Biochemical Pharmacology, Vol. 20, pp. 2231–2238. Pergamon Press, 1971. Printed in Great Britain

EFFECT OF HYDRAZINE ON UREA CYCLE ENZYMES *IN VITRO* AND *IN VIVO*

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(*Received* 5 *November* 1970; *accepted* 22 *January* 1971)

Abstract—The effect of hydrazine on urea cycle enzymes was studied in adult male rats. Addition of different concentrations of hydrazine to homogenates of rat liver produced a gradual inhibition of citrulline and urea synthesis. A daily injection of subconvulsive doses (32 mg/kg) of this toxic substance for 4 days provoked an increase of citrulline and urea contents in different tissues. The activities of urea cycle enzymes were not affected by such a treatment except for argininosuccinase where an increase was noted. The activity of ornithine-ketoacid transaminase was greatly inhibited. The results suggest that the inhibition of ornithine-ketoacid transaminase provokes an accumulation of ornithine. The presence of a high concentration of this amino acid together with an increased ammonia production stimulates urea synthesis. Under these physiological conditions, the condensation reaction of citrulline with aspartic acid may become rate-limiting in the operation of the urea cycle with the resultant accumulation of citrulline.

HYDRAZINE has long been under investigation to ascertain its biological toxicity. This substance has been shown to be a convulsant agent[1–3] and its toxicity, like that of various hydrazine compounds, can be prevented by pyridoxin[4,5] or by arginine, α-ketoglutarate and oxaloacetate.[3,6] Several studies have indicated that hydrazine produces liver and muscle glycogenolysis,[7,8] inhibition of glucogenesis,[9,10] hypoglycemia,[9,11] elevation of lactate, pyruvate, citrate, malate and oxaloacetate in blood,[8,10,11] rise in free fatty acids and appearance of a fatty liver.[7,12] Other investigations have shown that hydrazine injections produce an amino acid imbalance in experimental animals.[13–15] According to Banks,[16] this general mobilization of amino acids is made by skeletal muscle "protein reserves", since protein biosynthesis in liver is increased by such treatment.[16–18]

In the present study, we have investigated the effect of different concentrations of hydrazine on the synthesis *in vitro* of citrulline from NH_4^+, HCO_3^- and ATP and of urea from citrulline and aspartic acid using liver homogenates. In addition, the levels of citrulline, arginine and urea were determined in different tissues of rats injected with a daily subconvulsive dose of hydrazine. Activities of urea cycle enzymes and ornithine-ketoacid transaminase in rat livers were also estimated.

MATERIAL AND METHODS

Effects of various concentrations of hydrazine on the synthesis of citrulline and urea were carried out *in vitro*. The preparation of rat liver homogenate, the composition of the incubation media and the incubation samples in Warburg vessels were prepared

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according to the methods of Charbonneau and Berlinguet.[19,20] Hydrazine sulfate (Fisher Scientific Company) solution prepared just before use was added in calculated amounts after neutralizing with KOH. The reaction was stopped by addition of 5 ml of 0·5 M perchloric acid. Aliquots of the protein-free filtrate were analyzed for citrulline by the method of Archibald[21] as modified by Ratner.[22] Standard colorations obtained with citrulline and urea were not affected by hydrazine concentrations. The results are expressed in micromoles of product per sample per hour.

Male white rats (200–300 g), Wistar strain, used for the experiments *in vivo*, received a daily subconvulsive dose of hydrazine (1 m-mole/kg/day) by intraperitoneal injection. Hydrazine sulfate was prepared and neutralized with an NaOH solution just before use. After 4 days of treatment, rats were killed by decapitation. Blood was collected and other tissues (brain, liver and kidneys) were quickly removed and homogenized in cold double-distilled water (10%, w/v). The homogenates were centrifuged at 105,000 *g* for 30 min. Proteins were precipitated from aliquots of blood and supernatants by the addition of 0·5 ml of concentrated perchloric acid (70%). The method of Archibald[21] as modified by Ratner[22] was used for the estimation of urea; the method of Sakaguchi,[23] for arginine and that of Hunninghake and Grisolia,[24] for citrulline were employed. The results are expressed as micrograms of citrulline, arginine and urea per milliliter of blood or per gram of fresh tissue.

