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STUDIES ON ASCORBIC ACID METABOLISM IN PHENYLHYDRAZINE-TREATED RATS

Kasturi SENGUPTA and Ajay K. CHATTERJEE

Department of Physiology, Calcutta University College of Science, 92, Acharya Prafulla Chandra Road, Calcutta 700009, India

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Summary Phenylhydrazine administered to rats at a dose of 4 mg/100 g body weight per day for 3 days, diminished the total ascorbic acid level in liver, kidney and spleen tissues. The total ascorbic acid level in blood was, however, elevated after phenylhydrazine treatment. The other effects of phenylhydrazine treatment were a reduction in the activities of D-glucuronoreductase, L-gulonooxidase, dehydroascorbatase and urono-lactonase in liver. The dehydroascorbatase activity in kidney was also diminished after phenylhydrazine treatment. The changes in the activities of liver enzymes were found to be accompanied by a diminution in the level of reduced ascorbic acid and an elevation in the dehydroascorbic acid level. It was concluded that the biosynthesis of L-ascorbic acid from both D-glucuronolactone and L-gulonolactone as substrates, was reduced in phenylhydrazine-treated rats. The degradation of L-ascorbic acid by both liver and kidney was diminished by the phenylhydrazine treatment. It was further concluded that phenylhydrazine-treated rats tried to conserve the level of substrate for the synthesis of L-ascorbic acid, by reducing the hydrolytic cleavage of D-glucuronolactone. The elevated blood ascorbic acid level under the same condition was ascribed to the diminished catabolism of L-ascorbic acid.

Various drugs, including a variety of foreign compounds, influence the metabolism of ascorbic acid (*1–8*). Recently, the effects of hydrazine on ascorbic acid metabolism were demonstrated (*9*). The biosynthesis as well as the degradation of L-ascorbic acid was found to be affected by the hydrazine treatment. Phenylhydrazine, which is an aromatic hydrazine, is known to cause a hemolytic anemia (*10–13*). Ascorbic acid administration, on the other hand, was reported to cause a marked improvement in anemia induced by phenylhydrazine (*14*), while pernicious anemia was found to be accompanied by a reduction in plasma ascorbic acid level (*15*). It appears, therefore, phenylhydrazine may have an impact on the metabolism of ascorbic acid and, accordingly, the present investigation was intended to study the effects of phenylhydrazine *in vivo* on the metabolism of ascorbic acid.

MATERIALS AND METHODS

Male Wistar rats, 60–110 g, were divided into two groups of equal average body weight. The animals of one group were injected intraperitoneally with phenylhydrazine (hydrochloride solution adjusted to pH 7.4 with $NaHCO_3$) at a dose of 4 mg/100 g body weight per day for 3 days. The animals of the other group served as pair-fed controls. The animals were maintained on an 18% casein diet. The other ingredients of the diet were the same as reported elsewhere (*7, 8, 16*). Fat-soluble and water-soluble vitamins were supplied in the diet. Water-soluble vitamins were furnished according to BERG (*17*).

After the last injection, the animals were fasted overnight and killed under light ether anaesthesia. Blood was collected in heparinized tubes. Various tissues (liver, kidneys, spleen, testes and adrenals) were removed, chilled in ice and weighed.

For another set of experiments involving measurement of reduced ascorbic acid and dehydroascorbic acid contents of liver, animals were grouped, maintained and treated as above.

Estimation of total ascorbic acid. A portion of each tissue was weighed and homogenized with cold 5% (w/v) metaphosphoric acid. The extract was made up to a known volume and filtered. Blood was deproteinized with 6% trichloroacetic acid. The protein-free filtrates of both tissues and plasma were treated with liquid bromine to convert ascorbic acid to dehydroascorbic acid. The total ascorbic acid contents were determined by the dinitrophenylhydrazine method of ROE and KUETHER (*18*).

