Sources of Ammonia for Mammalian Urea Synthesis

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The initial rate of incorporation of [^{15}N]alanine into the 6-amino group of the adenine nucleotides in rat hepatocytes was about one-eighteenth of the rate of incorporation into urea. Thus the purine nucleotide cycle cannot provide most of the ammonia needed in urea synthesis for the carbamoyl phosphate synthase reaction (EC 2.7.2.5). On the other hand, contrary to the view expressed by McGivan & Chappell [(1975) *FEBS Lett.* **52**, 1–7], the experiments support the view that hepatic glutamate dehydrogenase can supply the required ammonia.

When urea is synthesized in the mammalian liver, half of the nitrogen of urea must pass through the stage of ammonia and the other half through the stage of aspartate. Until 1975 it was the generally accepted view that a major part of the required ammonia is released by the glutamate dehydrogenase reaction, as was first suggested by Braunstein (1957). The validity of this concept was then questioned by McGivan & Chappell (1975), because experiments on isolated mitochondria suggested that the activity of the glutamate dehydrogenase system *in situ* is too low because of the slow entry of glutamate into the mitochondrial matrix. These workers also noted that leucine accelerates glutamate dehydrogenase in sonicated mitochondria, whereas it inhibits urea synthesis in intact hepatocytes. These observations led McGivan & Chappell (1975) to the tentative conclusion that glutamate dehydrogenase plays no major role in connection with the synthesis of urea. As for an alternative source of ammonia, they expressed the view that the deamination of AMP and the purine nucleotide cycle (Raggi *et al.*, 1969; Lowenstein, 1972) might be the normal pathway of hepatic ammonia formation.

We have tested the latter assumption with the help of [^{15}N]alanine by comparing the rates of incorporation of ^{15}N into the 6-amino group of the adenine nucleotides and into urea. If the purine nucleotide cycle operates in the synthesis of urea then the 6-amino group of AMP must turn over at the rate of 1 molecule of AMP formed from IMP/molecule of urea formed. If it can be shown that urea is synthesized from alanine at a more rapid rate than the rate of labelling of the 6-amino group, then a major role of the purine nucleotide cycle can be excluded.

Experimental

Materials and Methods

^{15}N -labelled L-alanine was prepared by the method of Greenaway *et al.* (1977) and supplied by these authors. The sample used contained 95 atoms % excess ^{15}N and was practically free from [^{15}N]glutamate.

For the measurement of the incorporation of ^{15}N into the 6-amino group of the adenine nucleotides and into urea it is necessary to isolate a derivative from which gaseous nitrogen can readily be prepared for direct isotopic analysis in a mass spectrometer. A suitable derivative is ammonia. This was obtained from urea with urease (EC 3.5.1.5) and from adenine nucleotides by the combined action of potato apyrase (EC 3.6.1.5) and AMP deaminase (EC 3.5.4.6). In the early attempts to isolate ammonia irregular results were obtained, which were traced to the contamination of the samples with external ammonia. Such contamination dilutes adenine nucleotide N to a much greater extent than urea N (on a percentage basis) because the amounts of urea N are much greater under the test conditions. The results became consistent only when all sources of ammonia contamination had been minimized. The distilled water used was freshly prepared and then passed through a mixed-bed-resin deionizer. The HCl solutions used in the Conway units were prepared immediately before use from a BDH volumetric solution ampoule. This solution contains HgCl_2 and therefore could be used only in the Conway dishes. An alternative source of low-ammonia HCl, used for neutralization of solutions to which enzymes had to be added, was the 5M-HCl analytical volumetric

solution, BDH code no. 19066, selected for low ammonia content and diluted with ammonia-free water. The enzyme preparations used were chosen for their low or negligible ammonia content. They were obtained from Sigma (London) Chemical Co. Ltd., Poole, Dorset BH17 7NG, U.K. The urease preparation (grade VI freeze-dried powder) contained less than $1\mu\text{g}$ of ammonia/unit of enzyme activity. Adenylate deaminase (grade IV in 66% glycerol) contained no measurable amount of ammonia, and apyrase (grade I freeze-dried powder) contained $0.008\mu\text{mol}$ of ammonia in the $20\mu\text{l}$ used. All solutions were kept stoppered when not in use, and where possible fresh solutions were prepared for each experiment and checked for the ammonia content. The Conway units were rinsed with alkali, then with distilled water, and finally with deionized water before they were dried at 110°C .

Isolated hepatocytes

Isolated rat hepatocytes, which readily synthesize urea from alanine, served as experimental material. They were prepared as described by Krebs *et al.* (1974) according to the principles introduced by Berry & Friend (1969). Cell suspensions were incubated in 25ml conical flasks. Each flask contained 4ml of suspension, which included about 150mg wet wt. of cells, 6mM-L-[^{15}N]alanine, 1mM-sodium oleate, 0.1mM-L-ornithine and 2.5% dialysed bovine serum albumin. Flasks were incubated for 15, 30 and 60min in pairs, which were later combined to provide sufficient material.

