THE EFFECT OF BENZENE AND OF CARBON TETRACHLORIDE ON THE CONCENTRATION OF CERTAIN VITAMINS, FAT, AND NITROGEN IN THE LIVER OF THE RAT*

BY

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This study is concerned with the effects of short term moderate dosages of benzene and of carbon tetrachloride on the content of the liver with respect to vitamins, fat, and nitrogen. The experiments were performed because of our interest in the problem of whether exposures to certain toxic agents of industrial concern alter the nutritional needs of the organism. A change in the ability of an organ or tissue to retain normal amounts of a nutrient is one mechanism by which nutritional needs may be altered. That this mechanism actually exists has been demonstrated with certain carcinogens. Thus Goerner and Goerner (1939) found that the hepatic stores of vitamin A were diminished by dibenzanthracene. A similar effect on riboflavin stores of liver in animals treated with butter yellow (Kensler, Young, and Rhoads, 1942; Miller, 1947), and with acetylaminofluorene has been noted (Griffin, Cook, and Luck, 1949; Wase and Allison, 1950).

Experimental Methods and Procedure

Analytical Methods.—The following methods were used.

Nitrogen.—The micro-Kjeldahl determination was employed with the distillate collected in 2% boric acid and ammonia measured titrimetrically.

Lipid.—Fatty acids and unsaponifiable matter were measured gravimetrically using a procedure essentially that of Leathes and Raper (1925) adapted to a small scale.

Vitamin A.—The procedure was essentially that of Lemley, Brown, Bird, and Emmett (1947).

Thiamine (Vitamin B₁).—Bound thiamine was released from liver tissue by treatment with clarase (diastase) in acetate buffer as described by Cheldelin, Eppright, Snell, and Guirard (1942). The vitamin was measured fluorometrically by a modification of the procedure of Hennessy (1941). To help avoid interfering substances which may not be adequately compensated for in the usual non-ferricyanide treated blank, we have employed benzene sulfonyl chloride as suggested by Urban and Goldman (1944). This substance prevents the formation of thiochrome, and its use allows both the blank and the unknown to be treated with alkaline ferricyanide. In treating the blank, the benzene sulfonyl chloride is added before ferricyanide, whereas with the unknown it is added after ferricyanide.

Riboflavin.—Bound riboflavin was released in the manner described for thiamine and the free vitamin was determined by the microbiological method of Snell and Strong (1939).

Niacin.—Homogenized liver was suspended in 1 N sulphuric acid autoclaved at 15 lb. pressure for 15 minutes, cooled, and brought to volume. When ready for assay, an aliquot was neutralized and assayed by the microbiological procedure of Krehl, Strong, and Elvehjem (1943).

Procedure.—Three groups of male rats* were maintained on a pellet stock diet† from weaning until they had reached 60 days of age. They were then placed on a purified diet (Diet 11) of the following composition: casein, 20‡; corn oil, 10; cerelese, 66; salts, 4§; with vitamins incorporated per 100 g. of this

* Rats were randomly distributed from seven litters with as nearly equal distribution as possible of litter-mates among the three groups. Since some of the litters were not matched in each group, the statistical analysis employed treated the mean values of the groups as coming from independent samples.

† Rockland Rat Diet ("D" free).

‡ Borden B3F.

§ USP XIII No. 2.

* This study was aided by the Division of Research Grants and Fellowships of the U.S. Public Health Service.

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basal diet as follows: thiamine, 1 mg.; riboflavin, 1 mg.; pyridoxine, 1 mg.; niacin, 1 mg.; calcium pantothenate, 2.5 mg.; folic acid, 0.2 mg.; biotin, 0.002 mg.; choline, 50 mg.; percomorph oil, 0.12 ml.

Eight days later the treatments were started. Group I, the control group, received 0.3 ml. per rat of an 0.9% sodium chloride solution subcutaneously on the same days that group II was injected subcutaneously with 0.3 ml. of benzene per 100 g. of bodyweight, and group III received subcutaneously 0.1 ml. carbon tetrachloride per 100 g. bodyweight. Injections were given on seven of the following nine days. On the tenth day all rats were killed by exsanguination and their livers weighed. Small samples of liver were taken for histological examination and the remainder immediately wrapped and frozen in solid CO₂ until analyzed.

At the conclusion of the experiment Group I consisted of nine rats, Group II of 11 rats, and Group III of 11 rats. Group I rats were restricted in their food intake to maintain weight at approximately the levels of the benzene and CCl₄ treated rats.