The method of Brown and Cohen[25] was used for the preparation of the enzymatic source used in the study of the urea cycle enzymes. The enzymatic activities were determined according to the methods described by Charbonneau *et al.*[26] The method of Raïhä and Kekomäki[27] was used for the determination of the specific activity of ornithine-ketoacid transaminase (EC 2.6.1.13, L-ornithine: 2-oxoacid amino-transferase). The assay medium consisted of 75 μmoles L-ornithine, 75 μmoles α-ketoglutarate and 0·4 ml of liver homogenate (2%, w/v) in a total volume of 2·0 ml. The specific activity of ornithine-ketoacid transaminase was expressed in units per gram of soluble protein (1 unit catalyzes the formation of 1 μmole of Δ^1-pyrroline-5-carboxylic acid/min).

RESULTS

The effects of hydrazine on the synthesis of citrulline and urea in the presence of liver homogenates are given in Tables 1 and 2. An increase in the concentration of hydrazine in the incubation medium resulted in a decrease in citrulline and urea formation and, at high concentrations (80–90 μmoles per assay), total inhibition of urea formation was noted.

A daily injection of a subconvulsive dose of hydrazine resulted in a drastic fall of rat growth as shown in Fig. 1. The animals died 6 and 7 days after initiation of treatment with hydrazine. A parallel fall in body weight was also noted in fasting rats.

A treatment period of 4 days was selected for the estimation of citrulline, arginine and urea in different tissues and for the determination of the activities of liver enzymes. Table 3 shows that citrulline levels were increased by 3-fold in liver of rats treated with hydrazine. Lesser increases were also found in blood and kidneys. The concentration of arginine was not affected, while urea levels were significantly enhanced: about 3-fold in blood, 6-fold in liver, 3-fold in brain and 2-fold in kidney.

Table 4 presents the effect of daily injections of hydrazine on the activities of all the enzymes of the urea cycle. Four enzymes, namely carbamoyl-phosphate synthetase

TABLE 1. FORMATION OF CITRULLINE FROM NH_4^+, HCO_3^- AND ATP BY LIVER HOMOGENATES IN THE PRESENCE OF HYDRAZINE*

Hydrazine added (μmoles)	Citrulline synthesized (μmoles/sample/hr)	Inhibition (%)
0	17·5	—
10	14·5	17
20	9·5	46
30	8·2	53
40	7·3	58
50	7·0	60
60	7·0	60
70	5·3	70
80	4·8	72
90	4·8	72

* Incubation mixture contains: 0·5 ml homogenate (1:2) in a total volume of 4 ml; $NaHCO_3$, 28 μmoles; NH_4Cl, 24 μmoles; L-ornithine, 35 μmoles; L-glutamic acid, 28 μmoles; ATP, 7 μmoles; hydrazine at concentrations indicated; potassium phosphate buffer (pH 7·3) was added to give an isotonic concentration. Final volume, 4·0 ml; incubation time, 1 hr at 37°. Coloration of product was not affected by the addition of such concentrations of hydrazine.

TABLE 2. FORMATION OF UREA FROM CITRULLINE AND ASPARTIC ACID BY LIVER HOMOGENATES IN THE PRESENCE OF HYDRAZINE*

Hydrazine added (μmoles)	Urea synthesized† (μmoles/sample/hr)	Inhibition (%)
0	19	—
10	17·8	6
20	16·6	13
30	14·0	26
40	11·5	40
50	10·0	48
60	8·0	58
70	4·6	76
80	0·0	100
90	0·0	100

* Incubation mixture contains: 0·5 ml homogenate (1:2) in a total volume of 4 ml; ATP, 5 μmoles; $MgSO_4$, 10 μmoles; L-citrulline, 20 μmoles; fumarate, 20 μmoles; urease, 5 mg; L-aspartic acid, 20 μmoles; hydrazine at concentrations indicated. Final volume, 4·0 ml; incubation time, 1 hr at 37°. Coloration of product was not affected by the addition of such concentrations of hydrazine.

