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THE PROTECTIVE ACTION OF N-ACETYL AND N-CARBAMYL DERIVATIVES OF GLUTAMIC AND ASPARTIC ACIDS AGAINST AMMONIA INTOXICATION

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Abstract—The effect pretreatment with a number of amino acids and derivatives on mortality of mice injected intraperitoneally with 560–580 mg/kg NH_4Cl (LD_{70-90}). Significative protection was produced by N-acetyl-glutamate, N-acetyl-aspartate, N-carbamyl-glutamate and N-carbamyl-aspartate administered in doses of 5 mM/kg, 6 mM/kg, 5 mM/kg and 6 mM/kg respectively. N-benzoyl-derivatives of glutamic and aspartic acid, N-acetyl-glycine, N-acetyl-alanine, N-carbamyl-asparagine, glutamine, asparagine, α-acetoglutarate, diacetyl and urea were inactive. In other experiments the effect of these active compounds upon blood ammonia was studied in rats given a sublethal dose of NH_4Cl (320 mg/kg). Ammonia estimations performed 15 min after NH_4Cl administration showed a significant decrease in the blood ammonia level. The mechanism of the protective action is discussed in relation to the urea cycle and to pyrimidine and glutamine synthesis.

INTRODUCTION

EXPERIMENTAL and clinical research[1–6] established the protective action of arginine in experimental and endogeneous ammonia intoxication, and suggested its possibly favourable effect in hepatic coma which many authors found to be associated in a great number of cases with increased blood ammonia level.[7]

Since then therapy with arginine alone or in association with other medicaments is currently used in hepatic coma, chronic hepatic diseases and has recently been recommended for cases of professional intoxication with hydrasine (propellant fuel).[32]

Salvatore and Bocchini[8] reported the protective action of ornithine in rats and of the association of ornithine + aspartate against intoxication induced by ammonium acetate (LD_{50}). This protection was accompanied by a lowering of blood ammonia level.

Tomitaro Kita *et al.*[9] obtained protection in rats given ammonium chloride (LD_{80}) by previous administration of L-arginine, DL-ornithine and γ-guanidine β-hydroxibutiric acid. The protected animals showed a lower ammonia level and an increased production of urea.

These experimental data *in vivo* are in agreement with the biochemical data *in vitro* of Krebs and Henseleit[10, 11] which showed that the intermediates of urea cycle (citrulline, ornithine and arginine) increased urea formation from CO_2 and NH_3.

The catalytic action of N-acetyl-glutamate in carbamyl-phosphate synthesis, a necessary step for ammonia entrance into the urea cycle, demonstrated *in vitro* in

mammal and frog liver, suggested to us the investigation of its possible protective action in experimental and clinical ammonia intoxication.

The similar action *in vitro* of carbamyl-glutamate, formyl-glutamate and chloracetyl-glutamate[12, 13] as well as the possibility of a direct synthesis of arginine succinate from ornithine and carbamyl-aspartate (suggested recently by Della Pietra[14]) and the role of carbamyl-aspartate in ammonia incorporation in pyrimidine compounds[15] led us to study several other N-acyl derivatives of glutamic and aspartic acids.

EXPERIMENTAL

Some of the compounds used in this work were synthesized in our laboratory according to the methods found in the literature; N-acetyl-L-glutamic acid according to Knopp and Oestelin,[17] N-acetyl-L-aspartic acid according to Gordon, Martin and Synge,[18] N-carbamyl-DL-aspartic acid according to Nik and Mitchell,[19] N-benzoyl-L-glutamic acid according to Fischer,[20] N-acetyl-alanine (L and DL) according to Karrer, Escher and Widmer,[21] pyrrolidone-carboxylic acid according to Menozzi and Appiani[22] and α-methyl-glutamic acid according to Gall *et al.*[23]

NH_4Cl, glutamic acid, aspartic acid, asparagine, and urea were obtained from E. Merk Darmstadt, lysine, glutamine and citrulline from Serva-Heidelberg, ornithine HCl and uridine from N.B.C. Cleveland, fumaric acid from B.D.H. Laboratories, arginine-HCl from Difco Laboratories, and α-cetoglutaric acid from Loba-Chemie-Wien.

The action of different amino acids and derivatives in lethal convulsions induced by a LD_{70-90} was studied in male mice (R.A.P. strain) weighing 18–21 g.

In other experiments performed on male Wistar rats (weight 100 ± 5 g), we examined the effect of some of these compounds on the blood ammonia increase induced by NH_4Cl.