Results

In Table 1 are listed the average bodyweights of the three groups during the experiment together with average liver weights expressed on the basis of total bodyweight and per 100 g. of bodyweight. The control animals lost weight slightly as a result of food restriction. The benzene group decreased in weight to a greater extent, while the CCl₄ group maintained weight. The CCl₄ treated group had the heaviest livers, but the benzene-treated rats also had livers which were heavier than those of the controls. Calculation of liver weights per 100 g. of bodyweight did not change this order. Statistical analysis of the liver weights by the "t" test indicated that the differences among

TABLE 1
AVERAGE BODY AND LIVER WEIGHTS* OF THE EXPERIMENTAL GROUPS

Group	Control	Benzene	CCl ₄
Number of rats	9	11	11
At 1st day	176	199	187
„ 6th „	168	190	192
„ 10th „	170	172	192

* Liver and body weights expressed in grams ± = standard error of the mean.

Total liver weight 5.72 ± 0.23 7.16 ± 0.23 9.68 ± 0.29

Liver weight per 100 g. bodyweight 3.36 ± 0.060 4.17 ± 0.096 5.08 ± 0.180

Difference (g.) 0.81 1.72 0.91

Significance (P) <0.001 <0.001 <0.001

groups were highly significant on the basis of both the total and per 100 g. bodyweight (Table 1).

In Table 2 are summarized the results of the analyses for thiamine, riboflavin, niacin, vitamin A, nitrogen, and lipid, all expressed per gram of liver. It is clear that the benzene treatment had no significant effect on the concentration of the vitamins and of the fat; the nitrogen concentration of this group was decreased slightly but significantly below that of the controls. Carbon tetrachloride treatment, on the other hand, caused a significant drop in the concentration of all vitamins and of nitrogen. As was expected, the lipid content increased markedly, and this increase in fatty material undoubtedly accounts for some of the

TABLE 2
EFFECT OF BENZENE AND CARBON TETRACHLORIDE ON LIVER CONCENTRATION OF VITAMINS, NITROGEN, AND LIPID*

Group	No. of Rats	Thiamine (γ)	Riboflavin (γ)	Niacin (γ)	Vitamin A (I.U.)	Nitrogen (mg.)	Lipid (mg.)
I. Control	9	14.6 ± 0.85	38.3 ± 1.84	195.9 ± 11.6	838.4 ± 47.1	27.9 ± 0.55	45.6 ± 1.1
II. Benzene	11	12.8 ± 0.94	33.8 ± 2.79	180.7 ± 5.7	836.1 ± 35.3	25.3 ± 0.79	45.0 ± 1.6
III. CCl ₄	11	9.4 ± 0.52	23.3 ± 2.53	134.5 ± 4.9	676.5 ± 36.1	20.1 ± 0.64	166.9 ± 5.9
Mean Differences between Groups and their Significance							
Between control and benzene	diff. P	1.8 0.18	4.5 0.18	15.2 0.23	2.3 0.55	2.6 0.02	0.6 0.55
Between control and CCl ₄	diff. P	5.2 <0.001	15.0 <0.001	61.4 <0.001	161.9 0.01	7.8 <0.001	121.3 <0.001
Between benzene and CCl ₄	diff. P	3.4 <0.001	10.5 0.01	46.2 <0.001	159.6 0.004	5.2 <0.001	121.9 0.001

* Mean per g. of liver ± Standard error of the mean.

differences in vitamin and nitrogen concentrations.

Calculations made on the basis of estimated fat-free liver weight brings the nutrient concentrations of the CCl_4 -treated rats closer to those of the controls (Table 3). However, with the exception of vitamin A, the differences are still considerable. Further calculation on the basis of equal nitrogen content per gram of liver brings the concentrations of the water-soluble vitamins of the two groups much more closely into line with each other (Table 3).

TABLE 3
LIVER CONCENTRATION OF VITAMINS AND NITROGEN OF CCl_4 TREATED RATS* (PERCENTAGE OF CONTROL)

Basis	Thiamine (%)	Riboflavin (%)	Niacin (%)	Vitamin A (%)	Nitrogen (%)
Wet weight*	64.4	61.0	68.8	80.7	72.1
Fat-free†	74.9	71.0	80.3	95.3	83.9
Equal N‡	90.0	84.9	95.0	112.7	100.0

* As actually determined from Table 2.

† Fat free concentrations calculated by multiplying concentrations on wet weight basis by factor of $100 \div (100 - \text{mg. neutral fat})$. Neutral fat value taken as 110% of lipid value as given in Table 2.

‡ The observed values of CCl_4 treated livers (Table 2) were multiplied by 1.39; i.e. N content of controls $\div$ N content of CCl_4 treated rats.

Despite the differences observed in concentration of nutrients per gram of liver, calculation of the total liver content revealed that the CCl_4 group had as much or more of each of the nutrients compared to the benzene and control groups (Table 4). The content of the water-soluble vitamins was not significantly different in the three groups, but there was a significantly greater amount of fat, vitamin A, and nitrogen in the larger liver of the CCl_4 -treated rats. The same tendency was noted for the latter three substances in the livers of the benzene-treated animals; fat and vitamin A content were significantly higher than in the controls while the nitrogen increase was of doubtful significance ($P = .07$).

