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SEZIONE II

(Fisica, chimica, geologia, paleontologia e mineralogia)

Chimica biologica. — *Studies on urea synthesis: comparison between* NH_3 *produced* in vitro *and urea excreted* (*). Nota di FRANCESCO CEDRANGOLO, PATRIZIA GALLETTI e ANTONIO FEDERICO, presentata (**) dal Corrisp. F. CEDRANGOLO.

RIASSUNTO. — In queste ricerche che rappresentano la continuazione di altre precedenti, pure comunicate all'Accademia [7], si sono utilizzati ratti, divisi in 3 gruppi: A) controlli (a dieta normale): B) a dieta iperproteica per 24 h; C) a digiuno anche per 24 h. Per ogni ratto si è misurata l'ammoniaca prodotta *in vitro* e l'urea escreta. L'ammoniaca è stata dosata negli omogenati di fegato, rene, cervello tenuti a 37° per 60 minuti: gli organi erano prelevati dall'animale alla fine delle 24 h di esperimento. Durante questo stesso tempo l'urina era raccolta e utilizzata per il dosaggio dell'urea. I risultati hanno dimostrato: 1) nei ratti a dieta iperproteica, quale che sia l'omogenato di organo sperimentato e il tempo, al quale viene effettuato il dosaggio, l'ammoniaca non supera mai il corrispondente valore riscontrato nel gruppo dei controlli come in quello dei ratti a digiuno; 2) i ratti a dieta iperproteica eliminano invece urea in quantità enormemente più grande rispetto agli altri due gruppi: si è calcolato infatti un aumento medio del 483% rispetto ai controlli e addirittura del 570% rispetto ai ratti a digiuno. Il paragone tra il comportamento dell'NH_3 prodotta *in vitro* e quello dell'urea escreta, porta, pertanto, anche nel caso di queste ricerche, a ritenere poco probabile la via ureogenetica, che vorrebbe l'ammoniaca un intermediario obbligatorio.

INTRODUCTION

In this Laboratory several evidences have been collected suggesting the existence of an alternative urea pathway, aside from the classical cycle of Krebs-Henseleit [1–6]. All performed experiments are indicative for the absence of " free " ammonia as an intermediate in the overall pathway that from the amino-nitrogen of proteins leads to urea.

In order to substantiate this view, ammonia production *in vitro* from homogenates of several rat tissues has been compared to the urea excretion in the same animals. Three groups of rats have been employed for these

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experiments: (*i*) control animals fed with normal diet; (*ii*) rats fed with high protein diet for 24 hrs; (*iii*) animals fasted for the same period of time.

Preliminary results of this work have been previously reported [7].

Chemicals

The chemicals employed were analytical-grade preparations obtained from the usual commercial sources. Glass-distilled water was passed through ion exchange resin as Amberlite IR 120 (H^+ form) in order to avoid the presence of traces of ammonia. Urease (E.C. 3. 5. 1. 5.) purified from jack beans was obtained from Worthington Biochemical Corporation. Nessler's reagent was prepared following Vanselow's directions [8]. Saturated borate–NaOH buffer, for ammonia microdiffusion, was prepared according to Reinhold and Chung [9].

Methods

Ammonia was assayed by the Conway–Seligson [10–11] microdiffusion technique modified by Cedrangolo *et al.* [12]. The diffusion apparatus was modified to enclose the rotator for the diffusion bottles in a temperature controlled incubator. This device gives excellent recovery of ammonia by a careful control of temperature (37° C) and of speed rotation (50 rpm). The pH during microdiffusion was controlled at 11 ± 0.2 to obtain complete recovery of free ammonia present in the tissue and to avoid formation of artifactual ammonia from unstable amides, proteins etc. [11–12]. Urea was determined by dosing ammonia formed after treatment of the samples with urease.

Wistar male rats (Morini, Reggio Emilia, Italy), weighing about 250 gr, were used in all experiments. They were routinely maintained on a diet *ad libitum* until 24 hrs before use. At this time the rats were divided in three groups and placed in individual metabolic cages. One group was fed with normal diet, the second was fasted and the third one was fed *ad libitum* with high protein diet (raw cow meat). During this period of experimentation urine was collected under toluene. Urea was determined on 100 µl aliquots of urine with nesslerization after treatment with urease. After 24 hrs of dietary treatment, the animals were killed by decapitation and liver, brain and kidney were rapidly removed and weighed. The organs were rinsed with cold buffer and immediately homogenized for 2 minutes in phosphate buffer (KH_2PO_4–K_2HPO_4) 0.1 M at pH 7.4 (30 % *v/v*). The whole procedure was performed in the cold room at 2° C. The homogenate was made in a glass Potter–Elvehjem apparatus with teflon pestle at 1,000 rpm. 1 ml samples were transferred in 25 ml flasks and incubated at 37° C in a Dubnoff shaking-bath. After various times of incubation, ammonia was measured by microdiffusion.

