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A Study of Carbon Tetrachloride

VI. Aminoaciduria in Response to Carbon Tetrachloride Inhalation[1]

HERBERT H. CORNISH AND RICHARD C. RYAN

Department of Industrial Health, School of Public Health and Institute of Industrial Health, The University of Michigan, Ann Arbor, Michigan

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A number of studies including those of Cornish and Block (1960) have measured the marked increase in serum enzyme levels following acute exposure to CCL_4 vapor. If it is assumed that these enzymes are being released from damaged tissue, one would anticipate that smaller molecular weight compounds, such as amino acids, would also be released. Increased urinary excretion of free amino acids or alteration of the amino acid excretory pattern might serve as a sensitive means of detecting tissue damage, due to CCL_4 inhalation. For these reasons, it seemed advisable to measure the free amino acid excretion of rats exposed to various concentrations of CCL_4 vapor and to identify the amino acids excreted before and after such an exposure. Simultaneous studies of the free amino acids of the blood would provide information which could be correlated with the pattern of urinary amino acid excretion.

MATERIALS AND METHODS

Specimen collection. Groups of four Sprague-Dawley (Holtzman) male rats, weighing 150–200 g were placed in individual metabolism cages for the collection of urine uncontaminated by feces. The animals were maintained on a normal protein test diet obtained from the Nutritional Biochemical Corporation, Cleveland, Ohio. Twenty-four-hour control urine samples were collected every day for a period of 5 days. The individual urine specimens were adjusted to a pH of 7.0 and made up to a uniform volume of 10 ml. A drop of toluene was added to inhibit bacterial action, and the specimen was refrigerated until used.

Animal exposure. After collection of control urine samples, each group of four animals was exposed to CCl_4 vapors in a dynamic exposure chamber as previously described (1960a). After 6 hours' exposure, the animals were returned to their metabolism cages and 24-hour urine samples again collected for a period of 5 days. Individual groups of 4 rats were exposed to the following levels of CCl_4 vapor: 1000, 2000, 4000, 4500, 9000, 12,000 parts per million.

Analysis. The total α-amino acid nitrogen of the urine was determined by the colorimetric ninhydrin method of Khachadurian *et al.* (1960). Each urine was also analyzed for creatinine content, using the Jaffe reaction. The results are also expressed as the ratio of α-amino acid nitrogen to creatinine nitrogen. The standard deviation was calculated for each control series.

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Preparation of serum. The serum of each rat was dialyzed against distilled water for 16–20 hours. The dialyzate containing the amino acids was then reduced, under vacuum, to the original serum volume. These samples and the urine specimens were used for chromatographic studies. All samples were kept frozen when not in use.

Chromatography. One- and two-dimensional ascending chromatography was carried out on 18 × 22 sheets of Whatman no. 1 paper. The solvent systems utilized were: methanol, *n*-butanol, benzene, water (2:1:1:1); buffer-saturated phenol and *n*-butanol, acetic acid, water (4:1:1). The first two solvents were used for two-dimensional chromatography. Spray reagents included 0.2% ninhydrin in water-saturated *n*-butanol and diazotized sulfanilic acid (Berry *et al.*, 1951; Aronoff, 1956).

Twenty-five reagent grade amino acids in water and in 2% NaCl solution were used as reference solutions. The R_f values of this series of compounds were utilized in the identification of specific amino acids.

Measured amounts of the individual urines were applied to the paper in 3 to 5 separate applications forming approximately an 8-mm spot at the origin. The chromatograms were placed in the solvent and allowed to develop for 16–20 hours. They were removed from the chromatography jar, air dried under heat lamps, and sprayed with a suitable reagent. For two-dimensional chromatography the chromatograms were air dried and then placed in the second solvent for an additional 16–20 hours.

RESULTS

The normal volume of urine produced in 24 hours by one rat was 8–10 ml. This volume increased to 15–20 ml for the first 24 hours after exposure. Subsequent 24-hour samples were of normal volume. The increase in urine volume on the first day after exposure was a consistent finding in all the CCl_4 exposures except at 1000 ppm.

Two of the four rats exposed to 12,000 ppm of CCl_4 died during the inhalation period, and the other two expired within 36 hours.