Biosynthesis of L-ascorbic acid. The biosynthesis of L-ascorbic acid from D-glucuronolactone and L-gulonolactone was studied by the method as adopted earlier (*7, 8*). The D-glucuronoreductase activity was assayed in a test system containing sodium phosphate buffer, pH 7.4 (20 mM), D-glucuronolactone (10 mM), NaCN (50 mM) and liver homogenate (in 0.15 M KCl) equivalent to 100 mg fresh tissue. The mixture was incubated at 37°C for 60 min using a Dubnoff shaker. The reaction was stopped by the addition of 0.5 ml of 30% HPO_3 (cold). It was filtered and the amount of L-ascorbic acid synthesized was determined by titrating the filtrate against standardized 2,6-dichlorophenolindophenol solution.

Another test system containing sodium pyrophosphate (50 mM), NaCN (1 mM), sodium phosphate buffer, pH 7.4 (20 mM), L-gulonolactone (5 mM) and liver homogenate as stated above was used to determine the L-gulonooxidase activity. The subsequent procedures were like that of the assay system of D-glucuronoreductase.

Catabolism of L-ascorbic acid. The dehydroascorbatase activity of liver or kidney tissue was studied by the method reported elsewhere (*8*). The incubation mixture contained Tris-maleate buffer, pH 6.8 (66 mM), reduced glutathione (0.1 mM), $MgCl_2$ (6.6 mM), dehydroascorbic acid (3.3 mM) and liver homogenate (in 0.15 M KCl) equivalent to 100 mg wet tissue or kidney homogenate (in 0.15 M KCl) equivalent to 50 mg wet tissue. The mixture was incubated at 37°C for 15 min. The reaction was stopped by the addition of 1 ml of 20% (w/v) HPO_3 containing 2% (w/v) $SnCl_2$. The dehydroascorbic acid remained after the reaction was rapidly reduced

with H_2S. Then a requisite amount of thiourea was added. It was mixed and filtered and the excess H_2S was removed from the filtrate by bubbling a stream of CO_2. An aliquot was taken for the estimation of 2,3-dioxogulonic acid thus formed by reacting with 2,4-dinitrophenylhydrazine according to the method of KAGAWA *et al.* (*19*).

Hydrolysis of D-*glucuronolactone.* The activity of liver uronolactonase which catalyzes the hydrolysis of D-glucuronolactone to its free acid was assayed by the method of EISENBERG and FIELD (*20*). The incubation mixture contained potassium phosphate buffer, pH 7.4 (50 mM), D-glucuronolactone (12 mM) and liver homogenate (in 0.15 M KCl) equivalent to 100 mg wet tissue. The mixture was incubated at 37°C for 60 min in air. The system was inactivated by the addition of 1 ml of 10% (w/v) trichloroacetic acid. The disappearance of D-glucuronolactone was noted by measuring in the filtrate the amount of D-glucuronolactone remained after enzymatic hydrolysis. Glucuronolactone was measured by adopting the hydroxamic procedure of LIPMAN and TUTTLE (*21*) described by EISENBERG and FIELD (*20*).

The method of ROE *et al.* (*22*) was employed for the determination of reduced ascorbic acid and dehydroascorbic acid contents of liver.

The protein content of tissue homogenates used for assaying the activities of enzymes was determined by the biuret method (*23*).

RESULTS AND DISCUSSION

The average body weight of phenylhydrazine-treated rats was not found to be different from that of pair-fed control rats (Table 1). This suggests that phenylhydrazine has no direct effect on the body weight of animals. The weights of liver, kidneys, spleen and adrenals per 100 g body weight were, however, increased after phenylhydrazine treatment (Table 1). Among the organs spleen showed, as expected, maximum enlargement. The weight of the testes per 100 g body weight was, however, not altered by the phenylhydrazine treatment. It is known that phenylhydrazine when administered to animals including lower forms of animals caused hemolysis with increasing breakdown of erythrocytes (*24–27*). It was also recognized that the hemolytic activity of phenylhydrazine was accompanied by splenic erythrophagocytosis resulting in increased spleen size (*24, 25*). In the present investigation, therefore, the marked increase in spleen size was an effect of hemolytic action of phenylhydrazine. It was suggested that the increased size of the organs (liver, kidney *etc.*) caused by hydrazine treatment might arise from accumulation of fat (*28, 29*). It is, therefore, possible that the enlargement of other organs (liver, kidney *etc.*) in phenylhydrazine-treated rats might be the result of accumulation of fat.