RESULTS AND DISCUSSION

In Table I are reported the amounts of NH_3 present in livers, brains and kidneys of the three groups of animals. Kidney appears the organ most rich in NH_3 (per *g* weight). This finding is in accordance with the known specific function of this organ, i.e. ammonia excretion.

No sensible variation is observable between the three groups of animals: i.e. the levels of ammonia present in the organs of rats fed with high protein diet are of the same order of magnitude when compared with that of controls or animals fasted. This first result is suggestive for an urea cycle without the partecipation of " free " ammonia: otherwise an increase of ammonia content would be expected in the animals treated with high protein diet. These considerations are particularly valid for the liver, where all the enzymes of the urea cycle are virtually confined [13]. On the other hand several experiments by Schimke *et al.* [14–15] demonstrated that the liver content of urea cycle enzymes is directly related to the daily consumption of protein.

TABLE I.

Ammonia content of rat tissues.

ORGAN	μmoles NH_3/gr fresh tissue		
	A	B	C
Liver	5.8	4.7	7.7
Brain	4.5	4.1	4.9
Kidney	8.1	7.1	7.2

A = Controls (rats fed with normal diet).
B = Rats fed with high protein diet for 24 hrs.
C = Rats fasted for 24 hrs.

A several times increase in all urea cycle enzymes was demonstrated after raising dietary concentration of protein [14–15]. Therefore the lack of increase in liver ammonia content in animals with increased protein intake can also be explained with an induction of the urea cycle enzymes. To investigate in an unambiguous way this problem, the ammonia formation *in vitro* from the organs of the three groups of rats has been measured. The results are reported in figs. 1–3 and are corrected for NH_3 present at O time.

Fig. 1 shows that in liver, at different incubation times, ammonia formation in animals with high protein intake does not exceed the ammonia produced in the other two groups of rats. On the contrary a decreased

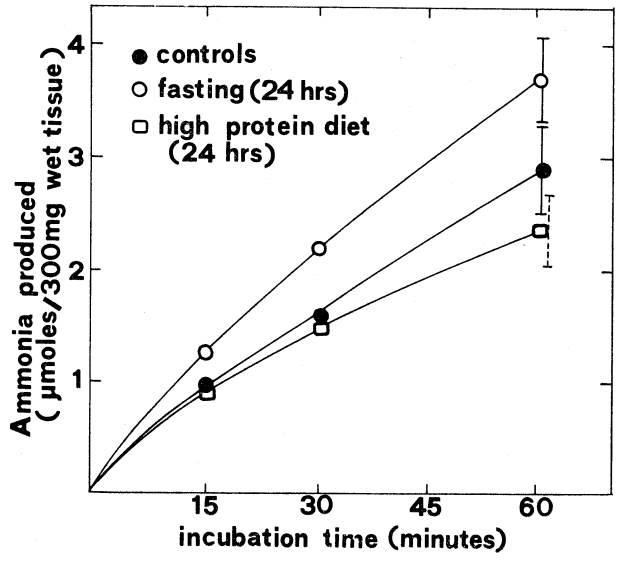

Fig. 1. – Ammonia production from rat liver homogenates.
Vertical bars indicate standard deviation.

production of NH_3 is observable in this group of animals if compared to the others. The result concerning the increased ammonia production in fasted rats (see fig. 1) cannot be explained at present; an adequate interpretation requires further and different experimentation.

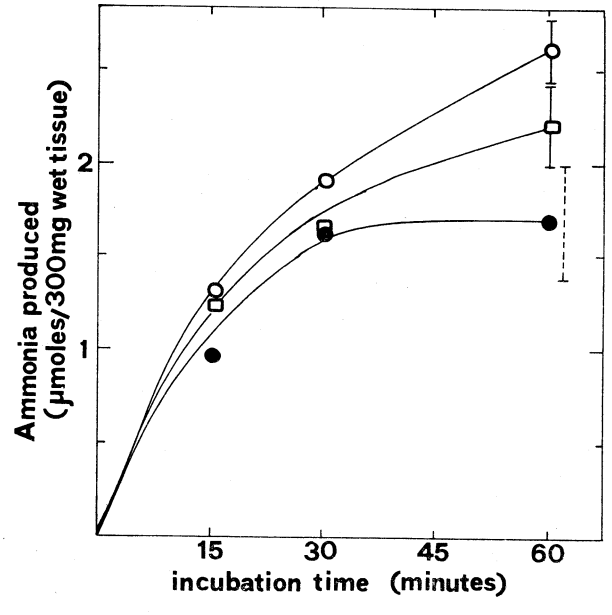

Fig. 2. – Ammonia production from rat kidney homogenates.
Vertical bars indicate standard deviation. For the symbols see fig. 1.