† Each micromole of citrulline disappearing from the system is converted to a corresponding amount of urea.[28]

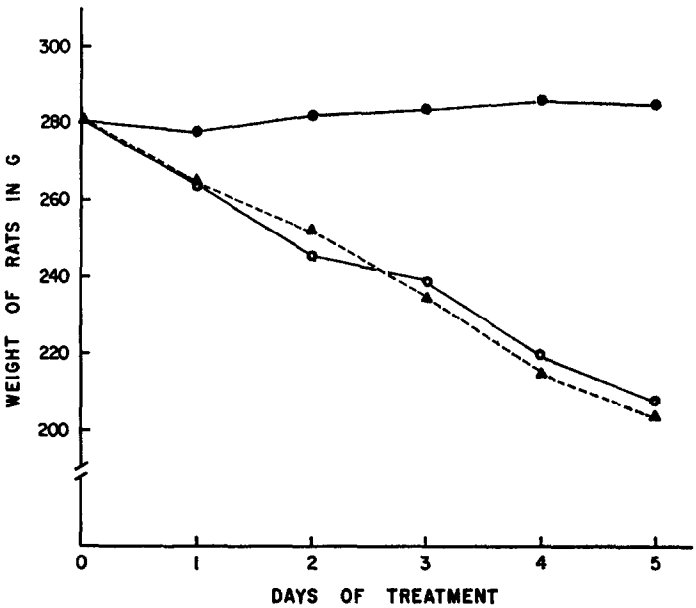

FIG. 1. Effects of a daily injection of hydrazine on the weights of rats. Injection i.p., 1 m-mole/kg. Control rats, O—O; Rats treated with hydrazine, ▲ - - - ▲; fasting rats, O—O.

TABLE 3. CITRULLINE, ARGININE AND UREA LEVELS IN DIFFERENT TISSUES OF RATS TREATED WITH HYDRAZINE

Substances determined	Hydrazine*	Blood	Liver	Brain	Kidneys
Citrulline	Minus	37 ± 2·7†	93 ± 4·0	47 ± 2·4	93 ± 4·0
	Plus	70 ± 7·0‡	282 ± 34·0‡	57 ± 3·6	149 ± 14·0‡
Arginine	Minus	111 ± 9·0	167 ± 5·3	163 ± 10·0	752 ± 30·0
	Plus	103 ± 7·1	161 ± 6·6	161 ± 6·7	739 ± 41·0
Urea	Minus	368 ± 12·0	374 ± 13·0	278 ± 9·0	1586 ± 63·0
	Plus	1209 ± 150·0‡	2463 ± 284·0‡	987 ± 129·0‡	3297 ± 152·0‡

* Injection i.p., 1 m-mole hydrazine/kg/day; treatment, 4 days.

† Each value is the mean of 12 animals; four determinations are made per rat. Results are expressed in micrograms of citrulline, arginine or urea per ml of blood or per g of fresh tissue.

‡ $P < 0{\cdot}01$.

and transferase, argininosuccinate synthetase and arginase, were not affected by treatment of rats with hydrazine. The activity of argininosuccinase was greatly increased. Starvation during the same period increased significantly the activities of carbamoyl-phosphate synthetase and transferase and of argininosuccinase. Nevertheless, the increase noted in the activity of this last enzyme was less pronounced (about 0·5-fold) as compared to that of rats treated with hydrazine (about 3-fold).

