Structural Requirements of Nitrogenous Substances Which Have Protective Effects Against Acute Hydrazine Toxicity in Mice.* (30271)

EUGENE ROBERTS, DAISY G. SIMONSEN, AND ETHEL ROBERTS
Department of Biochemistry, City of Hope Medical Center, Duarte, Calif.

A detailed study is being made in this laboratory of modes of protection against hydrazine toxicity as well as of the mechanism of toxic action of hydrazine in mice. As a result of an analysis of the data in the literature it was first suggested by us that a number of the effects of hydrazine could be rationalized if it were assumed that the primary action of this substance were on the structure of some essential membrane component or components in the affected tissues (liver and/or brain). Various considerations pointed to the possibility that hydrazine might displace the guanidino groups of arginyl residues in keratin-like proteins of nerve or liver cell membranes from interaction with adjacently-located negatively-charged groups. On this basis, L-arginine was tested as a possible protective agent against acute hydrazine toxicity and was found to be effective(1). Subsequent studies on a semi-empirical basis led to the final development of a protective mixture which contained L-arginine, L-glutamate, α-ketoglutarate, and oxalacetate(2,3). The present study is an approach to the elucidation of the mechanism of hydrazine toxicity at the cellular level and the mechanism of the action of the protective substances.

One of the possibilities that needed to be tested was whether the protective action of arginine could be attributable to an effect on ammonia toxicity secondary to the liver pathology caused by hydrazine or whether arginine was, perhaps, acting by blocking some membrane and/or enzyme receptor sites and, therefore, competing with the hydrazine for attachment. Another possibility was that a portion of the arginine which is drained off for the biosynthesis of creatine might be critical and that sparing endogenous arginine by administering creatine precursors might also have a protective action. It was possible to obtain some *preliminary* answers about these questions and several others in the present work.

Materials and methods. Male Swiss mice of an inbred Swiss stock, weighing approximately 25 g, were used in all of the present experiments. Previously no sex differences were observed in acute hydrazine toxicity. Every experiment to be reported was performed with at least 10 animals in each experimental group. The animals were fasted for approximately 18 hours prior to a single intraperitoneal injection of about 0.1 ml of a freshly prepared hydrazine solution (pH 7.0). The injection of hydrazine was preceded by 30 minutes by the injection of physiological saline (0.1-0.4 ml) or by a similar volume of neutral test solution. In each experiment to be reported all of the animals received the same volume of saline or of test solution. After initiation of the experiment the animals were observed continuously for a 3-hour period and at 0.5-hour intervals for an additional 3 to 4 hours. The final observations were made 24 hours after injection of hydrazine. The various substances tested were of the highest degree of purity commercially available. The amino acids were all of the L-configuration. The differences between experimental and control groups were tested for significance with the aid of a 2×2 contingency table and those showing P values of 0.05 or less indicated(4).†

Results. Effects on hydrazine toxicity of compounds related to urea and pyrimidine metabolism (Table I). It is seen from the data in Table 1 that both arginine and ornithine at a level of 4 mM/kg protected against hydrazine toxicity. On the other hand, citrulline did not afford any protection in the first experiment or in several other similar ones. Arginine, ornithine, and citrulline are all intermediates in the well-known Krebs-

* This investigation was supported in part by the Air Force Office of Scientific Research Grant AF 4(609)-2614 and by the Hector Escobosa fund.

† We are indebted to Mr. Walter Knowles for these calculations.

TABLE I. Effects of Hydrazine Toxicity of Compounds Related to Urea and Pyrimidine Metabolism.

Exp No.	No. of mice per group	Group	Compounds	Cumulative deaths, % 30 min	60 min	24 hr
1*	15	1	Saline	46.7	73.5	73.5
	"	2	Arginine (2 mmoles/kg)	26.6	53.3	60
	"	3	" (4 ")	0 ‡	40	40
	"	4	Ornithine (2 ")	33.3	60	60
	"	5	" (4 ")	0 ‡	6.7‡	26.7†
	"	6	Citrulline (2 ")	33.3	60	60
	"	7	" (4 ")	40	66.7	66.7
	"	8	Urea (2 ")	66.7	73.5	73.5
	"	9	" (4 ")	40	66.7	66.7
2*	12	10	Saline	41.7	58.3	83.3
	13	11	Arginine (4 mmoles/kg)	0 †	15.4†	23.1‡
	"	12	Carbamylaspartic acid (1 mmole/kg)	46.2	69.3	69.3
	"	13	" " (2 mmoles/kg)	61.6	61.6	69.3
	"	14	" " (3 ")	46.2	61.6	61.6
	"	15	" " (4 ")	23.1	61.6	61.6
	"	16	Arginine (4 mmoles/kg) + carbamylaspartic acid (4 mmoles/kg)	7.7	30.8	46.2
	"	17	Uridine (4 mmoles/kg)	69.3	92.4	92.4
	"	18	Arginine (4 mmoles/kg) + uridine (4 mmoles/kg)	23.1	53.9	53.9