In both types of experiment the compounds tested for ammonia protection were injected intraperitoneally in doses of 2–6 mM/kg one hr before NH_4Cl administration, in the form of Na salts at pH 7·3. Arginine, and ornithine were used as hydrochlorides, and citrulline as an uncombined base. The total volume injected was 0·1–0·2 ml in mice and 0·5–1 ml in rats. An equal quantity of saline solution was injected in controls under the same conditions. An 8 or 5% solution of NH_4Cl was administered intraperitoneally in mice in doses of 560–580 mg/kg which gave an LD_{70-90}. In rats NH_4Cl was injected in a dose of 320 mg/kg, which was sufficient to determine convulsions generally non lethal.

Blood ammonia determinations in rats were made 15 min after NH_4Cl administration, this time co-incidencing with the maximum convulsions. After decapitation of the animals, the blood was collected on a small quantity of Na F in tubes maintained at 0°.

A modified microdiffusimetric method was used for ammonia determination.[16] One ml of blood was introduced into the larger compartment, and one ml of K_2CO_3 in the other compartment of the cell (Fig. 1). A fixed quantity of N H_2SO_4 was deposited on the glass rod. The agitation was performed in a rotative rocking device, the cells being mounted in horizontal position. Using this model of cell the diffusion is started simultaneously in all samples and is complete after 12 min. For ammonia estimation the Berthelot–Luboschinsky colorimetric reaction was selected in preference to Nessler's reaction because of its greater sensitivity. This consists in the blue colour reaction which the ammonium ion gives with Na phenolate in the presence of nitroprusside

and hypochlorite in alkali medium. We devised this method, which has a good reproductibility, after a comparative study of other diffusimetric methods (Conway's, Selygson's, Ternberg's and Girard's method).

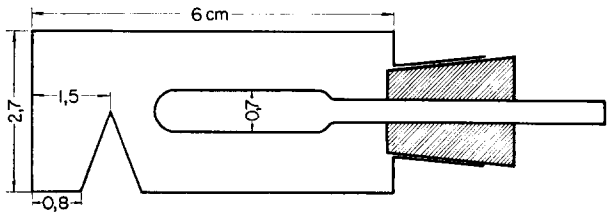

FIG. 1. Schematic drawing of the microdiffusion cell.

For statistical evaluation of data in mice the confidence intervals of percentage mortalities were determined for $P < 0{\cdot}05$ and $P < 0{\cdot}01$.

For blood ammonia determinations in rats the significance of the difference between the mean level of protected and control animals was estimated by the "normal deviate test". 37 control animals were used. With 18–25 animals/group protected with the above mentioned compounds we obtained for all protectors a significative lowering of blood ammonia level at a $P < 0{\cdot}001$.

RESULTS

The results obtained in mice are given in Table 1 which represents the data of 18 experiments with groups containing 20–40 animals.

The results concerning the blood ammonia level in rats protected with N-acetyl L-glutamate, N-carbamyl-L-glutamate, N-acetyl-L-glutamate + N-acetyl-L-aspartate and arginine are shown in Fig. 2.

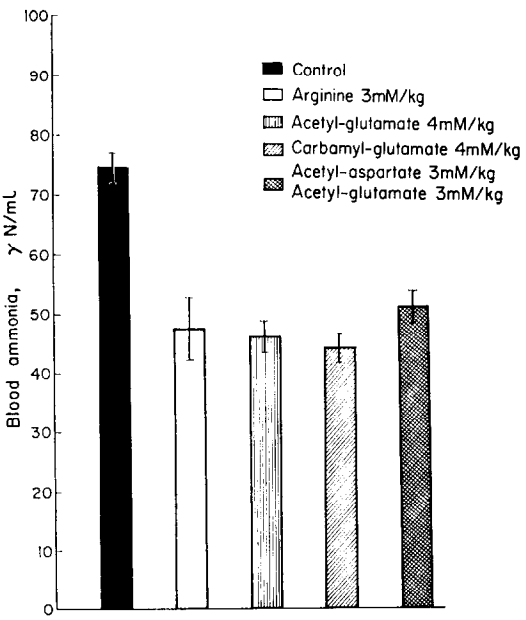

FIG. 2.

TABLE 1. THE EFFECT OF SOME AMINOACIDS AND DERIVATIVES UPON MORTALITY IN MICE GIVEN NH_4Cl, IN THE DOSE OF 560–580 MG/KG.

