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Effect of Liver Necrosis and Thyrotoxicosis on the Synthesis of L-Ascorbic Acid by Rat-Liver Microsomes

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The administration of carbon tetrachloride can neither increase the liver-tissue reserve of ascorbic acid nor bring about an enhanced urinary excretion of ascorbic acid in rats (N. C. Ghosh & N. C. Kar, unpublished work). Lindan & Work (1953) observed that the ascorbic acid content of the livers of rats given a necrogenic diet was significantly lower than that of livers of rats given a 25% casein diet. They found that the preneurotic liver had a slightly diminished ascorbic acid content, and that when necrosis developed ascorbic acid could not be detected in the liver.

Various investigators have shown that the synthesis of ascorbic acid as evidenced from the urinary excretion is correlated with the activity of the endocrine glands. Dumm & Ralli (1949) observed that the amount of ascorbic acid excreted by rats after adrenalectomy falls to a very low value. Sure & Theis (1938) reported that, when thyroxine at a toxic dose is fed to rats, the ascorbic acid content of the adrenals, thymus and kidney is greatly decreased. Burns, Evans & Trousof (1957) demonstrated that barbital and Chloretone have little or no effect on ascorbic acid excretion in

hypophysectomized rats, suggesting a possible hormonal control of ascorbic acid synthesis in response to drugs.

The enzyme system catalysing the conversion of D-glucuronolactone and L-gulonolactone into L-ascorbic acid in animals is entirely located in the microsomal fractions of the rat- and goat-liver homogenates (Burns, Peyser & Moltz, 1956; Chatterjee, Ghosh, Ghosh & Guha, 1957*a, b*, 1958). Chatterjee, Kar, Ghosh & Guha (1961) also reported that the enzymic activity of liver microsomes for the conversion of L-gulonolactone into L-ascorbic acid is significantly decreased in vitamin E deficiency. The activity could be restored by the addition of α -tocopherol or vitamin K₁. A similar reactivation by α -tocopherol has also been obtained by Carpenter, Kitabchi, McKay & Caputto (1959), who observed an increased peroxide formation in the microsomes from vitamin E-deficient rats. They concluded that α -tocopherol functions by inhibiting the peroxide formation. Chatterjee *et al.* (1961) further showed that compounds other than antioxidants equally stimulate the synthesis of L-ascorbic acid by the microsomes from vitamin

E-deficient rats. Thus the action of antioxidants did not appear to be specific. Similar results have also been obtained with microsomes from vitamin A-deficient, thiamine-deficient and riboflavin-deficient rats (Chatterjee *et al.* 1961; Chatterjee & Kar, 1960). The present paper reports the effects of liver damage caused by carbon tetrachloride poisoning and by giving a necrogenic yeast diet, and also the effect of thyrotoxicosis, on the microsomal synthesis of L-ascorbic acid by rat-liver tissues. Vitamin B₁₂ has some protective action against liver damage and thyrotoxicosis (Kasbekar, Lavate, Rege & Sreenivasan, 1959; Bosshardt, Paul, O'Doherty, Huff & Barnes, 1949), and the effect of this vitamin in relation to L-ascorbic acid synthesis has therefore been investigated in rats treated with carbon tetrachloride and with thyroglobulin.

MATERIALS

L-Gulonolactone was prepared as described by Chatterjee, Chatterjee, Ghosh, Ghosh & Guha (1960). D-Glucuronolactone was purchased from California Corp. for Biochemical Research, U.S.A. L-Thyroxine (sodium salt) (Eltroxin) was purchased from Glaxo Laboratories (India) Ltd. Thyroglobulin was supplied by Martin and Harris Ltd., Calcutta, India. Vitamin B₁₂ was purchased from Sigma Chemical Co., U.S.A.

The solutions of substrates, sodium pyrophosphate and potassium cyanide were freshly prepared as described by Chatterjee *et al.* (1960).

METHODS

Preparation of microsomes. Rat-liver microsomes were prepared by the method described by Chatterjee *et al.* (1960).

