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THE EFFECT OF CARBON TETRACHLORIDE, ETHIONINE AND CHLORETONE ON THE ASCORBIC ACID CONTENT OF RAT LIVER

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Abstract—In rats with acute CCl_4 poisoning, the ascorbic acid content of the liver is very low. Chloretone (chlorobutanol) given to normal rats much increases the ascorbic acid content of the liver, but when given to rats poisoned with CCl_4, it does not. In rats which have received injections of ethionine, the ascorbic acid of the liver is within the normal range. Chloretone, given to such rats, lowers the ascorbic acid value of the liver. This apparent reversal of the action of Chloretone, which occurs when the synthetic mechanism for ascorbic acid is not disturbed, is at present not explained.

INTRODUCTION

THERE are several studies of the effect of CCl_4 on the vitamin content of the liver. Shils *et al.*[1] found a decrease of vitamin A and of certain of the vitamin B group in the livers of CCl_4-poisoned rats. Using liver slices and mitochondrial preparations, Christie and Judah[2] concluded that CCl_4 damages the mitochondria. Vitamin E has also been studied in relation to CCl_4 poisoning.[3] Ascorbic acid, however, has not been studied, possibly because it is not a vitamin for the rat.

Homogenates of most normal tissues form fatty acid peroxides during incubation and these react with 2-thiobarbituric acid to give a red color. Ascorbic acid is necessary for the oxidation,[5] and the amount of color formed in homogenates can usually be increased by the addition of ascorbic acid to the incubating mixture. It was noted that the color formed in liver homogenates of rats with acute CCl_4 poisoning was much reduced, and that the addition of ascorbic acid to the mixture before incubation resulted in a marked increase of color. This suggested a low ascorbic acid content in the poisoned tissues. Therefore, the ascorbic acid concentration in rats livers after CCl_4 administration was determined and compared to that of the livers of rats given ethionine. The effect of Chloretone (chlorobutanol), which is known to stimulate the synthesis of ascorbic acid, was also investigated.

EXPERIMENTAL

Five groups of rats were used. Groups 1, 2 and 3 consisted of a mixed lot of Wistar rats of different ages and sexes. The animals of group 1 were injected intraperitoneally with a single dose of CCl_4, 1 mg/g or sometimes less; they were killed by a blow on the head, usually 17 hr later. Those of group 2 were injected with Chloretone, 30 mg/rat, dissolved in 0·1 ml ethyl alcohol, and the rats were killed at 17 hr. The animals

of group 3 received both substances and were similarly treated. Groups 4 and 5 consisted of female rats which were fasted for 12 hr and then were given ethionine, 75 mg/100 g, divided into three doses at intervals of 2½ hr as described by Koch-Weser *et al.*;[6] they were killed 48 hr later. The animals of group 5 were injected with Chloretone 17 hr before death.

The livers were removed and iced, and 1 g was weighed and homogenized with 9 ml of water, or, for ascorbic acid determinations, with 5% metaphosphoric acid.

The ascorbic acid in the liver was determined by the method of Roe[7]. The metaphosphoric acid homogenate was centrifuged and 1 ml of the supernatant fraction, which was clear or slightly opalescent, was treated with 9 ml of a solution of 2:6-dichlorophenolindophenol in sodium acetate solution and the color was read at once in the Evelyn colorimeter at 520 mμ. The dye was then decolorized completely by the addition of excess ascorbic acid and the value given by the blank was subtracted from the experimental values. The amount of ascorbic acid corresponding to the amount of reduced dye was read from a standard curve.

In order to estimate the total ascorbic acid (the reduced and the dehydroform), the method described by Roe[8] was used. Ascorbic acid was oxidized by treatment with activated Norit, and the color formed with dinitrophenylhydrazine-thiourea and sulfuric acid was estimated in the Evelyn colorimeter at 540 mμ. For this determination the liver homogenate was diluted 1 : 25 with 6% trichloracetic acid, centrifuged and Norit was added. After filtration, 4 ml were used for the determination.

The total nitrogen values were determined on the liver water homogenates, using 2 ml of a 1 : 1000 dilution. The usual Kjeldahl procedure was employed, followed by Nesslerization, and the color was read at 420 mμ. For dry weights, a 3 ml sample of the 1 : 10 water homogenate was dried and weighed.

TABLE 1. THE EFFECT OF CCl_4 ON THE ASCORBIC ACID CONCENTRATION IN RAT LIVER

	Dry weights g/100 g liver	Nitrogen g/100 g liver	Ascorbic acid mg/100 g liver	mg ascorbic acid / g nitrogen
Normal male rat	27·6	2·7	23·0	8·5
Normal female rat	30·7	3·5	27·0	7·8
Male rats after CCl_4	27·4	3·9	11·5	2·9
	26·9	3·3	14·0	4·2
Female rats after CCl_4	20·6	2·15	7·8	3·6
	20·0	2·15	7·0	3·3

RESULTS

Table 1 shows the results of ascorbic acid determinations on the livers of rats with acute CCl_4 intoxication. A definite reduction was found in the amount of ascorbic acid in these livers.