Experiment No.	Number of animals/ group	Group	Compounds tested	Doses in m Moles/ kg.	Animals dead after 3 hr	Percent survived %	Protection
1	20	1	Control	—	16	20	—
		2	N-Acetyl-L-glutamate	4 mM	4	80	$P < 0.01$
		3	N-Acetyl-L-aspartate	4 mM	12	40	—
		4	N-Acetyl-L-aspartate + N-Acetyl-L-glutamate	3 mM + 3 mM	6	70	$P < 0.05$
		5	N-Acetyl-glycine	6 mM	16	20	—
		6	L-Glutamate	6 mM	17	15	—
		7	L-Asparagine	6 mM	18	10	—
		8	L-Arginine	3 mM	0	100	$P < 0.01$
2	40	9	Control	—	35	12·5	—
		10	N-Acetyl-L-aspartate	6 mM	21	47·5	$P < 0.05$
		11	N-Acetyl-L-glutamate	5 mM	19	32·5	$P < 0.05$
		12	N-Acetyl-L-aspartate + N-Acetyl-L-glutamate	3 mM + 3 mM	13	67·5	$P < 0.01$
		13	L-Arginine	3 mM	13	67·5	$P < 0.01$
3	22	14	Control	—	20	9·1	—
		15	L-Arginine	2 mM	17	22·7	—
		16	L-Arginine + N-Acetyl-L-glutamate	2 mM + 3 mM	9	59·1	$P < 0.05$
		17	DL-Asparagine	6 mM	20	9·1	—
		18	N-Acetyl-DL-alanine	6 mM	15	31·8	—
		19	Diacetyl	4 mM	21	4	—
4	20	20	Control	—	15	25	—
		21	L-Arginine	2 mM	7	65	—
		22	L-Arginine	3 mM	5	75	$P < 0.05$
		23	L-Arginine + N-Acetyl-L-aspartate	2 mM + 3 mM	1	95	$P < 0.01$
		24	L-Arginine + N-Acetyl-L-aspartate	3 mM + 3 mM	1	95	$P < 0.01$
5	20	25	Control	—	18	10	—
		26	L-Arginine	2 mM	13	35	—
		27	L-Arginine + L-Aspartate	2 mM + 3 mM	15	25	—
6	20	28	Control	—	15	25	—
		29	N-Acetyl-L-aspartate + N-Acetyl-L-glutamate	3 mM + 3 mM	5	75	$P < 0.05$
		30	Fumarate	6 mM	10	50	—
		31	Fumarate + DL-Ornithine	3 mM + 3 mM	8	60	—
		32	L-Aspartate	6 mM	17	15	—

TABLE 1—*continued*

Experiment No.	Number of animals/ group	Group	Compounds tested	Doses in m Moles/ kg	Animals dead after 3 hr	Percent survived %	Protection
		33	Control	—	13	35	—
		34	N-Carbamyl-DL-aspartate	3 mM	15	25	—
		35	N-Carbamyl-L-glutamate	3 mM	10	50	—
7	20	36	N-Carbamyl-DL-aspartate + L-Aspartate	3 mM + 3 mM	10	50	—
		37	N-Carbamyl-DL-aspartate + L-Glutamate	3 mM + 3 mM	9	55	—
		38	N-Carbamyl-L-glutamate + L-Aspartate	3 mM + 3 mM	12	40	—
		39	Control	—	23	8	—
		40	N-Carbamyl-DL-aspartate	4 mM	19	24	—
		41	N-Carbamyl-L-glutamate	4 mM	9	64	$P < 0.01$
8	25	42	N-Carbamyl-DL-aspartate	6 mM	13	48	$P < 0.05$
		43	N-Carbamyl-DL-aspartate + N-acetyl-L-glutamate	3 mM + 3 mM	13	48	$P < 0.05$
		44	Uridine	50 mg/kg	20	20	—
		45	Control	—	19	5	—
		46	L-Arginine	3 mM	6	70	$P < 0.01$
		47	L-Arginine + L-Aspartate	3 mM + 3 mM	7	65	$P < 0.01$
9	20	48	L-Arginine + L-Glutamate	3 mM + 3 mM	4	80	$P < 0.01$
		49	L-Arginine + N-Acetyl-L-aspartate	3 mM + 3 mM	5	75	$P < 0.01$
		50	L-Arginine + N-Acetyl L-glutamate	3 mM + 3 mM	6	70	$P < 0.01$
		51	Control	—	17	15	—
		52	N-Benzoyl-L-aspartate + N-Benzoyl-L-glutamate	3 mM + 3 mM	14	30	—
10	20	53	DL-Ornithine + N-Benzoyl-L-aspartate	3 mM + 3 mM	13	35	—
		54	DL-Ornithine + N-Benzoyl-L-glutamate	3 mM + 3 mM	13	35	—
		55	Control	—	32	20	—
11	40	56	DL-Ornithine + L-Aspartate	3 mM + 3 mM	16	60	$P < 0.05$
		57	DL-Ornithine + N-Acetyl-L-aspartate	3 mM + 3 mM	16	60	$P < 0.05$
		58	Control	—	18	10	—
		59	DL-Citrulline	3 mM	9	55	$P < 0.05$
12	20	60	DL-Citrulline + L-Aspartate	3 mM + 3 mM	8	60	$P < 0.05$
		61	DL-Citrulline + L-Glutamate	3 mM + 3 mM	6	70	$P < 0.01$
		62	DL-Citrulline + N-Acetyl-L-aspartate	3 mM + 3 mM	6	70	$P < 0.01$
		63	DL-Citrulline + N-Acetyl-L-glutamate	3 mM + 3 mM	0	100	$P < 0.01$