The results presented in Table 2 reveal that phenylhydrazine treatment reduced the total ascorbic acid levels of liver, kidney and spleen. The fall in total ascorbic acid level in the liver of phenylhydrazine-treated rats was accompanied by reduced activities of both D-glucuronoreductase and L-gulonooxidase (Table 3). This indicates that the fall in total ascorbic acid level in the liver of phenylhydrazine-treated rats resulted from diminished synthesis of L-ascorbic acid. It appears that

Table 1. Effect of phenylhydrazine on body and organ weights.

Groups of animals	Body weight (g)	Organ weight (per 100 g body weight)				
		Liver (g)	Kidney[a] (g)	Spleen (g)	Testis[a] (g)	Adrenal[a] (mg)
Pair-fed control	90.9 ± 2.4 (7)	3.92 ± 0.12 (7)	0.832 ± 0.031 (6)	0.612 ± 0.050 (7)	1.944 ± 0.100 (6)	22.64 ± 2.25 (6)
Treated	82.8 ± 6.4 (6) $p > 0.05$	5.29 ± 0.42 (6) $p < 0.01$	1.008 ± 0.020 (6) $p < 0.001$	1.779 ± 0.140 (6) $p < 0.001$	1.973 ± 0.129 (6)	34.81 ± 0.73 (6) $p < 0.001$

The values are means ± S.E.M.
[a] Organs of both sides. The figures in the parentheses indicate the number of animals.

Table 2. Effect of phenylhydrazine on total ascorbic acid content of various tissues and blood.

Groups of animals	Liver	Kidney	Spleen (μg/100 mg tissue)	Testis	Adrenal	Blood (mg/100 ml)
Pair-fed control	23.27 ± 1.15 (6)	15.71 ± 0.69 (6)	52.68 ± 1.66 (7)	32.93 ± 1.61 (6)	390.1 ± 49.2 (6)	1.528 ± 0.202 (7)
Treated	17.52 ± 1.21 (6) $p < 0.01$	9.28 ± 0.92 (6) $p < 0.001$	24.28 ± 1.43 (6) $p < 0.001$	35.55 ± 0.73 (6) $p > 0.05$	293.0 ± 32.1 (6) $p > 0.05$	2.349 ± 0.110 (6) $p < 0.01$

The values are means ± S.E.M. The figures in the parentheses indicate the number of animals.

Table 3. Effect of phenylhydrazine on the biosynthesis of L-ascorbic acid by the liver.

Groups of animals	D-Glucuronoreductase activity (L-ascorbic acid synthesized)		L-Gulonooxidase activity (L-ascorbic acid synthesized)	
	μmoles/g protein	μmoles/g tissue	μmoles/g protein	μmoles/g tissue
Pair-fed control	8.80 ± 0.80 (5)	1.48 ± 0.09 (5)	74.76 ± 5.23 (6)	12.31 ± 0.98 (6)
Treated	4.43 ± 0.68 (5) $p<0.01$	0.63 ± 0.09 (5) $p<0.001$	44.07 ± 2.20 (6) $p<0.001$	6.88 ± 0.48 (6) $p<0.001$

The values are means ± S.E.M. The figures in the parentheses indicate the number of animals.

Table 4. Effect of phenylhydrazine on reduced ascorbic acid and dehydroascorbic acid contents of liver.