* Hydrazine (3 mmoles/kg) † $P < .05$ ‡ $P < .01$

Henseleit urea synthetic mechanism in liver. In the work of Greenstein *et al*(5) all 3 of the above compounds were found to protect against ammonia toxicity. The fact that only 2 of the substances, arginine and ornithine, protected against hydrazine toxicity suggested that the mechanism of hydrazine toxicity could not be exerted solely through the inhibition of the urea synthetic pathway. Since urea itself might be important in some, as yet unknown, biological mechanisms, urea itself was tested. At the levels employed urea had no effect on the toxicity of hydrazine.

An important aspect of ammonia metabolism is utilization of ammonia in the biosynthesis of pyrimidine precursors. Ammonia is used for the synthesis of the carbamyl group of carbamyl phosphate. An early intermediate in pyrimidine biosynthesis is carbamylaspartic acid. In Experiment 2 it is seen that carbamylaspartic acid at the levels employed had little or no effect on the toxicity of hydrazine. It is, therefore, unlikely that the inhibition of the synthesis of carbamyl phosphate is a primary mechanism of toxicity of hydrazine. Also, at the level employed uridine appeared to enhance the toxicity of hydrazine.

Effects on hydrazine toxicity of compounds related to creatine metabolism (Table II). Arginine furnishes the guanidino group for synthesis of creatine. An experiment was performed (Exp. 3) in which the effects on hydrazine toxicity were tested of compounds which are precursors of creatine. Administration of glycine or methionine did not decrease hydrazine toxicity. Guanidinoacetic acid, which might be expected to spare endogenous arginine, also showed no protective action. Administration of combinations of arginine, glycine, and methionine, or methionine and guanidinoacetic acid appeared to have no ameliorative influence on the lethal hydrazine effect. Administration of creatine, itself, also did not affect hydrazine toxicity significantly. It may, therefore, be tentatively concluded that the area of creatine metabolism does not represent a labile point with regard to the acute toxic action of hydrazine.

Comparative protective effects of several substances on hydrazine and ammonia toxicity (Table III). In the course of our large screening studies it was found that some diamino compounds had protective action against hydrazine in mice. Experiment 4 shows a rather detailed evaluation of the

structural requirements for this protection. As found in many of our previous studies, Exp. 4 shows that arginine and ornithine can exert a protective effect. However, the most effective protective agent in this series of experiments was α,γ-diaminobutyric acid. Likewise, α,β-diaminopropionic acid appeared to be as effective as arginine and ornithine, while lysine and α,γ-diaminoglutaric acid showed no protection. This hierarchy of effectiveness was generally repeatable in many experiments. A lack of effectiveness of lysine is further shown in Exp. 7, Table IV. When the same substances employed in Exp. 4 were tested for their protective action against ammonia toxicity (Exp. 5), it was found, as expected, that arginine and ornithine were protective, but that the other substances tested showed no significant protection. The results of Exp. 4 and 5 show clearly that the protective action of several substances against hydrazine toxicity can be separated from an effect on ammonia toxicity. The particularly noteworthy difference is that α,γ-diaminobutyric and α,β-diaminopropionic acids are effective against hydrazine but not against ammonia. The latter observations suggest that there may be some physical action of the diamino compounds in which arginine and ornithine also might be participants. α,γ-Di-

TABLE II. Effects on Hydrazine Toxicity of Compounds Related to Creatine Metabolism.

Exp No.	No. of mice per group	Group	Compounds	Cumulative deaths, % 30 min	60 min	24 hr
3*	13	19	Saline	61.6	92.4	100
	14	20	Arginine	0 ‡	7.1‡	21.4‡
	14	21	Glycine	78.5	93	93
	14	22	Methionine	78.5	93	100
	12	23	Guanidinoacetic acid	58.3	83.3	100
	14	24	Arginine + glycine + methionine	50	78.5	100
	14	25	Methionine + guanidinoacetic acid	93	100	100
	14	26	Creatine	42.8	78.5	100
	14	27	Ornithine	7.2‡	21.4‡	35.7‡
	14	28	Ornithine + glycine + methionine	64.3	71.5	93

* Hydrazine (3 mmoles/kg); all other substances with exception of methionine, 3 mmoles/kg. Methionine, 2 mmoles/kg.
‡ $P < .01$.