Handling of incubated cell suspension

Separation of cells and medium. At the end of the incubation the cell suspension was centrifuged in the separating tubes described by Hems *et al.* (1975). The cells containing the adenine nucleotides were collected in the bulb filled with 1.5ml of 4% (w/v) HClO_4 . After centrifugation the supernatant, containing most of the urea but no adenine nucleotides, was decanted and mixed with 0.1 vol. of 20% (w/v) HClO_4 .

Treatment of separated cells: isolation of ammonia derived from the 6-amino group of adenine nucleotides. The contents of the bulbs of duplicate incubations, about 3.0ml, were combined and centrifuged. The supernatant was collected and, after addition of Universal indicator, the pH was adjusted to 10 with 30% (w/v) KOH. The KClO_4 was removed and, in order to remove all contaminating ammonia, the solution was re-adjusted to pH10 and dried on a rotary evaporator. The residue was then taken up in 1ml of water and the pH was adjusted to 7.0 with ammonia-free HCl. Two units of apyrase (Sigma) and 0.6 unit of AMP deaminase (Sigma) were then

added. The mixture was incubated for 2h, by which time the deamination of the adenine nucleotides had reached its maximum value. Recovery tests gave a yield of 93%. This small deficit was probably due to the dephosphorylation of AMP; adenosine formed by this reaction escapes deamination. For the isolation of the ammonia the solution was cooled to 0°C and transferred quantitatively to the outer ring of a Conway unit (Conway, 1962). The centre well contained 1ml of freshly prepared ammonia-free 1M-HCl. Then $50\mu\text{mol}$ of NaOH was added to the outer ring to adjust the pH to 11 as checked by the indicator. The Conway units were kept at room temperature for 3h with occasional shaking. The HCl solution was then analysed for total ammonia (Bernt & Bergmeyer, 1970) and ^{15}N atoms % excess.

This procedure, which pools the ammonia derived from the 6-amino group of all three adenine nucleotides; was used because the amounts of AMP in hepatocytes are normally too low (about $0.3\mu\text{mol/g}$ wet wt.) for the present analytical work. Owing to the high activity of adenylate kinase (about $100\mu\text{mol}$ of ATP formed from ADP at $38^\circ\text{C}/\text{min per g}$ of liver), AMP is bound to exchange the purine ring system with ATP and ADP almost instantaneously. This exchange would occur even when there is no turnover of ATP as a result of oxidative phosphorylation. In fact, the turnover rate through oxidative phosphorylation in rat liver is high. At the basal rate of oxygen consumption of $2.5\mu\text{mol}/\text{min per g}$, the rate of ATP synthesis is $2.5 \times 6 = 15\mu\text{mol}/\text{min per g}$. The steady-state concentration of ATP in the liver is about $2\mu\text{mol/g}$; thus this amount is dephosphorylated to ADP and rephosphorylated every 8s, and during the 30min incubation period ATP would turn over 225 times.

This conclusion was tested experimentally by introducing ^{14}C -labelled adenosine into the hepatocytes and measuring the rate of labelling of AMP, ADP and ATP. Added AMP does not enter hepatocytes readily, but added adenosine does so and is rapidly phosphorylated to AMP (Lund *et al.*, 1975). Hepatocytes were incubated with [U- ^{14}C]adenosine for 5, 10 and 20min and the suspension was then centrifuged in the separating tubes of Hems *et al.* (1975). The deproteinized extract was neutralized with KOH. The adenine nucleotides were separated by paper chromatography (Krebs & Hems, 1953) and their amounts and specific radioactivities were measured. As shown in Table 1, the values for the specific radioactivity of the three adenine nucleotides even after 5min were close enough to justify this procedure.

Treatment of supernatant of cell suspension: conversion of urea into ammonia. A 5ml sample of the neutralized deproteinized supernatants was evaporated to dryness at pH10 and the residue was taken up in 1ml of water. The pH was adjusted to 7.0 and

1.5 units of Sigma type IV urease were added. The tubes were then incubated for 2 h at 25°C. Ammonia was distilled in Conway units as described above for the pellet extracts.

Isotope mass spectrometry. The samples of NH₄Cl from the experiments were diluted with a known amount of AnalaR NH₄Cl solution (BDH) and treated with LiOBr *in vacuo* (Ross & Martin, 1970). The resulting nitrogen gas was introduced into an isotope mass spectrometer (AEI MS20) for analysis. The isotopic abundances were compared with those of the diluting NH₄Cl.

Metabolite assays. Glutamine was determined as glutamate after hydrolysis by glutaminase (Lund, 1974). The other metabolites were determined as described by Cornell *et al.* (1974) and Lund *et al.* (1975).

