THE FORMATION OF FREE AMMONIA FROM GLUTAMATE OXIDATION IN RABBIT RETICULOCYTES; EVIDENCE FOR THE INSIGNIFICANCE OF OXIDATIVE DEAMINATION

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1. Introduction

The role of oxidative deamination by way of the glutamate dehydrogenase reaction has remained controversial. Evidence for this reaction may be furnished by measurement of ammonia formation from glutamate. Earlier studies had shown that amino acids are the main respiratory substrate of reticulocytes [1]. Their ammonia formation exceeds that of mature erythrocytes two to tenfold, the great variability being caused by differences in respiratory rate of the cells and various experimental conditions [2–6]. The amounts of ammonia formed are much less than would be expected from the utilisation of the carbon of the amino acids, particularly so if one considers that preferably short-chain aliphatic amino acids are oxidized, while the cyclic amino acids do not contribute significantly. Thus one would expect a ratio of 3–4.5 between the moles of oxygen consumed and ammonia formed. The actual ratios observed after 2–6 hr of incubation are however between 6 and 9. The discrepancy becomes even greater if the considerable amounts of ammonia which are derived from the deamination of nucleotides [7, 8] as evidenced by the formation of hypoxanthine are deducted. Furthermore there is a possibility that ammonia measurements performed by alkalinisation techniques may liberate ammonia from labile compounds.

Recent studies on glutamate oxidation [9, 10] have indicated that a high proportion of the glutamate is metabolised by way of transamination with oxalacetate. In order to obtain direct evidence the ^{15}N-ammonia formation from ^{15}N-glutamate by reticulocytes was measured. In addition the ammonia was determined enzymatically.

2. Materials and methods

The preparation of leucocyte free reticulocyte rich red blood cell suspensions, the incubation in Warburg vessels, the measurements of $^{14}CO_2$ formation and of the corresponding O_2 uptake were performed as previously described [11].

The ammonia was assayed with the glutamate dehydrogenase reaction [12]. Immediately after the precipitation of the incubation mixture with 5% trichloroacetic acid the supernatant fluid was carefully neutralised at 0° with saturated $KHCO_3$ to a pH of 6.0 under vigorous stirring. Glutamate at higher concentrations interferes with the enzymic ammonia measurement due to the equilibrium of the reaction and therefore was removed by anion exchange by Dowex 1X8 [13].

To measure directly the proportion of ammonia which originates from glutamate the cells were incubated with 30 mM DL-glutamate (^{15}N), with a $^{15}N/^{14}N$ ratio of 93.6%, which was additionally labelled with L-glutamate (U-^{14}C).

The ammonia present in the acid extract was isolated by ion exchange chromatography with the amino acid analyzer BC 200 (Biocal Instruments, München, GFR). The separation was performed with the short column of 14 X 0.9 cm filled with BioRad A 5 resin, 11.5–15.5 μm at 30°. The freshly prepared 0.2 M citrate buffer pH 4.25 [14] was freed from traces of ammonia by passage through the "buffer wash column" inserted in the buffer line (18 X 0.9 cm, filled with BioRad AG 50 WX 8 resin, 50–100 mesh). In 0.2 ml aliquots of the 2 ml fractions collected the ammonia peak was localised with the ninhydrin

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reaction. Since the citrate interferes with the subsequent procedures, the ammonia was transferred into 2 ml of 0.1 N HCl by steam destillation. The solution was brought to dryness and the residue was redissolved in 30 µl H_2O. The ammonia was oxidized to N_2 with CuO/CaO [15]. The isotope ratio $^{15}N/^{14}N$ was measured with the ^{15}N-Analyser developed in the Institut für Stabile Isotope, Leipzig (GDR) by ultraviolet emission spectrometry [16].

In the calculations of $^{15}NH_3$ and $^{14}CO_2$ only the L-glutamate was considered. DL-glutamate (^{15}N) was from VEB Berlin-Chemie (GDR), glutamate (U-^{14}C) from The Radiochemical Centre, Amersham, and ammonia-free glutamate dehydrogenase from Boehringer, Mannheim (GFR).

3. Results and discussion

As can be seen in fig. 1 the ammonia formation were actually lower with 15 mM glutamate as substrate than without substrate. At this glutamate concentration about 20% of the total respiration substrate originated from the added glutamate [18]. The ratio of O_2-uptake/NH_4^+ formation in the two experiments shown was about 8 and 10 on substrate-free incubation, as has been observed before. The results of the experiment with ^{15}N- and ^{14}C-labelled glutamate are given in table 1. The ^{15}N-ammonia found after the various incubation periods represents directly that ammonia which is derived from the added glutamate alone. The glutamate oxidized is calculated from the $^{14}CO_2$ formation on the assumption, that each mole of [U-^{14}C] glutamate deaminated is degraded into 5 moles of $^{14}CO_2$. The ratio $^{15}NH_4^+/[^{15}N]$ glutamate oxidized shows that only 8.5% (after 30 min incubation) to 13% (after 240 min incubation) of the amino groups of the [^{15}N] glutamate which were oxidized, were converted into free ammonia.

A secondary removal of ammonia liberated by the glutamate dehydrogenase reaction could not have changed the isotope ratio $^{15}N/^{16}N$ in the ammonia pool of the cell.

These results represent direct evidence that in intact reticulocytes oxidative deamination only plays a subordinate role. The initial step is rather transamination with oxaloacetate.

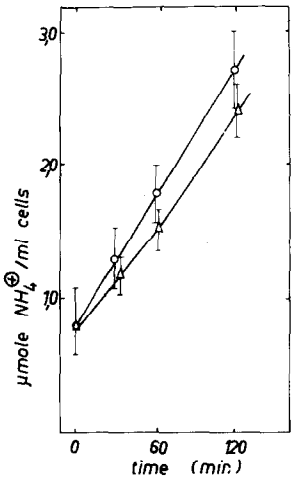

Fig. 1. Ammonia formation in rabbit reticulocytes with and without 15 mM L-glutamate. The results of two experiments are given. The O_2 uptake was 9.5 and 9.8 µmoles O_2/ml cells/hr. (Δ) With glutamate; (○) without glutamate.

Table 1
$^{15}NH_3$ formation from DL-glutamate (^{15}N; ^{14}C) in reticulocytes.

Incubation period (min)	30	60	120	240
NH_4^+ found*	1.25	1.60	2.60	3.20
$\frac{^{15}N}{N}$ (%)	0.8	1.17	1.51	2.60
$^{15}NH_4^+$ formed*	0.011	0.020	0.042	0.089
Glutamate oxidized* ($^{14}CO_2/5$)	0.13	0.22	0.38	0.67
$\frac{^{15}NH_4^+ \text{ found}}{\text{Glutamate oxidized}}$ (%)	8.5	9.1	11	13

* Values given as µmoles/ml cells.

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