It is also interesting to note the lack of linearity between ammonia formation and incubation time. This result can be caused either by denaturation of ammonia-forming enzymes during the course of incubation or by the limited concentration of endogenous substrates. The same kind of results, with minor differences, are obtained with kidney and brain (figs. 2–3).

Even by taking into consideration an induction of urea cycle enzymes in the rats fed with high protein diet, the data reported in figs. 1–3 are indicative for a mechanism of urea synthesis without involvement of " free " ammonia. In fact the lack of addition of ATP and aspartate in the homogenates and the virtual absence of endogenous ATP in our experimental

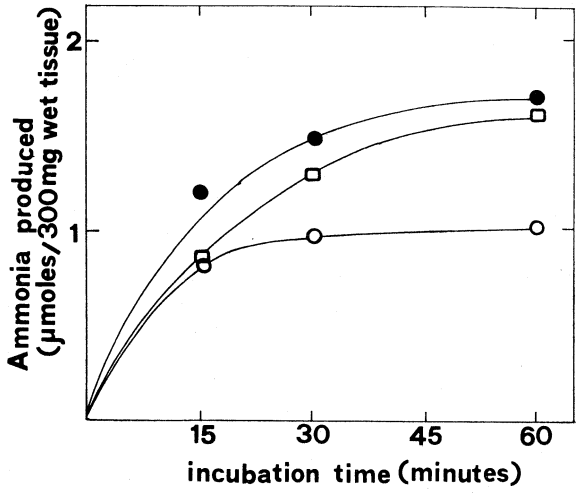

Fig. 3. – Ammonia production from rat brain homogenates.
For the symbols see fig. 1.

conditions prevents the activity of carbamyl phosphate synthetase and argininosuccinate synthetase. Therefore if ammonia was an obligatory intermediate in the pathway of urea biosynthesis, it should be produced in larger amounts in the homogenates from the rats fed with high protein diet.

In Table II is compared the urea excretion *in vivo* in the three groups of animals with ammonia formation *in vitro* in the same animals. The results differ slightly from previous data obtained in this laboratory [7] since the present data of ammonia formation have been obtained after 1 hr of incubation, whereas previously [7] they were extrapolated from results after 10′ incubation. Moreover the dietary conditions are different. The results reported in Table II, either for NH_3 produced *in vitro* or for urea excreted, are expressed as NH_3 formed/kg body weight in 60 minutes: for the calculations the weights of organs and the body weights of rats have been taken into account. From Table II it clearly appears that there is no relation between the 6–times increase of urea excretion in animals fed with high protein diet and the absence of a parallel increase of ammonia formation.

TABLE II.

Comparison between urea excretion in vivo *and ammonia formation* in vitro *in rat.*

	A	B	C
NH_3 formed from 3 organs/hr/kg body weight (μmoles)	457 ± 18	406 ± 21	546 ± 25
Urea excreted expressed as NH_3/hr/kg body weight (μmoles)	3,141 ± 220	18,341 ± 1,418	2,736 ± 250

A = Controls (rats fed with normal diet).
B = Rats fed with high protein diet.
C = Rats fasted for 24 hr.

These results are suggestive for a mechanism of urea synthesis without the partecipation of " free " ammonia. This remark is valid either considering the classical formulation of the urea cycle (Krebs-Henseleit), where ammonia is the precursor of both nitrogens of urea, or taking into consideration the cycle as it is now generally accepted. In the latter formulation, in fact, NH_3 is the precursor of the first nitrogen of urea molecule, whereas the second one could originate either from " free " NH_3 or from the amino-nitrogen of amino acids through transport reactions.

Our conclusions are in close agreement with data previously obtained in this laboratory [1–7] and with recent studies on congenital disorders of urea cycle enzymes [16–19]. In fact in patients affected with citrullinemia, argininosuccinic aciduria, etc., in which some enzymes of urea cycle are absent, the plasma level of urea is within the normal limits.

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