Results

In the course of a large number of preliminary experiments the optimal conditions for measuring the relative rates of incorporation of ¹⁵N from L-[¹⁵N]alanine were explored and the techniques of avoiding contamination with external ammonia were elaborated. These tests showed that the rate of incorporation of ¹⁵N into the 6-amino group of the

adenine nucleotides was by no means negligible. As was to be expected, the differences between the abundance of [¹⁵N]urea and the 6-amino group decrease with time and are liable to disappear eventually. It is therefore important to measure the rate of incorporation at the early stage of exposure to [¹⁵N]alanine. The final and crucial experiment recorded in Table 2, carried out after the perfection of the technique, shows that under the test conditions the ¹⁵N atoms % excess in urea after 15 min was 18 times greater than in the 6-amino group (47.7% against 2.72%). After 60 min the ¹⁵N atoms % excess was still 95 % in urea and 50 % in the 6-amino group.

The absolute values of ¹⁵N atoms % excess (in contrast to the relative values) showed some variations between experiments because contamination with extraneous ammonia could not be eliminated entirely. Ideally such experiments should be carried out in a special laboratory not available to us. This should be kept free from all sources of ammonia. A troublesome source of contamination within this laboratory proved to be the (NH₄)₂SO₄ solutions in which commercial enzyme preparations are kept.

Alternative precursors of hepatic ammonia

If AMP cannot be a significant immediate precursor of hepatic ammonia, the question of other reactions not involving glutamate dehydrogenase has to be considered. The direct liberation of ammonia in the degradation of glutamine, asparagine, serine, threonine and histidine must be ruled out, because the rate of supply of these potential precursors is too low. The only major source is the portal blood, which contains ammonia (and alanine) derived predominantly from deamidation of glutamine and aminotransferase reactions respectively in the intestinal wall (Windmueller & Spaeth, 1974). However, the major immediate precursor of hepatic ammonia is glutamate. This is provided from three sources: the hydrolysis of protein, hepatic intracellular (including intramitochondrial) transamination reactions of alanine, aspartate, tyrosine, phenylalanine, lysine and ornithine and the degradation of histidine, proline and arginine.

Table 1. Rate of incorporation of ¹⁴C into adenine nucleotides on addition of [U-¹⁴C]adenosine to isolated hepatocytes

A suspension (4 ml) containing 60 mg wet wt. of cells was incubated with 0.1 mM-[U-¹⁴C]adenosine, 6 mM-alanine, 2 mM-lactate and 0.5 mM-ornithine. The radioactivity counting efficiency was 96%.

Period of incubation (min)	Concn. of adenine nucleotides (μmol/g wet wt. of cells)			10 ⁻⁴ × Specific radioactivity of adenine nucleotides (c.p.m./μmol)		
	ATP	ADP	AMP	ATP	ADP	AMP
5	1.98	1.35	0.34	42.2	32.4	41.5
10	2.36	1.54	0.33	51.9	47.3	61.2
20	3.55	1.26	0.22	54.4	57.2	55.0

Table 2. Incorporation of ¹⁵N from [¹⁵N]alanine into urea and the adenine nucleotides

Each incubation flask contained 4 ml of the standard incubation medium with 2.5 mM-[¹⁵N]alanine, 1.0 mM-oleate, 0.1 mM-ornithine and 149 mg wet wt. of hepatocytes.

Time of incubation (min)	Urea			Adenine nucleotides				¹⁵ N atoms % excess in 6-amino group
	(μmol/flask)	(μmol/h per g)	(¹⁵ N atoms % excess)	ATP (μmol/g)	ADP (μmol/g)	AMP (μmol/g)	Total (μmol/g)	
15	1.84	49.5	47.7	1.81	0.35	0.20	2.36	2.72
30	3.09	41.6	94.2	2.78	0.54	0.14	3.46	6.59
60	4.83	32.5	95.4	2.83	0.73	0.14	3.70	15.0

McGivan & Chappell (1975) justified their exclusion of glutamate as a major precursor of hepatic ammonia by the following statements.

(1) Leucine inhibits urea synthesis from alanine and increases the formation of glutamate and glutamine in hepatocytes; therefore they suggest that glutamate dehydrogenase is 'primarily involved in N storage'.

(2) Leucine does not affect the synthesis of citrulline from ornithine, bicarbonate and ammonia in isolated mitochondria (McGivan *et al.*, 1973). Quoting results of Mendes-Mourão *et al.* (1975), they state that there is no evidence that leucine acts directly on the urea cycle.

(3) The rate of glutamate deamination by intact mitochondria is too low (4.3 nmol/mg of mitochondrial protein at 30°C; McGivan *et al.*, 1974). Claims of higher rates by other authors are not valid, because they neglected the limited permeability of the inner mitochondrial membrane for glutamate.