Analytical

The normal creatinine nitrogen and α-amino nitrogen values for the 28 animals used in this study are compiled in Table 1. The last column indicates the ratio of α-amino N to creatinine N for each animal. Creatinine nitrogen values are quite constant and average 2.80 mg/24 hours. Free amino acid nitrogen values range from 160 to 280 μg/24 hours and average 220 μg/24 hours. The α-amino nitrogen to creatinine nitrogen ratios calculated from the previous two values average 0.081.

Table 2 illustrates the daily amino acid nitrogen to creatinine nitrogen ratios of a series of rats before and after CCl_4 exposure. Each control value represents the 5-day average obtained on each of 4 rats prior to CCl_4 exposure. The subsequent values are the daily averages of the 4 rats after exposure. A 6-hour exposure to 1000 ppm of CCl_4 vapor did not significantly alter the average α-amino acid N to creatinine N ratio of the exposed animals. After exposure to 2000 ppm of CCl_4 vapor two of the four animals had elevated indexes of amino acid excretion, a rise that is discernible in the slightly elevated average ratio on the first day after exposure.

At 4000 ppm and above, all animals had significantly elevated indexes of amino acid excretion on the first, and usually on the second, day after exposure. After an exposure to 9000 ppm of CCl_4 for 6 hours, the α-amino N to creatinine N ratio is

approximately 4 times the normal level on the first day and is still elevated on the second day after exposure. Only two of the four animals exposed to 12,000 ppm of CCl_4 vapor survived for more than 24 hours. The index of amino acid excretion in the two surviving animals, however, is markedly elevated, being approximately 5 times the control levels.

Chromatographic

Paper chromatographic studies of the urinary amino acids allowed an identification of 10 amino acids on the basis of R_f values in the three different solvents, using both

TABLE 1

URINARY CREATININE NITROGEN AND α-AMINO ACID NITROGEN EXCRETION OF NORMAL RATS[a]

Rat no.	Creatinine N, mg/24 hr	α-Amino acid nitrogen	
		mg/24 hr	mg/mg Creatinine N
1	2.85	0.230	0.081
2	2.30	0.190	0.083
3	3.00	0.280	0.093
4	2.40	0.270	0.112
5	2.90	0.250	0.086
6	2.70	0.215	0.080
7	3.00	0.260	0.087
8	2.75	0.205	0.074
9	2.90	0.235	0.081
10	2.65	0.215	0.081
11	2.95	0.230	0.078
12	3.00	0.250	0.083
13	2.85	0.250	0.088
14	3.20	0.220	0.069
15	3.30	0.175	0.053
16	3.65	0.220	0.060
17	2.95	0.160	0.054
18	2.80	0.205	0.073
19	3.00	0.175	0.058
20	3.30	0.190	0.058
21	2.30	0.235	0.102
22	2.45	0.280	0.114
23	2.55	0.245	0.096
24	2.65	0.230	0.087
25	2.55	0.220	0.086
26	2.65	0.215	0.081
27	2.20	0.200	0.091
28	2.40	0.160	0.067
Mean ± SD	2.80 ± 0.30	0.220 ± 0.050	0.081 ± 0.016

[a] Each value is the average of 5 daily determinations.

one- and two-dimensional chromatography. Figure 1 illustrates a typical paper chromatogram of equivalent amounts of rat urine before and after exposure of the animals to 9000 ppm of CCl_4 vapor for 6 hours. Although all amino acids in urine are not increased proportionately, there is a general overall increase in amino acid excretion of exposed over control rats as illustrated in the paper chromatograms (Fig. 1). Since these animals do not eat during the first 24 hours following exposure, it is not an

TABLE 2

URINARY α-AMINO ACID NITROGEN:CREATININE NITROGEN RATIOS OF RATS AFTER CCl_4 EXPOSURE

Days after exposure	1000[a]	2000	4000	4500	9000	12,000
Control	0.086 ± 0.016	0.083 ± 0.016	0.081 ± 0.016	0.062 ± 0.016	0.067 ± 0.016	0.086 ± 0.019
1	0.086	0.118	0.140	0.126	0.245	0.393
2	0.102	—	0.113	0.108	0.153	—
3	—	0.100	0.089	0.094	0.086	—
4	0.097	0.081	0.097	0.086	0.073	—
5	0.086	0.100	0.078	0.078	0.091	—

[a] Level of CCl_4 vapor in parts per million.