TABLE 1—*continued*

Experiment No.	Number of animals/ group	Group	Compounds tested	Doses in m Moles/ kg.	Animals dead after 3 hr	Percent survived %	Protection
		64	Control	—	4	20	—
		65	α-Cetoglutarate	6 mM	2	10	—
13	20	66	α-Cetoglutarate + L-Aspartate	3 mM + 3 mM	1	5	—
		67	α-Cetoglutarate + L-Glutamate	3 mM + 3 mM	3	15	—
		68	α-Cetoglutarate + N-Acetyl-L-aspartate	3 mM + 3 mM	3	15	—
		69	α-Cetoglutarate + N-Acetyl-L-glutamate	3 mM + 3 mM	7	35	—
		70	Control	—	18	10	—
		71	L-Lysine	3 mM	12	40	—
14	20	72	L-Lysine + L-Arginine	3 mM + 3 mM	2	90	$P < 0.01$
		73	L-Lysine + N-Acetyl-L-aspartate	3 mM + 3 mM	1	95	$P < 0.01$
		74	L-Lysine + N-Acetyl-L-glutamate	3 mM + 3 mM	3	85	$P < 0.01$
		75	Control	—	15	25	—
		76	N-Carbamyl-L-asparagine	3 mM	15	25	—
		77	N-Carbamyl-L-asparagine + L-Aspartate	3 mM + 3 mM	18	10	—
15	20	78	N-Carbamyl-L-asparagine + L-Glutamate	3 mM + 3 mM	18	10	—
		79	N-Carbamyl-L-asparagine + N-Acetyl-L-aspartate	3 mM + 3 mM	12	40	—
		80	N-Carbamyl-L-asparagine + N-Acetyl-L-glutamate	3 mM	14	30	—
		81	Control	—	16	20	—
		82	Urea	3 mM	16	20	—
16	20	83	Urea	6 mM	13	35	—
		84	L-Glutamine	3 mM	12	40	—
		85	L-Glutamine	6 mM	10	50	—
		86	L-Arginine	3 mM	3	85	$P < 0.01$
		87	Control	—	16	33·3	—
		88	N-Acetyl-L-alanine	6 mM	9	62·5	—
17	24	89	α-Methyl-DL-glutamate	6 mM	4	83·3	$P < 0.05$
		90	N-Acetyl-L-glutamic-ethyl-ester	6 mM	18	25	—
		91	Control	—	18	25	—
18	24	92	Carbamyl-L-asparagine	5 mM	17	29·2	—
		93	Na-Pyrrolidone-carboxylic	6 mM	16	33·3	—

Our findings reveal an evidently protective action for the N-acetyl derivatives of L-glutamic and L-aspartic acids and for N-carbamyl-L-glutamic and N-carbamyl-DL-aspartic acids. The minimum protective doses were 4–5 mM/kg, 6 mM/kg, 4–5 mM/kg and 6 mM/kg respectively as compared with 3 mM/kg for arginine. The protection afforded by these compounds was associated with a lower blood ammonia increase (Fig. 2). The benzoyl-derivatives of the same amino acids were without effect.

A mixture of sub-protective doses of two of the N-acetyl or N-carbamyl derivatives was protective in the case where the total amount administered was about 5–6 mM/kg. Similarly the association of a subprotective dose of arginine with a subprotective dose of one of these four aminoacid derivatives was also active.