Groups of animals	Reduced ascorbic acid (μg/100 mg tissue)	Dehydroascorbic acid (μg/100 mg tissue)
Pair-fed control (6)	17.27 ± 1.36	2.73 ± 0.30
Treated (6)	10.52 ± 1.03 $p<0.01$	3.73 ± 0.20 $p<0.02$

The values are means ± S.E.M. The figures in the parentheses indicate the number of animals.

phenylhydrazine treatment reduced the synthesis of L-ascorbic acid from both D-glucuronolactone and L-gulonolactone as substrates. That phenylhydrazine treatment causes reduced synthesis of L-ascorbic acid seemed also evident from the diminished level of reduced ascorbic acid in the liver (Table 4). The dehydroascorbatase activity of both liver and kidney was depressed by the phenylhydrazine treatment (Table 5). This demonstrates that the conversion of dehydroascorbic acid to 2,3-dioxogulonic acid was reduced after phenylhydrazine treatment. The elevated level of dehydroascorbic acid in the liver of phenylhydrazine-treated rats (Table 4) also suggests a diminished conversion of dehydroascorbic acid to 2,3-dioxogulonic acid. Inspite of diminished degradation of L-ascorbic acid, the lowering of total ascorbic acid level in the liver of phenylhydrazine-treated rats could be ascribed to the fact that the biosynthesis of L-ascorbic acid was adversely affected since the activities of both the enzymes involved in the biosynthesis of L-ascorbic acid were retarded. Phenylhydrazine treatment also reduced the activity of uronolactonase in liver (Table 6), suggesting a diminished hydrolysis of D-glucuronolactone. These studies indicate, therefore, that phenylhydrazine-treated rats showed a tendency to adapt to the altered situation by reducing the hydrolysis of D-

Table 5. Effect of phenylhydrazine on the catabolism of L-ascorbic acid.

Groups of animals	Dehydroascorbatase activity (2,3-dioxogulonic acid formed)			
	Liver		Kidney	
	μmoles/g protein	μmoles/g tissue	μmoles/g protein	μmoles/g tissue
Pair-fed control	294.2 ± 3.8 (6)	50.72 ± 2.39 (6)	332.4 ± 8.0 (6)	47.89 ± 2.02 (6)
Treated	247.4 ± 2.8 (6) $p < 0.001$	39.46 ± 1.12 (6) $p < 0.01$	247.6 ± 14.4 (5) $p < 0.001$	33.16 ± 1.54 (5) $p < 0.001$

The values are means ± S.E.M. The figures in the parentheses indicate the number of animals.

Table 6. Effect of phenylhydrazine on uronolactonase activity of liver.

Groups of animals	D-Glucuronolactone hydrolyzed	
	μmoles/g protein	μmoles/g tissue
Pair-fed control (6)	871.9 ± 10.5	142.7 ± 2.1
Treated (6)	793.8 ± 26.2 $p < 0.02$	122.7 ± 4.7 $p < 0.01$

The values are means ± S.E.M. The figures in the parentheses indicate the number of animals.

glucuronolactone, in an attempt to raise the level of the endogenous substrate for the synthesis of L-ascorbic acid, and also by bringing in a mechanism which caused a diminution in the breakdown of L-ascorbic acid.

Although the synthesis of L-ascorbic acid by the liver was diminished, the total ascorbic acid level of whole blood was elevated by the phenylhydrazine treatment (Table 2). This elevation in blood ascorbic acid level might arise from the diminished degradation of L-ascorbic acid. In the face of elevated blood ascorbic acid level and diminished degradation of L-ascorbic acid, the fall in total ascorbic acid level in kidney and spleen might be ascribed to the enlargement of the organs or the diminished uptake of ascorbic acid by the tissues from the blood.

Thus, the present studies demonstrate that rats respond to phenylhydrazine treatment by showing a variety of changes in ascorbic acid metabolism.

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