inconsistent finding to note also that an occasional urinary amino acid appears to be decreased 24 hours after exposure. In Table 3 are shown the qualitative changes in amino acid composition as visually detected on paper chromatograms of urine and blood serum. The qualitative scale is from one plus (barely detectable) to four plus (maximum intensity) as seen with the ninhydrin spray reagent. A minus sign indicates

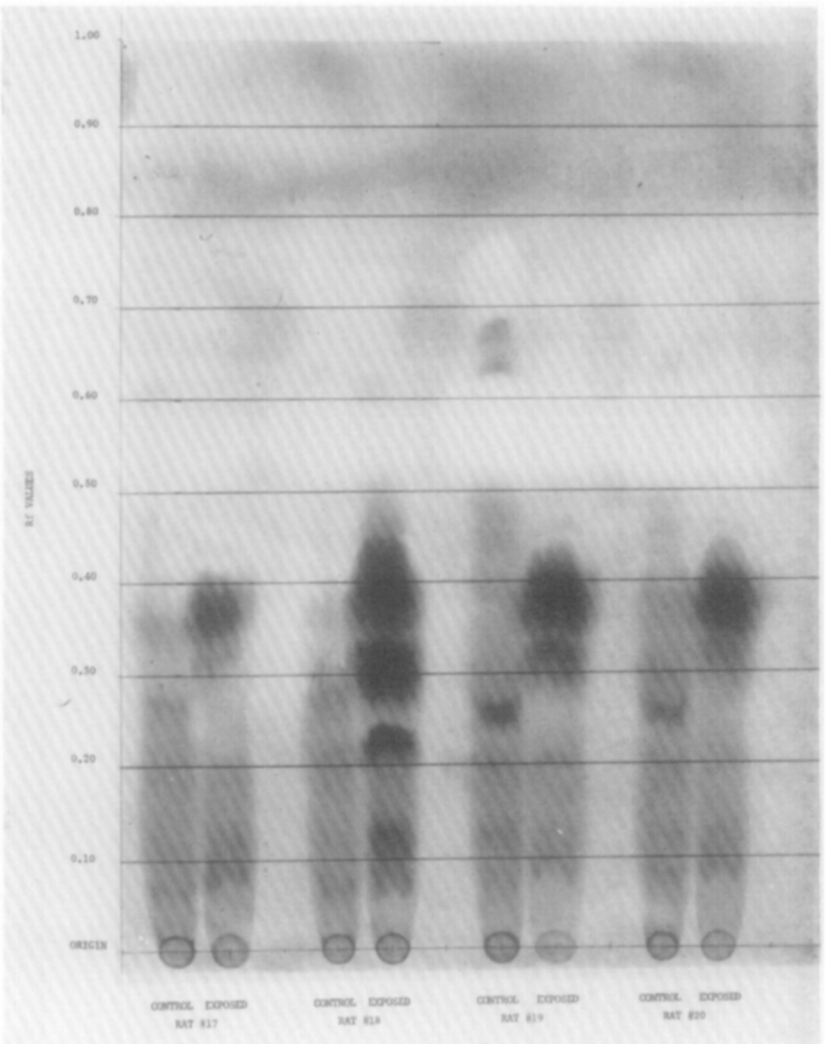

FIG. 1. Paper chromatogram of equivalent amounts of rat urine before and after exposure of rats to 9000 ppm of CCl_4 vapor for 6 hours. Color developed with ninhydrin spray reagent. Solvent: methanol, *n*-butanol, benzene, water (2:1:1:1).

that this amino acid was not detected under the chromatographic conditions utilized in this study; it does not imply that this free amino acid does not exist in the urine or serum samples. The amino acids identified in urine show a generalized increase following exposure to 9000 ppm of CCl_4 for 6 hours. It is of interest to note that there occurs simultaneously an increase in the free amino acid concentration of the serum (Table 3) and that the specific amino acids increased in the urine are also

found to be elevated in the serum. Glutamic acid, glycine, threonine, and alanine are among those amino acids showing major increases in both the urine and the serum.

DISCUSSION

Since the 24-hour creatinine excretion is relatively constant for any one animal, the α-amino nitrogen to creatinine nitrogen ratio serves as a direct index of the variation in amino acid excretion. The α-amino N:creatinine N ratio as determined in this study (0.081 ± 0.016) is approximately 50% of the value (0.17 ± 0.045) reported by Khachadurian *et al.* (1960) for a series of human subjects.