Conversely, the association of one of these derivatives in subprotective doses with glutamate or aspartate yielded no protection although the total amount would have been effective for one of the N-acyl-derivatives alone. The same is true for the association of arginine with L-aspartate or L-glutamate. Other results (Exp. No. 12) confirmed the protective action of citrulline found by Tomitaro Kita *et al.* and of the mixture of ornithine + aspartate found by Salvatore and Bocchini.[8]

The data listed in experiment No. 12 suggest a more effective protection for the association of citrulline + N-acetyl-L-glutamate than for citrulline + L-aspartate.

L-glutamine, L-asparagine, L-carbamyl-asparagine alone, are in association with the active N-acyl derivatives, were ineffective. Similarly, N-acetyl-glycine, N-acetyl-alanine (DL or L), L-glutamic acid, L-aspartic acid, fumaric acid, α-cetoglutaric acid, pyrrolidone carboxylic acid, diacetyl, ethyl ester of N-acetyl glutamic acid, urea and uridine (50 mg/kg) were inactive. Lysine in doses of 3 mM/kg did not interfere with the protection induced by the active acetyl or carbamyl derivatives or by arginine.

An interesting observation is the protection given by α-metil-DL-glutamate in doses of 5–6 mM/kg. This latter compound is now being investigated more thoroughly.

DISCUSSION

The data obtained in this work demonstrate the protective action of N-acetyl-L-glutamate, N-acetyl-L-aspartate, N-carbamyl-L-glutamate and N-carbamyl-DL-aspartate against lethal convulsions induced by ammonia intoxication.

In this respect our data previously presented in a summary[24] and largely exposed in this paper are not in agreement with those obtained by Manning, Thorning and Falleta[25] who, when using monosodium acetyl-glutamate, did not find protection with 2 mM/kg and 10 mM/kg. According to our findings the negative result of these authors with 2 mM might be explained by a subliminal dose for protection. On the other hand the dose of 10 mM probable becomes toxic. In addition the use of NH_4Cl instead of $CH_3\ COONH_4$, may introduce further differences.

The inactivity of N-benzoyl glutamate may be explained by a steric effect of the benzoyl radical which probably interferes with the formation of the intermediate postulated by Jones and Spector[31] in carbamyl-phosphate synthesis.

The protection by N-acetyl-aspartic is difficult to explain. Tallan[26, 27] demonstrated the presence of this metabolite in great quantities in the brain. However, considering the inability of this compound to cross the blood brain barrier, we must admit an extracerebral mechanism for the protective action probably hepatic in nature through stimulation of the urea cycle. There are no biochemical studies concerning the possible role of this metabolite in the urea cycle as there are for acetyl-glutamate or carbamyl-glutamate.

The action of carbamyl-aspartate may be partially ascribed to a stimulation of the urea cycle by an increased rate of arginine succinate synthesis from ornithine and carbamyl-aspartate (as suggested by the findings *in vitro* of Della Pietra[14]). On the other hand the incorporation of ammonia in pyrimidines via carbamyl-aspartate and orotic acid is well known from biochemical studies and this pathway may play an important role in ammonia detoxication *in vivo*. There are no experimental data *in vivo* concerning the intervention of this pathway in correcting the metabolic disturbances in ammonia intoxication or in hyperammoniemia of hepatic diseases. Carbamyl-aspartate, representing a metabolic link between urea and pyrimidine synthesis, may control the relative efficiency of these two routes in ammonia detoxication. The L form being unavailable we did no further study on this compound. However a greater activity must be expected for the L-form than for the DL-form which in our experiment showed an important protective action.

Neither glutamine, nor asparagine have shown a protection against ammonia intoxication under our experimental conditions. These findings suggest different mechanisms for ammonia convulsions and for Pentamethylentetrazol or Megymide induced convulsions, which can be prevented by intraperitoneally or orally administered L-asparagine as demonstrated by Hawkins and Sarett[28] although according to Richter[29] and Benitez[30] the action of pentamethylentetrazol is accompanied by a rise of brain ammonia.

Our experiments revealed a greater activity *in vivo* for arginine and citrulline than for the N-acyl-derivatives of glutamic and aspartic acids. These findings suggest differences in the importance of the urea cycle for ammonia detoxication between these two groups of compounds. Other biochemical differences underlying the action of these compounds are very probable and are of interest for the understanding of the mechanism of ammonia metabolism.

A comparative study of the metabolic changes induced by these different protectors are now in progress in our laboratory.

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