The influence of the length of treatment given to rats on the development of argininosuccinase induction is given in Table 5. Our results show that, after the first day

TABLE 4. EFFECT OF DAILY INJECTIONS OF HYDRAZINE ON THE ACTIVITIES* OR UREA CYCLE ENZYMES IN RAT LIVERS

Enzymes	Treatment of rats		
	None	Hydrazine†	Starvation
Final body wt. (g)	238 ± 5·0 (227)‡	188 ± 4·1 (230)‡	193 ± 2·5 (240)‡
Liver wt. (g)	9·2 ± 0·38	7·2 ± 0·40	5·5 ± 0·10
Protein§ per g fresh wt. (mg)	116 ± 3·0	106 ± 8·0	120 ± 3·5
Carbamoyl-phosphate synthetase	4·2 ± 0·20	5·6 ± 0·27	8·2 ± 0·27‖
Carbamoyl-phosphate transferase	114·0 ± 4·5	109·0 ± 4·2	175·0 ± 3·9‖
Argininosuccinate synthetase	1·6 ± 0·07	1·7 ± 0·16	1·6 ± 0·05
Argininosuccinase	4·1 ± 0·12	15·1 ± 0·46‖	6·0 ± 0·12‖
Arginase	163·0 ± 7·8	140·0 ± 5·4	152·0 ± 5·4

* A unit of enzyme is defined as the amount catalyzing the formation of 1 μmole of product/hr under the conditions of the assay. The specific activity is defined as units per milligram of protein. Each value is the mean of 18 animals except for the group under starvation where only eight rats were used.

† Injection i.p., 1 m-mole hydrazine/kg/day; treatment, 4 days.

‡ Initial body weight.

§ Soluble protein content in the supernatant of liver homogenate.[25]

‖ $P < 0·01$.

TABLE 5. RELATION BETWEEN INDUCTION OF ARGININOSUCCINASE AND LENGTH OF TREATMENT

Days of treatment*	Body wt. (g)		Liver wt. (g)	Protein† per g fresh wt. (mg)	Sp.act. of argininosuccinase‡
	Initial	Final			
0	221 ± 3·3	223 ± 4·3	9·2 ± 0·9	88 ± 1·2	4·0 ± 0·19
1	234 ± 3·5	214 ± 4·1	10·0 ± 0·35	98 ± 4·0	8·2 ± 0·42
2	209 ± 1·8	174 ± 4·9	7·6 ± 0·17	92 ± 2·6	19·2 ± 0·50
3	196 ± 4·3	144 ± 3·2	6·9 ± 0·14	100 ± 5·0	18·8 ± 0·90
4	210 ± 8·8	147 ± 3·6	7·2 ± 0·18	86 ± 2·0	18·5 ± 0·60
5	220 ± 7·4	149 ± 3·7	7·4 ± 0·60	105 ± 8·1	17·7 ± 1·5

* Injection i.p., 1 m-mole hydrazine/kg/day; treatment as described in the table.

† Soluble protein content in the supernatant of liver homogenate.[25]

‡ For the definition of specific activity, see Table 4. Each value is the mean of six animals.

of treatment, the specific activity of this enzyme was increased significantly (1-fold). The maximum induction (4-fold) was observed after only 2 days (two injections). This was followed by a plateau for the subsequent days of treatment.

Table 6 shows that the induction of argininosuccinase by hydrazine was inhibited (from 14·0 to 6·8) by one injection of actinomycin D. The specific activity of argininosuccinase in livers of normal rats was not affected by actinomycin D.

Table 7 shows that daily injections of hydrazine greatly decreased the specific activity of ornithine-ketoacid transaminase.