Incubation media. The test system contained sodium phosphate buffer, pH 7.4 (20 mM), D-glucuronolactone (10 mM), microsomal dispersion (0.25 ml., equivalent to 250 mg. of fresh liver) and potassium cyanide (50 mM). The total volume was 2.5 ml., and the mixture was incubated at 37° for 1 hr.

When L-gulonolactone (5 mM) was used as substrate, cyanide (1 mM) was added to the test system to prevent the oxidation of L-ascorbic acid in the medium. Though cyanide at a higher concentration is inhibitory to the synthesis of L-ascorbic acid, a lower concentration of cyanide (1 mM) is not. The experiments were performed with sodium pyrophosphate (20 mM) unless otherwise stated.

Determination of ascorbic acid. Ascorbic acid was determined by the procedure described by Chatterjee *et al.* (1960).

Determination of protein. Protein was determined by the method of Chatterjee *et al.* (1960).

EXPERIMENTAL

Induction of liver damage by the administration of carbon tetrachloride. Male albino rats (150–200 g.) were maintained on stock diet containing: whole wheat, 86 parts;

whole-milk powder, 9.5 parts; yeast powder, 2.5 parts; cod-liver oil, 1 part; Steenbock salt mixture no. 40 (Steenbock & Nelson, 1923), 1 part. They were divided into three groups of which two were injected daily with a solution of carbon tetrachloride in liquid paraffin (0.03 ml./100 g. body wt./rat) intraperitoneally for 3 days. One of the carbon tetrachloride-treated groups was given daily oral doses of vitamin B₁₂ (20 µg./rat), this treatment commencing 2 days before the first injection of carbon tetrachloride and continuing throughout the experimental period. The control group received injections of liquid paraffin without carbon tetrachloride (0.5 ml./100 g. body wt./rat). As the injection of carbon tetrachloride decreased the food intake in both groups of rats equally, the control rats were therefore given the same amount of food as that consumed by the treated rats. The animals were decapitated 24 hr. after the last dose of carbon tetrachloride and the livers removed quickly. Small portions from different lobes of the livers were taken for histological studies and the remaining portions were chilled and homogenized, and the microsomes prepared by the method of Chatterjee *et al.* (1960).

Induction of liver damage by giving a necrogenic diet. Experimental liver necrosis in rats was produced by giving the necrogenic yeast diet described by Lindan & Work (1953). Four groups of rats, each rat weighing 35–45 g., were placed on the experimental diets as follows: (a) necrogenic diet without α -tocopherol; (b) necrogenic diet plus oral supplement of α -tocopherol (10 mg./rat/day); (c) necrogenic diet plus DL-methionine (0.11%) and L-cystine (0.13%); (d) necrogenic diet plus DL-methionine and L-cystine together with an oral supplement of α -tocopherol (10 mg./rat/day). One control group was placed on the stock diet mentioned above. The rats were killed after 4 weeks, their livers removed and the microsomes isolated by the method described by Chatterjee *et al.* (1960).

Induction of experimental thyrotoxicosis. Thyrotoxicosis in rats was produced by giving thyroxine or thyroglobulin mixed with the stock diet (mentioned above) in the proportions of 3 and 2 mg./100 g. respectively for 9 days. To one group of thyroglobulin-treated rats vitamin B₁₂ (20 µg./rat/day) was administered orally during the experimental period. The control group was given the stock diet only. The rats were killed and their liver microsomes prepared by the procedure of Chatterjee *et al.* (1960).

Histological examination of livers. Samples of liver from the animals were fixed in formal saline. Neutral lipid in the section was stained with Sudan III and Sudan IV. Paraffin sections of the livers were stained with Masson's trichrome for histological study.

Carbon tetrachloride-poisoned liver showed a high degree of fatty degeneration. There was destruction of the general structure of hepatic tissue. A definite necrosis of the cells has been observed in the central zone with relatively normal ones around the portal area. A similar type of degeneration has also been observed in carbon tetrachloride- and vitamin B₁₂-treated rats. On the average 60–70% of hepatic tissue was destroyed. The liver of the control rats was histologically normal and there was no fatty infiltration.