TABLE III. Comparative Protective Effects of Several Substances on Hydrazine and Ammonia Toxicity.

Exp No.	No. of mice per group	Group	Compounds	Cumulative deaths, % 30 min	60 min	24 hr
4* (hydrazine)	15	29	Saline	33.3	53.3	73.5
	"	30	Arginine	0 ‡	26.6	40.0
	"	31	Ornithine	0 ‡	13.3‡	53.3
	"	32	α, γ-Diaminobutyric acid	0 ‡	6.7§	6.7§
	"	33	α, β-Diaminopropionic acid	0 ‡	13.3‡	40.0
	"	34	Lysine	13.3	66.7	73.5
	"	35	α, γ-Diaminoglutaric acid	33.3	60.0	80.0
				15 min		
5† (ammonium acetate)	12	36	Saline	100		
	"	37	Arginine	16.7§		
	"	38	Ornithine	41.6§		
	"	39	α, γ-Diaminobutyric acid	92		
	"	40	α, β-Diaminopropionic acid	100		
	"	41	Lysine	92		
	"	42	α, γ-Diaminoglutaric acid	100		

* Hydrazine (2.8 mmoles/kg); other substances (4 mmoles/kg).
† Ammonium acetate (10.8 mmoles/kg); other substances (4 mmoles/kg).
‡ $P < .05$.
§ $P < .01$.

TABLE IV. Comparative Protective Effects on Hydrazine Toxicity of Arginine, Several Diamino-Monocarboxylic Acids, and Mono- and Diamines.

Exp No.	No. of mice per group	Group	Compounds	Cumulative deaths, % 30 min	60 min	24 hr
6*	11	43	Saline	81.8	81.8	100
	11	44	Arginine	0 ‡	27.3†	36.4‡
	12	45	Ornithine	0 ‡	16.7‡	41.7‡
	11	46	α, γ-Diaminobutyric acid	0 ‡	9.1‡	18.2‡
	11	47	α, β-Diaminopropionic acid	0 ‡	9.1‡	45.5‡
	11	48	1,2-Diaminoethane	18.2‡	18.2‡	45.5‡
	12	49	1,2-Diaminopropane	25 ‡	50	83.3
	11	50	1,3-Diaminopropane	27.3†	36.4†	54.6†
	11	51	1,4-Diaminobutane (Putrescine)	18.2‡	27.3†	36.4‡
7*	10	52	Saline	60	70	70
	12	53	Arginine (4 mmoles/kg)	0‡	8.3‡	16.7†
	"	54	Arginine (8 ")	0‡	8.3‡	25 †
	"	55	Lysine (4 ")	25	50	66.7
	"	56	Lysine (8 ")	25	83.3	83.3
8*	10	57	Saline	70	80	100
	"	58	α, γ-Diaminobutyric acid	10‡	20†	20‡
	"	59	1,2-Diaminoethane	0‡	0‡	60†
	"	60	1-Aminoethane	40	80	90
	"	61	1-Aminobutane	100	100	100

* Hydrazine (3 mmoles/kg); other substances (4 mmoles/kg) unless otherwise indicated.
† $P < .05$.
‡ $P < .01$.

aminobutyric acid is only slowly metabolized when administered to intact animals and appears to be concentrated in the liver(6).

Comparative protective effects on hydrazine toxicity of arginine, several diamino-monocarboxylic acids, and mono- and diamines (Table IV). The study on the structural requirements for protective action was continued (Exp. 6-8). In addition to the previously observed actions, it was found that 1,2-diaminoethane and 1,3-diaminopropane were protective. Some protective action was even shown by putrescine (1,4-diaminobutane).

As in other experiments, in Exp. 7 it was shown that lysine failed to protect against hydrazine. In Exp. 8, as in Exp. 6, 1,2-diaminoethane was shown to have some protective action, whereas 1-aminoethane and 1-aminobutane did not. Taken together, the above experiments suggest that for an aliphatic amine to have a protective action it must have at least 2 amino groups, and that for a diamino-monocarboxylic acid to have protective action the amino groups must be separated at the most by 4-carbon atoms.