Under the conditions of these experiments, the livers of female rats treated with CCl_4 were grossly larger and appeared more fatty than the livers of males. This impression was confirmed by the dry weights and total nitrogen content of the livers, as can be seen in Table 1. In the table, the data for two individual female and two male

rats are given, and experimental values were obtained for many others. For instance, in a group of five normal rats (three males and two females) the mean total nitrogen content of the livers was 3·2 g per 100 g wet weight, and ascorbic acid value was 23·6 mg, with a ratio of mg of ascorbic acid/g of total N of 7·4. The corresponding figures for six male rats treated with CCl_4 were, nitrogen, 3·3 g, ascorbic acid 14·1 mg, and a ratio of 4·4. For a group of eight similarly treated females, the nitrogen was 2·15 g, the ascorbic acid 7·8 mg, and the ratio 3·6. It can be seen that the ascorbic acid content of the CCl_4-treated animals is low, expressed either in terms of wet weight or nitrogen content. This is true for both males and females.

Dehydroascorbic acid is not estimated under the conditions employed for ascorbic acid, so it was possible that, in CCl_4-poisoned livers, the low values for ascorbic acid might be the result of a change of equilibrium between the two forms, and the oxidation of a greater part than normal, without change in the total amount. In order to test this, the total ascorbic acid was estimated after the oxidation of the ascorbic acid fraction with activated Norit. The values for two normal rats were, ascorbic acid, 18·6 and 22·0 and the total ascorbic acid 20·0 and 25·5 mg/100 g of liver, and for two rats which had been injected with CCl_4 the values were 4·0 and 3·7, and 4·2 and 4·0, respectively. Therefore, the low amounts of ascorbic acid found in the CCl_4-livers are not the result of oxidation of part of the acid, but represent a real lowering of the total amount present.

The reduction of the ascorbic acid content of the livers of rats treated with CCl_4 is rapid. A few experiments showed that the process is already beginning after 5 hr, and is maximal at from 12 to 15 hr after the CCl_4 injection. Recovery follows relatively slowly, and at 48 hr the ascorbic acid values were still lower than normal.

It seemed possible that fasting might contribute to changes in the concentration of ascorbic acid in the liver. Animals poisoned with CCl_4 eat little if any food, so some experiments were carried out to determine the ascorbic acid values on fasting rats. Food was removed for 15 hr during the night before they were killed. The values for ascorbic acid were as high as or higher than normal, and a group of five rats averaged 27·4 mg/100 g.

A remarkable list of drugs, chemically unrelated to one another, cause an increase in the urinary output of ascorbic acid in rats and other animals.[9] Among these Chloretone is especially effective and increases the excretion in rats to amounts up to 100 times the normal range. Therefore, the effect of this drug on the ascorbic acid content of CCl_4 livers was investigated.

Single doses of Chloretone (30 mg) were given by intraperitoneal injection to rats, which were killed 17 hr later. Table 2 shows that this drug increased the ascorbic acid content of the normal liver, a finding which was to be expected in view of its known effect on the urinary excretion of the acid. However, in rats receiving Chloretone with CCl_4 (1 mg/g) the stimulation of the production of ascorbic acid was prevented, and the values resembled those obtained after CCl_4 alone; smaller doses of CCl_4 caused this effect. For instance, in a series of twenty rats, four normal ones gave an average value for the ascorbic acid of the liver of 23·0 mg/100 g. Six rats were given 30 mg of Chloretone alone and the mean value found was 36·6 mg. For four rats which received 30 mg of Chloretone, and 1·0 mg of CCl_4 per g, the value was 7·5 mg; four receiving 0·5 mg of CCl_4 per g gave 10·4 mg/100 g, and two receiving 0·25 mg of CCl_4 per g gave 8·4 mg of ascorbic acid per 100 g. Even increasing the dose of

Chloretone up to 100 mg in three rats did not increase the low ascorbic acid values caused by 0·25 mg of CCl_4 per g.