The following observations were noted in the liver tissues of animals given various diets: (a) Necrogenic diet alone: pale staining of cytoplasm was observed; the nuclei of hepatic cells and Kupffer cells were normal, and there was

fatty infiltration in the mid-zonal area. (b) Necrogenic diet plus α -tocopherol: as described under (a). (c) Necrogenic diet plus methionine and cystine: normal staining of cytoplasm and absence of fatty infiltration were observed. (d) Necrogenic diet plus DL-methionine, L-cystine and α -tocopherol: as described under (c).

RESULTS

Effect of the administration of carbon tetrachloride, with and without vitamin B₁₂, on the conversion of D-glucuronolactone and L-gulonolactone into L-ascorbic acid by rat-liver microsomes. Table 1 shows that the microsomal conversion of D-glucuronolactone and L-gulonolactone into L-ascorbic acid by rat-liver tissue is greatly decreased after the injection of carbon tetrachloride. The administration of vitamin B₁₂ has no effect in restoring the enzymic activity.

Effect of giving a necrogenic diet on the conversion of L-gulonolactone into L-ascorbic acid by rat-liver microsomes. Table 2 shows that the livers of rats given the necrogenic diet without α -tocopherol have significantly decreased enzymic activity for the synthesis of L-ascorbic acid. Supplementation

with α -tocopherol slightly stimulates the activity. When sulphur-containing amino acids, namely DL-methionine and L-cystine, are added to the necrogenic diet complete restoration of the enzymic activity to values comparable with that of normal rat-liver tissue is obtained. But no synthesis of L-ascorbic acid can, however, occur unless sodium pyrophosphate is added to the test system. The administration of α -tocopherol together with the amino acids causes no further stimulation in the activity of the enzyme, but it abolishes the requirement for pyrophosphate.

Effect of giving L-thyroxine and thyroglobulin in the diet on the conversion of D-glucuronolactone and L-gulonolactone into L-ascorbic acid by rat-liver microsomes. Table 3 shows that giving L-thyroxine and thyroglobulin in the diet decreases the synthesis of L-ascorbic acid by rat-liver microsomes. The decreased synthesis is more pronounced in the thyroglobulin-treated rats. The administration of vitamin B₁₂ to the thyroglobulin-treated rats partially restores the synthesis, to a value almost equal to that obtained with the thyroxine-treated rats.

Table 1. *Effect of the injection of carbon tetrachloride, with or without the administration of vitamin B₁₂, on the conversion of D-glucuronolactone and L-gulonolactone into L-ascorbic acid by rat-liver microsomes*

Experimental details are given in the text. Each result is expressed as the mean $\pm$ S.D. of five experiments, each on a different animal. The mean recoveries of microsomal protein (in mg./g. of fresh liver) were: control rats, 19.72 ± 3.4 ; rats treated with CCl₄, 18.40 ± 1.8 ; rats treated with CCl₄ plus vitamin B₁₂, 18.50 ± 1.6 .

Source of microsome preparation	Ascorbic acid synthesized (μ mole/mg. of protein)	
	From D-glucuronolactone	From L-gulonolactone
Untreated rats (controls)	0.06 ± 0.01	0.19 ± 0.01
Rats treated with CCl ₄	0.02 ± 0.01	0.06 ± 0.01
Rats treated with CCl ₄ + vitamin B ₁₂	0.02 ± 0.004	0.06 ± 0.01

Table 2. *Effect of giving a necrogenic diet on the conversion of L-gulonolactone into L-ascorbic acid by rat-liver microsomes*

Experimental details are given in the text. Each result is expressed as the mean $\pm$ S.D. of six experiments, each on a different animal. The mean recoveries of microsomal protein (in mg./g. of fresh liver) were: control rats, 14.46 ± 3.4 ; rats given necrogenic diet, 11.06 ± 2.3 ; rats given necrogenic diet plus vitamin E, 11.23 ± 2.2 ; rats given necrogenic diet plus methionine plus cystine, 14.90 ± 4.3 ; rats given necrogenic diet plus methionine plus cystine plus vitamin E, 14.20 ± 2.5 .

