assuming a 'compartmentation' of nicotinamide nucleotides in the mitochondria. The cofactor specificity of glutamate dehydrogenase was tested under conditions when glutamate removal was due exclusively to the dehydrogenase pathway. The rate-limiting enzyme was glutamate dehydrogenase, and the permeability of the mitochondria to nicotinamide nucleotides was increased by mild pretreatment that hardly affected the latency of the enzymes (Table 11). Although the mitochondria were not 'intact' the rate of oxidation of glutamate without added cofactors (vessels 2 and 8) were comparable with that obtained with the untreated preparation (vessel 1). Thus probably no 'regrouping' of the nicotinamide nucleotides occurred. In these preparations the rate of the dehydrogenation of glutamate was increased by NAD^+ (about 30%) and the rate in the presence of NADP^+ was about half of that observed with NAD^+ . Thus the properties of glutamate dehydrogenase under conditions when the latency was hardly affected were similar to those observed with the enzymes completely 'unmasked' (Gutfreund *et al.* 1961). These results make it unlikely that the control of the two pathways of glutamate oxidation would result from an NADP -specificity of mitochondrial glutamate dehydrogenase.

The author thanks Professor Sir Hans Krebs, F.R.S., for the benefit of his advice and for his hospitality in allowing him to spend a month in his Laboratory, Dr D. Richter for his interest in the work and for criticism of the manuscript, and Dr W. N. Aldridge, Dr R. J. Haslam and Dr D. R. Dahl for valuable discussions and for great help in preparing the manuscript. Dr W. Bartley is thanked for his helpful suggestions. The technical assistance of Mrs W. Cocks is acknowledged.

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