For comparison, the ascorbic acid content of fatty livers produced by ethionine was determined, using female rats only. Forty hours after the injection, when maximum damage should have occurred,[6] the animals were killed and the livers homogenized. The mean value for the ascorbic acid for four rats was 18·4 mg/100 g of liver (compared to 23·6 for untreated rats and 7·8 for female rats after CCl_4), while the ratio

TABLE 2. THE EFFECT OF CHLORETONE ON THE ASCORBIC ACID CONTENT OF THE LIVER OF RATS TREATED WITH ETHIONINE OR CCl_4

	Nitrogen g/100 g liver	Ascorbic acid mg/100 g liver	mg ascorbic acid / g nitrogen
Chloretone	3·3	36·0	10·9
	3·1	32·5	10·5
	3·1	28·5	9·1
CCl_4	2·15	7·8	3·6
	2·15	7·0	3·3
	3·3	14·0	4·2
CCl_4 and Chloretone	3·1	17·0	5·5
	3·1	14·0	4·5
	3·1	10·5	3·4
Ethionine	2·67	19·3	7·2
	2·70	23·5	8·7
	2·36	15·7	6·6
	2·56	15·0	5·8
Ethionine and Chloretone	2·90	9·5	3·3
	2·67	12·0	4·5
	1·95	12·5	6·4
	2·59	10·0	3·9

ascorbic acid : nitrogen remained within the normal range (Table 2). The effect of ethionine is thus in marked contrast with that of CCl_4.

Chloretone was given to rats which had received ethionine 24 hr previously. The animals were killed 17 hr after the Chloretone injection. The results show that Chloretone, in contrast to its effect on normal or CCl_4 livers, definitely lowers the ascorbic acid content of the livers of the ethionine-treated animals (Table 2).

DISCUSSION

Christie and Judah[2] were able to show that the changes in the vitamin B complex reported by Shils *et al.*[1] in CCl_4-treated rats were related to the leakage of pyridine nucleotides from the mitochondria, and consequent disorganization of the enzymes of the Krebs cycle. The activity of these enzymes could be completely or partially restored by the addition of the coenzymes *in vitro*. Attempts have been made to protect animals by the addition of vitamins to the diet. Hove and Hardin[3], after finding evidence that vitamin E was deficient in CCl_4-poisoned animals, were able to increase the survival rate of rats receiving one intraperitoneal dose of CCl_4 by injecting them with vitamins E and B_{12}. Nowhere, however, is there reference to vitamin C and the

effect of injections of CCl_4 on the concentration of the vitamin in the liver, nor of attempts to alleviate the damage by giving it.

The results shown in this paper indicate that there is a rapid lowering of the vitamin C concentration in the CCl_4-poisoned liver of rats. A few hours after a single intraperitoneal injection of CCl_4 the concentration of ascorbic acid in the liver is reduced; the reduction is maximal between 12 and 17 hr; the values then begin to rise again, but recovery is not complete in 48 hr.

These results are in contrast to those obtained when ascorbic acid was measured in the livers of ethionine-treated rats. In these fatty livers, the vitamin C values per 100 g wet weight were slightly lower than normal, but the ratio of ascorbic acid per gram of nitrogen remained within normal limits. The fatty infiltration without necrosis, typical of ethionine poisoning, does not affect the synthesis of vitamin C by the liver.

Ethionine has been shown to be more toxic for the livers of female rats than for those of males.[10] There is a tendency for the same sex difference under the conditions of these experiments with CCl_4, but the effect is not so marked.

Of the many drugs which increase the urinary output of vitamin C, Chloretone was chosen because it increases the daily vitamin C content of rat urine, up to 100 times the normal range. In guinea pigs, which cannot synthesize vitamin C, the D-glucuronic acid content of the urine rises after barbital,[11] suggesting that there is an increased synthesis of substances which, in most animals, yield ascorbic acid. There is no obvious reason for the increased ascorbic acid formation. Increased formation of glucuronic acid from glucose does not have any known relationship to the metabolism of the group of drugs, which, indeed, have no common chemical structure. Some of them are metabolized, but some are excreted unchanged. Moreover, an increased utilization of glucuronic acid, such as occurs when α-naphthol or borneol are conjugated, does not alter the output of vitamin C. It has been suggested that the effect of the drugs is a hormonal one, for it does not occur in hypophysectomized animals.

In these experiments, Chloretone increased the concentration of vitamin C in the livers of normal rats. However, when given to animals which had received CCl_4, no increase was observed. Even one-quarter the dose usually given was enough to prevent the Chloretone effect.

The effect of Chloretone given to ethionine-treated rats is quite different. In these livers, in which, judging from their ascorbic acid content, the mechanism for the production of ascorbic acid is still intact, Chloretone causes a reduction, rather than an increase, in the amount present. Koch-Weser *et al.*[6] discuss in detail the differences between the lesions of CCl_4- and ethionine-treated animals. Their description shows clearly that the fatty accumulations following the injection of ethionine are not associated with necrosis, nor did they find changes in the activity of certain enzymes. After CCl_4, however, necrotic and regenerative changes were also present, and enzyme activities were altered. These include increased alkaline phosphatase and decreased esterase activity. However, the experiments reported in this paper show that, even in the ethionine-fatty liver, the process by which ascorbic acid is synthesized is not normal. This is also indicated in a recent paper[12] which shows that barbital, which, like Chloretone, increases the excretion of ascorbic acid, does not do so in ethionine-treated rats. The mechanism of the changes cannot be explained.

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