Source of microsome preparation	Ascorbic acid synthesized (μ mole/mg. of protein)	
	Pyrophosphate present	Pyrophosphate absent
Rats given normal diet (controls)	0.21 ± 0.03	—
Rats given necrogenic diet	0.10 ± 0.01	0
Rats given necrogenic diet + vitamin E	0.13 ± 0.03	0.07 ± 0.02
Rats given necrogenic diet + methionine + cystine	0.21 ± 0.03	0
Rats given necrogenic diet + methionine + cystine + vitamin E	0.21 ± 0.03	0.19 ± 0.01

Table 3. *Effect of giving thyroglobulin, with and without the administration of vitamin B₁₂, and thyroxine on the conversion of D-glucuronolactone and L-gulonolactone into L-ascorbic acid by rat-liver microsomes*

Experimental details are given in the text. Each result is expressed as the mean $\pm$ S.D. of five experiments, each on a different animal. The mean recoveries of microsomal protein (in mg./g. of fresh liver) were: control rats, 18.32 ± 2.2 ; rats treated with thyroglobulin, 22.68 ± 3.1 ; rats treated with thyroglobulin plus vitamin B₁₂, 23.84 ± 3.8 ; rats treated with thyroxine, 22.64 ± 2.1 .

Source of microsome preparation	Ascorbic acid synthesized (μ mole/mg. of protein)	
	From D-glucuronolactone	From L-gulonolactone
Untreated rats (controls)	0.07 ± 0.02	0.20 ± 0.02
Rats treated with thyroglobulin	0.02 ± 0.01	0.05 ± 0.01
Rats treated with thyroglobulin + vitamin B ₁₂	0.04 ± 0.01	0.09 ± 0.01
Rats treated with thyroxine	0.04 ± 0.01	0.09 ± 0.01

DISCUSSION

The stimulated synthesis of L-ascorbic acid by the administration of Chloretone is completely abolished in rats treated with carbon tetrachloride (N. C. Ghosh & N. C. Kar, unpublished work). Studies *in vitro* with D-glucuronolactone and L-gulonolactone as substrates reveal that the L-ascorbic acid-synthesizing capacity of the microsomal enzyme system of rat liver is greatly decreased after the injection of carbon tetrachloride. Vitamin B₁₂, which has some protective action against liver injury caused by carbon tetrachloride poisoning (Kasbekar *et al.* 1959), cannot counteract the inhibition of the synthesis of L-ascorbic acid by rat-liver microsomes. This irreversible loss of activity of the microsomes in the synthesis of L-ascorbic acid seems to indicate that carbon tetrachloride not only damages the mitochondria (Christie & Judah, 1954) but also affects the microsomes.

It appears that the liver necrosis caused by giving the necrogenic yeast diet free from vitamin E results in a decreased synthesis of L-ascorbic acid by rat-liver microsomes. This decreased synthesis is, however, not observed when the diet is supplemented with sulphur-containing amino acids, e.g. DL-methionine and L-cystine, for the net synthesis equals that observed in normal rat-liver microsomes. The administration of α -tocopherol in addition to the amino acids has no stimulatory effect on the synthesis, but it does, however, slightly stimulate the synthesis in the absence of the sulphur-containing amino acids. These findings are in keeping with the results obtained by Lindan & Work (1953), namely that there is lowering of the L-ascorbic acid content in the livers of rats given the necrogenic diet. Lindan & Work (1953) also showed by experiments *in vivo* that supplementation of the necrogenic diet with DL-methionine and L-cystine maintains the L-ascorbic acid content of the livers at a normal value. The de-

creased enzymic activity in the necrotic livers reported in the present paper may be due to the diminished synthesis of the enzyme involved in the biosynthesis of L-ascorbic acid as a result of the degeneration of the microsomes brought about by the deficiency of sulphur-containing amino acids; or it may be due to the increased activity of the thyroid gland (Hock & Bergner, 1959), which has an inhibitory effect on the synthesis of L-ascorbic acid in normal rats (Table 3).