Failure of several ω-amino, ω-guanidino, and ω-ureido compounds to protect against hydrazine toxicity (Table V). The results in Exp. 9 show that glycine, β-alanine and γ-aminobutyric acid do not exert protective action in the test system. Many studies have shown that γ-aminobutyric acid does not penetrate the blood-brain barrier in normal adult mammals. It may be concluded from Exp. 9 and the preceding data that the α-amino group of α,γ-diaminobutyric acid is essential for its protective action in intact animals. The results in Exp. 10 show clearly that the guanidino-monocarboxylic acids and ureido acids employed afforded no protection against hydrazine toxicity.

Discussion. Although studies, such as reported here, are subject to the many vicissitudes by which all pharmacological experiments in intact animals are limited, they show clearly that under our conditions of testing there are some optimal structural requirements for protection against hydrazine toxicity by amino acids. It is interesting that a relatively poorly metabolized amino acid, α,γ-diaminobutyric acid, appears to be the single most effective substance in counteracting hydrazine toxicity. The latter substance produces toxicity in its own right, but at later time intervals than employed for the

TABLE V. Failure of Several ω-Amino, ω-Guanidino, and ω-Ureido Compounds to Protect Against Hydrazine Toxicity.

Exp No.	No. of mice per group	Group	Compounds	Cumulative deaths, % 30 min	60 min	24 hr
9*	15	62	Saline	13.3	46.6	53.3
	"	63	Glycine	46.6	53.3	60
	"	64	β-Alanine	60	66.7	80
	"	65	γ-Aminobutyric acid	26.7	40	60
10†	10	66	Saline	80	90	100
	"	67	Guanidinoacetic acid	100	100	100
	"	68	Guanidinopropionic acid	80	90	100
	"	69	Guanidinobutyric acid	50	90	100
	"	70	Ureidobutyric acid	90	100	100
	"	71	Citrulline	90	100	100

* Hydrazine (2.8 mmoles/kg); other substances (4 mmoles/kg).
† Hydrazine (3.0 mmoles/kg); other substances (4 mmoles/kg).

present observations. It is known to lead to the destruction of the Purkinje cells in the cerebellum of several species. Indeed, within 48 hours of receiving the dose of α,γ-diaminobutyric acid employed in the present study some of the animals were found to show symptoms of cerebellar ataxia. The results suggest that there may be a common neuronal site to which various basic substances may attach. They may have different affinities for this site, as a result of which some may produce toxic symptoms while others may protect the site against toxicity and then dissociate from it without permanent damage. Further experiments are contemplated in which tritium labeled α,γ-diaminobutyric acid will be administered before and after hydrazine and its localization followed with autoradiography in order to test this concept. Numerous other related studies are in progress.

Since it is well known that the diamines are excellent chelating agents, it is possible that a chelation phenomenon at membrane sites may be involved in the protective action observed in the present studies.

Summary. A continuation of previous studies on the protective effects of various substances against acute hydrazine toxicity in mice has revealed several interesting structural features which are consistent with the provisional interpretation that the protective actions of L-arginine and L-ornithine can be attributable, at most, only in part to their roles in ammonia detoxication *via* urea biosynthesis in liver. That the protection against lethality which they afford also must have another basis is suggested by the finding that citrulline exerted no protective action, while α,γ-diaminobutyric and α,β-diaminopropionic acids and several diamines did. Likewise, α,γ-diaminobutyric, which was more effective against hydrazine than arginine or ornithine, gave no protection against ammonia poisoning under the same condition under which the latter substances showed their typical effects. The experiments indicate that for an aliphatic amine to have a protective action it must have at least 2 amino groups, and that for a diamino-monocarboxylic acid to be protective the amino groups must be separated at the most by 4-carbon atoms.

1. Roberts, E., Simonsen, D. G., Roberts, E., Biochem. Pharm., 1963, v12, 1445.
2. ———, ibid., 1964, v13, 1451.
3. ———, ibid., 1965, v14, 351.
4. Finney, D. J., Tables for Testing Significance in a 2x2 Contingency Table, E. S. Pearson, ed., Cambridge Press, Cambridge, England, 1963.
5. Greenstein, J. P., Winitz, M., Gullino, P., Birnbaum, S. M., Otey, M. C., Arch. Biochem. Biophys., 1956, v64, 342.
6. Mushahwar, I. K., Koeppe, R. E., J. Biol. Chem., 1963, v238, 2460.

Received March 22, 1965. P.S.E.B.M., 1965, v119.