Histological examination of the livers of a number of the rats was made following fixation in Bouin's solution and staining with hematoxylin-eosin. In all seven of the 11 rats treated with CCl_4 , whose livers were examined, there was marked fatty infiltration; six showed varying degrees of necrosis of the central half of the liver lobule while the seventh animal had all of the lobule involved except a narrow zone in the portal area. Of the six benzene-treated animals examined, the liver showed no obvious changes in four, while in two there was a slight fatty infiltration of cells about the central veins. Livers of the nine control rats appeared normal.

Discussion

The interesting point in this study is that despite rather marked reductions in the vitamin concentrations per gram of liver of CCl_4 -treated rats, the overall contents in the liver were as great as, or greater, than in the controls. One question that arises is whether the vitamins in the damaged livers are as available to the organism as those of normal animals. Some attention has been given to this problem by other investigators in the case of vitamin A. Popper, Steigmann, and Dyniewicz, (1942) studied the distribution of this vitamin, using fluorescence microscopy, in the livers of CCl_4 -treated rats. They noted that the vitamin tended to accumulate rather quickly in areas of fatty degeneration and less rapidly in normal sites. Chemically, less vitamin A was stored in the liver of vitamin A-deficient rats with CCl_4 intoxication than in non-poisoned control deficient rats following vitamin A feeding. They suggest that the CCl_4 -damaged liver releases its vitamin A more slowly than does normal hepatic tissue with resulting lowered plasma levels.

Haig and Post (1941) analyzed the livers of rats for vitamin A three months following an eight-week period of CCl_4 administration. They found no correlation between the concentration of vitamin A in the cirrhotic livers, the liver weight, or the degree of fibrosis; however, the livers of the

TABLE 4
EFFECT OF BENZENE AND CARBON TETRACHLORIDE ON NUTRIENT CONTENT OF TOTAL RAT LIVER*

Group	Mean Liver Weight (g.)	Thiamine (γ)	Riboflavin (γ)	Niacin (γ)	Vitamin A (I.U.)	Nitrogen (mg.)	Lipid (mg.)
I. Control	5.72	83 ± 5.1	219 ± 12	1114 ± 78	4789 ± 317	162 ± 7.4	257 ± 12
II. Benzene	7.16	91 ± 6.1	240 ± 19	1291 ± 51	5957 ± 251	181 ± 6.7	325 ± 10
III. CCl_4	9.68	91 ± 6.0	226 ± 15	1298 ± 55	6523 ± 468	193 ± 6.5	1622 ± 117

* (Mean ± Standard Error.)

treated animals contained much less vitamin A than did those of the controls. These studies suggest the need for serial studies of plasma vitamin A levels, liver vitamin A content, and histological structure in order to correlate vitamin availability and storage with the changing morphology of the damaged and healing liver.

One would expect much higher vitamin A concentrations in the livers of the CCl₄-treated animals than in those of the controls if vitamin A is deposited preferentially in areas of fatty degeneration, as Popper and others (1942) have described. Our results were to the contrary, but we draw no definite conclusions on this aspect, since vitamin A intake was not rigidly controlled in our study. It should be noted also that there did not appear to be any correlation between total liver fat and total vitamin A. The CCl₄-treated rats had an increase in total fat above the controls of over 500% associated with a vitamin A increase of 36% ; the benzene-treated rats had an increase in total fat above controls of only 26% associated with an increased vitamin A of 24%.

Summary

The effects of moderate subcutaneous doses of benzene and of carbon tetrachloride administered over a period of nine days have been investigated with respect to changes in the concentration and total content of thiamine, riboflavin, niacin, vitamin A, fat, and total nitrogen in the livers of rats.

Carbon tetrachloride caused a marked increase in liver weight and liver fat. On the other hand, there was a significant decrease in the concentration of the vitamins and nitrogen per gram of liver.

Benzene treatment had no significant effect on the concentration of vitamins, but was associated with a slight decrease in nitrogen concentration and some increase in liver weight.

Because of the increased weight of the livers in the CCl₄-treated rats, the total amounts of the water-soluble vitamins in the livers of this group were about the same as in the livers of the benzene and control groups. Total vitamin A was greater in the CCl₄- and benzene-treated groups than in the controls, but the increases were not proportional to change in total liver fat.

Histologically, the changes in nutrient concentration in the livers of the CCl₄-treated rats were associated with marked fatty infiltration and necrosis of the central half of the liver lobule. Of the benzene-treated rats whose livers were studied, there were no obvious changes in the majority, while some showed slight fatty infiltration about the central veins.

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