The microsomal conversion of L-gulonolactone into L-ascorbic acid by the liver tissues of rats given the necrogenic diet, either alone or supplemented with DL-methionine and L-cystine, is absolutely pyrophosphate-dependent. The administration of α -tocopherol completely abolishes this requirement in rats given the necrogenic diet supplemented with the sulphur-containing amino acids. Chatterjee *et al.* (1961) observed that in the absence of sodium pyrophosphate there was a diminished formation of L-ascorbic acid from L-gulonolactone by the rat-liver microsomes, and that the addition of sodium pyrophosphate to the test system augmented the synthesis. The experiments with the necrogenic diet have shown that deficiency of vitamin E causes the pyrophosphate requirement for the synthesis of L-ascorbic acid. Caputto, Trucco & Kitabchi (1961) have, however, observed that the activity of the microsomal enzyme that converts L-gulonolactone into L-ascorbic acid is diminished during vitamin E deficiency. They suggested that the impaired activity is due to the formation of lipid peroxide. A similar inactivation of the enzyme during vitamin E deficiency and the restoration of its activity by the addition of α -tocopherol or vitamin K₁ has also been observed by Chatterjee *et al.* (1961). Cyanide (1 mM), which also forms complexes with certain metals, has been found to be completely ineffective; EDTA (1 mM) when used instead of sodium pyrophosphate stimulates the synthesis, but only to a value 50–70% lower than that observed with sodium

pyrophosphate. This experiment has been done only with rats given the necrogenic diet supplemented with methionine and cystine. The role of vitamin E in the synthesis may be explained only if it is assumed that its deficiency causes the release of certain metals that interfere with the oxidation of L-gulonolactone to L-ascorbic acid, and that the function of sodium pyrophosphate is to form chelates with the metals so that normal synthesis is thereby obtained.

The results presented in Table 3 show that the synthesis *in vitro* of L-ascorbic acid by rat-liver microsomes from the L-thyroxine- and thyroglobulin-treated rats is significantly lowered. The depression in the synthesis is more marked in thyroglobulin-treated rats. Vitamin B₁₂, which under certain conditions antagonizes the action of L-thyroxine, can partially counteract the depression of the synthesis in the thyroglobulin-treated rats under the experimental conditions studied. The role of L-thyroxine in altering the enzymic activity of liver microsomes with regard to the synthesis of L-ascorbic acid cannot be properly explained unless it is assumed that there is a derangement of morphology of the microsomes caused by the higher metabolic rate. It has also been observed that there is an increase in the glucose 6-phosphatase activity of the liver microsomes of thyrotoxic rats (N. C. Ghosh & N. C. Kar, unpublished work).

SUMMARY

1. The activity of the enzyme system catalysing the conversion of D-glucuronolactone and L-gulonolactone into L-ascorbic acid by rat-liver microsomes is greatly diminished after the injection of carbon tetrachloride. The administration of vitamin B₁₂ before and with the carbon tetrachloride does not prevent the inhibition of synthesis.

2. The microsomal conversion of L-gulonolactone into L-ascorbic acid is significantly decreased in liver tissues of rats given a necrogenic diet free from vitamin E. Supplementation of the necrogenic diet with DL-methionine and L-cystine completely restores the enzymic activity. The syn-

thesis is, however, pyrophosphate-dependent. The administration of α -tocopherol in addition to the amino acids abolishes the requirement for pyrophosphate.

3. The synthesis of L-ascorbic acid from D-glucuronolactone and L-gulonolactone is markedly decreased in liver microsomes from thyrotoxic rats. The administration of vitamin B₁₂ can partially restore the synthesis in the thyroglobulin-treated rats.

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