(4) Ammonia is derived from some as yet unidentified source, possibly the purine nucleotide cycle. McGivan & Chappell (1975) were careful to emphasize the hypothetical nature of their conclusions. Their paper includes the passage: 'if the views expressed in this article are ultimately found to have a semblance to reality . . .'.

Our own experiments (Table 3) do not confirm the alleged effect of leucine as stated above (1). Leucine did not increase the formation of glutamate and glutamine from alanine. Whereas it is true that leucine inhibits urea synthesis from alanine, it does not affect the rate of removal of alanine but it causes the nitrogen to appear as ammonia instead of urea (Table 3). Rognstad (1977) showed that leucine inhibits urea formation not only from alanine but also from NH_4Cl , glutamine and asparagine, and he pointed out that the effects of leucine are fully explained by its inhibition of ornithine carbamoyl-transferase (EC 2.1.3.3), an effect discovered by Marshall & Cohen (1972). As for point (2), leucine does not inhibit citrulline synthesis or urea formation in the presence of ornithine (Table 3), because the inhibition by leucine is competitive with ornithine (Marshall & Cohen, 1972).

The low rates of ammonia release from glutamate in isolated mitochondria (point 3) are not relevant *in vivo*. Low rates are found under conditions where equilibrium in the glutamate dehydrogenase system is rapidly established, i.e. when the products of the reaction are not removed (Charles, 1968). Within the physiological pH and $[\text{free NAD}^+]/[\text{free NADH}]$ ranges the equilibrium does not favour dehydrogenation. Under physiological conditions the rates of removal of NADH, ammonia and α -oxoglutarate are sufficiently rapid to account for the postulated rate of ammonia production from glutamate (see Krebs & Lund, 1977).

Table 3. Effect of leucine on the fate of alanine nitrogen and carbon

Hepatocytes were prepared from a 48 h-starved rat. Incubation was for 60 min at 37°C. Initial concentrations of alanine and leucine were 5 mM and of ornithine 2 mM. Each flask contained 66 mg wet wt. of tissue in 4 ml of medium (results are for a representative experiment). All values refer to μmol of metabolites/4 ml cell suspension. The flux rate through glutamate dehydrogenase was calculated on the assumption that all ammonia and half the urea N formed arose through glutamate dehydrogenase. [The results differ from those of Mendes-Mourão *et al.* (1975, Table 1) in respect of the effects of leucine on the fate of alanine C and N.]

Substrates added	Alanine removed	Glucose formed	Lactate formed	Pyruvate formed	Urea formed	Ammonia formed	Glutamine formed	Glutamate formed	Aspartate formed	Calc. flux through glutamate dehydrogenase
None	—	0.55	0.02	0.02	0.74	0.17	0.20	0.16	0.1	—
Alanine	10.0	1.35	2.4	1.8	2.35	2.76	0.18	0.91	0.6	5.11
Alanine; leucine	9.8	1.25	2.3	2.5	1.53	5.4	0.11	0.96	0.6	6.93
Alanine; ornithine	8.3	1.52	3.0	2.1	4.50	0.43	0.21	1.39	0.4	4.93
Alanine; ornithine; leucine	9.6	1.49	4.1	2.7	5.88	0.48	0.19	1.48	0.4	6.36

Discussion

Sources of ammonia for carbamoyl phosphate synthesis

The experiments with [^{15}N]alanine prove that the purine nucleotide cycle is not a major source of ammonia in rat liver. The arguments raised by McGivan & Chappell (1975) against the major role of glutamate dehydrogenase in urea synthesis from amino acids have all been invalidated. The evidence supports the view that the ammonia required for the carbamoyl phosphate synthase reaction can be supplied by hepatic glutamate dehydrogenase. We would point out that *in vivo* there are additional sources of ammonia available to the liver, including that arising from the degradation of glutamine in the intestinal wall (Windmueller & Spaeth, 1974).

Turnover of 6-amino group of adenine nucleotides

The present experiments show for rat liver that the turnover of the 6-amino group of AMP is by no means negligible (about 15% in 60 min under the test conditions). As the cells contain about 3.5 mm total adenine nucleotides the turnover of the 6-amino group was about $0.009\ \mu\text{mol}/\text{min}$ per g. This is extremely low compared with the turnover of the pyrophosphate bonds of ATP, which at 'rest' is about $12\ \mu\text{mol}/\text{min}$ per g and under conditions of maximal biosynthetic activity about $36\ \mu\text{mol}/\text{min}$ per g. Kalckar & Rittenberg (1947) found that the 6-amino group of the adenine nucleotides of skeletal muscle *in vivo* turns over much more rapidly than the purine-ring N. Their experiments do not, however, yield the necessary quantitative data from which the rate of turnover can be calculated.

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