A report by Knauf and Windsheimer (1960) showed that plasma amino acid and urine amino acid levels of rats were increased following an intraperitoneal injection of 0.55 ml CCl_4 per 100 g of body weight. However, this study does not give any indication as to the dose-response relationships or of the possible use of these findings in detecting tissue damage due to CCl_4 inhalation.

TABLE 3

FREE AMINO ACID LEVELS BEFORE AND AFTER EXPOSURE TO CCl_4 VAPOR[a]

	Urine		Serum	
Amino acid	Control	Exposed	Control	Exposed
Glutamic acid	+	++++	—	++
Glycine	++	++++	+	++
Alanine	+	+++	—	++
Threonine	+	+++	—	+
Aspartic acid	+	++	—	+
Histidine	+	++	+	++
Taurine	++	++	—	++
Tyrosine	+	++	—	++
Tryptophan	—	++		
Leucine-isoleucine	+	++	—	+

[a] Exposure to 9000 ppm for 6 hours.

In the present studies α-amino nitrogen to creatinine nitrogen ratios are not altered in animals exposed to 1000 ppm of CCl_4 for 6 hours. From 2000 to 4500 ppm, there is a gradual increase of the ratio above control values. At higher levels of exposure, 9000 and 12,000 ppm for 6 hours, the α-amino nitrogen to creatinine nitrogen ratio is markedly increased with ratios as great as 5 times those of the controls. Although amino acid excretion is definitely elevated following CCl_4 exposure, the present findings would indicate that this is not as sensitive an index of CCl_4 exposure as serum enzyme changes. A 6-hour exposure to 1000 ppm of CCl_4 vapor was without effect on total α-amino acid excretion, whereas a 4-hour exposure to 1000 ppm results in a marked increase in serum enzyme levels (Cornish and Block, 1960).

Identification of the amino acids excreted after CCl_4 exposure indicated that the excretory pattern was not unusual. Both before and after CCl_4 exposure, the amino acids excreted in the greatest amounts include glutamic acid, glycine, threonine, alanine, histidine, tyrosine, and taurine.

The increased excretion of free amino acids in the urine could result from kidney damage or from the elevation of free amino acids in the blood; thus the findings in the serum are of considerable interest. Since the serum free amino acids were also ele-

vated, this could well account for the increased urinary excretion of these compounds. A number of studies have demonstrated a relationship between serum and liver enzyme activities in CCl_4-exposed animals; thus one would also suspect a similar origin for the elevated free amino acids of serum. Tissue damage (presumably liver), due to the CCl_4 exposure could account for the release of free amino acids into the blood stream and the subsequent increased excretion of amino acids in the urine. It is interesting to note that the major free amino acids of tissues, including liver are, in general, the same group of amino acids found to be elevated in the serum and urine of rats exposed to CCl_4 vapor.

Although the free amino acids of the blood and urine are considerably elevated after exposure of rats to CCl_4 vapors, a relatively high exposure is necessary before these variations can be detected. The simultaneous increase of free amino acids in both serum and urine would suggest that at this time, the increased free amino acid excretion is not due to kidney damage and failure to reabsorb, but merely reflects the elevated free amino acid levels in the blood stream. The specific free amino acids excreted in increased quantities in the urine are those which are found to be elevated in the serum. One obvious source of these serum free amino acids is their release from damaged tissue cells and, on the basis of previous studies, one would suggest that damaged liver is the major source of these amino acids. Whether such a release of free amino acids is due to altered permeability or to actual cell death cannot be determined.

SUMMARY

The α-amino acid nitrogen to creatinine nitrogen ratio of normal rats on an ad libitum diet is quite constant and provides a reliable method for determining alterations in amino acid excretion.

Exposure of rats to high levels of CCl_4 vapor results in increased urinary excretion of free amino acids. There is a simultaneous increase in the corresponding free amino acids of the blood suggesting that increased urinary excretion is a reflection of elevated levels of free amino acids in the blood. The suggestion is made that the source of the elevated serum free amino acids is damaged tissue and, on the basis of the hepatotoxic action of CCl_4, one would expect damaged liver cells to be the major source of these free amino acids.

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