TABLE 6. EFFECT OF INJECTION OF ACTINOMYCIN-D ON THE INDUCTION OF ARGININOSUCCINASE BY HYDRAZINE IN RAT LIVER

Treatment of rats*	Injection of actinomycin-D	Body wt. (g)		Liver wt. (g)	Protein† per g fresh wt. (mg)	Sp.act. of argininosuccinase
		Initial	Final			
Control	Minus	257 ± 3·5	259 ± 4·2	11·2 ± 0·46	84 ± 2·5	4·0 ± 0·15
Control‡	Plus	257 ± 5·4	237 ± 7·2	9·0 ± 0·65	101 ± 1·5	4·5 ± 0·18
Hydrazine§	Minus	248 ± 7·0	221 ± 10·0	10·4 ± 0·60	104 ± 2·7	14·0 ± 0·75
Hydrazine‖	Plus	238 ± 6·2	210 ± 8·7	9·3 ± 0·52	95 ± 2·0	6·8 ± 0·31

* Twelve rats per group.
† Soluble protein content in the supernatant of liver homogenate.[25]
‡ A single injection of actinomycin-D (200 μg/kg) was given and animals were killed 24 hr later.
§ Two successive days of hydrazine injection (1 m-mole/kg).
‖ Same as §, except that a single injection of actinomycin-D was given after the first day of treatment.

TABLE 7. EFFECT OF DAILY INJECTIONS OF HYDRAZINE ON THE ACTIVITY OF ORNITHINE-KETOACID TRANSAMINASE IN RAT LIVER

Treatment	Activity (units*/g of soluble liver protein)
None	16·6 ± 0·67
Hydrazine†	2·4 ± 0·18

*A unit is defined as the amount catalyzing the formation of 1 μmole of Δ^1-pyrroline-5-carboxylic acid/min. Each value is the mean of 12 animals.

† Injection i.p., 1 m-mole hydrazine/kg/day; treatment, 4 days.

DISCUSSION

It seems well established by several investigations that the action of hydrazine on a variety of B_6 enzymes is due to binding of the coenzyme pyridoxal phosphate by this substance.[11,28–31] It is not surprising that hydrazine injections produce an amino acid imbalance in experimental animals.[13–16] Simonsen and Roberts[14] suggested a partial blockage of argininosuccinate synthetase to explain the increased level of liver citrulline, while that of liver ornithine noted by Banks[16] could be the result of a reduction in the conversion of ornithine to citrulline due to inhibition of carbamoyl-phosphate synthetase by hydrazine.[32]

Addition of different concentrations of hydrazine to homogenates of rat liver produces a gradual inhibition of citrulline and urea synthesis (Tables 1 and 2). A daily injection at a dose level of 1 m-mole/kg (32 mg/kg) decreases the body weight of rats and this fall in growth seems to be due to starvation[7] (Fig. 1). Treatment of rats with subconvulsive doses (32 mg/kg) for 4 days increases citrulline and urea contents in all tissues examined (Table 3). These increases seem not to be due to a complete or partial inhibition of carbamoyl-phosphate and argininosuccinate synthetases, since our results (Table 4) show that the activities of urea cycle enzymes are not affected by such treatment, except for argininosuccinase where an increase is noted. The activity of this enzyme reaches a maximum after 2 days of treatment (Table 5) and seems to be due to *de novo* synthesis, since actinomycin D produces marked inhibition of the increase in its activity.[33]

The inhibition of ornithine-ketoacid transaminase (Table 7) provokes an accumulation of ornithine in the liver of hydrazine-treated rats.[14,16] The presence of a high concentration of this amino acid together with an increased ammonia production[34] stimulates urea synthesis. Under these physiological conditions, the citrulline–aspartate reaction is the rate-limiting step in the urea cycle enzymes,[25,26] which gives this consequent accumulation of citrulline as suggested by Simonsen and Roberts.[14]

The question arises now as to whether there is any relation between inhibition of ornithine-ketoacid transaminase and induction of argininosuccinase and whether this increase in activity is due to a substrate induction or to the action of hydrazine on the enzyme itself.

Acknowledgements—This work was made possible by a grant from the Medical Research Council of Canada and by financial assistance from "Centre de recherche in nutrition, Université de Laval."

REFERENCES

1. F. P. UNDERHILL, *J. biol. Chem.* **10,** 159 (1911).
2. S. KROP, *Archs ind. Hyg.* **9,** 199 (1954).
3. E. ROBERTS, D. G. SIMONSEN and E. ROBERTS, *Biochem. Pharmac.* **12,** 1445 (1963).
4. M. A. MEDINA, *J. Pharmac. exp. Ther.* **140,** 133 (1963).
5. H. H. CORNISH, *Ann. N.Y. Acad. Sci.* **166,** 136 (1969).
6. E. ROBERTS, D. G. SIMONSEN and E. ROBERTS, *Biochem. Pharmac.* **14,** 351 (1965).
7. J. S. AMENTA and E. H. JOHNSTON, *Lab. Invest.* **11,** 956 (1962).
8. G. P. TAYLOR, SAM-TR-66-12, January (1966).
9. S. R. FORTNEY, D. A. CLARK and E. STEIN, *J. Pharmac. exp. Ther.* **156,** 277 (1967).
10. P. D. RAY, R. L. HANSON and H. A. LARDY, *J. biol. Chem.* **245,** 690 (1970).
11. S. R. FORTNEY, *Aerospace Med.* July 727 (1967).
12. D. L. TROUT, *Biochem. Pharmac.* **14,** 813 (1965).
13. H. B. LEWIS and S. IZUME, *J. biol. Chem.* **71,** 33 (1926).
14. D. G. SIMONSEN and E. ROBERTS, *Proc. Soc. exp. Biol. Med.* **124,** 806 (1967).
15. H. H. CORNISH and C. E. WILSON, *Toxic. appl. Pharmac.* **12,** 265 (1968).
16. W. L. BANKS, *Biochem. Pharmac.* **19,** 275 (1970).
17. W. L. BANKS and E. R. STEIN, *Proc. Soc. exp. Biol. Med.* **120,** 1 (1965).
18. W. L. BANKS, *Toxic. appl. Pharmac.* **11,** 71 (1968).
19. R. CHARBONNEAU and L. BERLINGUET, *Can. J. Biochem. Physiol.* **41,** 2297 (1963).
20. L. BERLINGUET and R. CHARBONNEAU, *Can. J. Biochem. Physiol.* **42,** 1317 (1964).
21. R. M. ARCHIBALD, *J. biol. Chem.* **156,** 121 (1944).
22. S. RATNER, in *Methods in Enzymology* (Eds. S. P. COLOWICK and N. O. KAPLAN), Vol. II, p. 356. Academic Press, New York (1955).
23. S. SAKAGUCHI, *J. Biochem., Tokyo* **5,** 133 (1925).
24. D. HUNNINGHAKE and S. GRISOLIA, *Analyt. Biochem.* **16,** 200 (1966).
25. G. W. BROWN and P. P. COHEN, *J. biol. Chem.* **234,** 1769 (1959).
26. R. CHARBONNEAU, A. ROBERGE and L. BERLINGUET, *Can. J. Biochem. Physiol.* **45,** 1371 (1967).
27. N. C. RAÏHÄ and M. P. KEKOMÄKI, *Biochem. J.* **108,** 521 (1968).
28. S. RATNER, *Archs Biochem. Biophys.* **102,** 249 (1963).
29. K. F. KILLAM and J. A. BAIN, *J. Pharmac. exp. Ther.* **119,** 255 (1957).
30. D. B. MCCORMICK, B. M. GULRABAD and E. E. SNELL. *Proc. Soc. exp. Biol. Med.* **104,** 554 (1960).
31. E. ROBERTS, J. WEIN and D. G. SIMONSEN, *Vitam. Horm.* **22,** 503 (1964).
32. S. MCKINLEY, C. D. ANDERSON and M. E. JONES, *J. biol. Chem.* **242,** 3381 (1967).
33. I. MERITS, *Biochem. biophys. Res. Commun.* **10,** 254 (1963).
34. H. MCKENNIS, A. S. YARD, E. J. ADAIR and J. H. WEATHERBY, *J. Pharmac. exp. Ther.* **131,** 152 (1961).