BIOCHEMICAL CHANGES IN CARBON TETRACHLORIDE FATTY LIVER

Concentration of Carbon Tetrachloride in Liver and Blood

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Christie and Judah,[1] and Dianzani[2-5] have pioneered in the application of modern biochemical methods to the investigation of the hepatocellular lesions occurring in experimental liver injury. However, the main conclusion reached by these workers—viz., that the primary action of a hepatotoxic agent such as carbon tetrachloride is to cause a breakdown of mitochondrial function in the liver cells—has not been confirmed in a number of laboratories. Calvert and Brody[6] found very definite fatty infiltration of the liver within 5 hours after the administration of carbon tetrachloride; yet these same workers found it difficult to observe functional alterations in the mitochondria before 20 hours. Intraperitoneally administered ethylenediamine tetraacetate (EDTA) provided partial protection against the disturbances in mitochondrial function seen at 20 hours, but fatty degeneration was nevertheless observed. In an interesting study of biochemical changes in experimentally induced liver injury, Neubert and Maibauer[7] found in carbon tetrachloride poisoned rats that a microsomal enzyme catalyzing the detoxication of aminopyrine was strongly depressed whereas mitochondrial oxidative phosphorylation remained intact. In a series of investigations[8-10] carried out in this laboratory on biochemical alterations in carbon tetrachloride poisoning, the development of marked pathologic changes in the liver mitochondria has been amply confirmed. However, the time of onset of the increase in liver fat consistently precedes by many hours the time of onset of the mitochondrial alterations. One of the difficulties in this complex problem is the uncertainty as to the optimum time following intoxication when the experimental measurements should be made, especially with reference to biochemical studies designed to reveal the earliest changes at the enzymic level.

From this point of view, it appeared to us that a study of the concen-

This work was supported by research grants A-329 (C4) and A-1489 from the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, United States Public Health Service.

Received for publication, August 3, 1959.

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tration of carbon tetrachloride in the liver as a function of the time following administration would serve as a useful frame of reference for the orientation and interpretation of studies of morphologic and biochemical changes accompanying the development of the hepatic lesions. This communication presents the results of such a study.

Experimental Methods

Determination of Carbon Tetrachloride

A number of analytic procedures have been described for the quantitative estimation of carbon tetrachloride and other halogenated hydrocarbons.[11-14] These methods are based on the colorimetric determination of the Fujiwara chromogen formed when carbon tetrachloride in pyridine is heated in the presence of aqueous sodium hydroxide. Initially, we experienced poor replication and wide variation in color development from day to day. The difficulties were traced to the instability of the Fujiwara chromogen, the marked effects of impurities on the course of the reaction, and the volatility of carbon tetrachloride. The final procedure developed was as follows:

Pyridine (Baker Analyzed Reagent, 5 pounds) was redistilled from 30 gm. of sodium hydroxide (Mallinckrodt Analytical Reagent pellets), until colorless. Sulfur-free toluene was prepared by extracting 10 volumes of toluene (Merck reagent grade) with 1 volume of concentrated sulfuric acid 3 times. The toluene was then redistilled twice, first with anhydrous potassium carbonate and then from calcium hydride. Acetone (Merck reagent grade) was redistilled, first from potassium permanganate and then from potassium carbonate. The pyridine, toluene and acetone were stored in dark bottles. The carbon tetrachloride used was Mallinckrodt analytical reagent (low sulfur).

Carbon Tetrachloride Standard Solution. An aqueous carbon tetrachloride reference solution was necessary to carry out recovery experiments. The reference solution was prepared by adding 10 ml. of carbon tetrachloride below 100 ml. of de-ionized water in a glass-stoppered bottle, previously cleaned with dichromate-H_2SO_4. Use of detergents yielded unreliable reference solutions, presumably due to fine aqueous carbon tetrachloride emulsions. Jarring or agitation of the solution was avoided. Diffusion equilibrium at the above ratio of carbon tetrachloride to water was reached in 72 hours. Reference solutions prepared by vigorous mixing of 10 ml. of carbon tetrachloride and 100 ml. of water, although they eventually reached the same concentration as reference solutions prepared by diffusion equilibrium, were not used since they steadily declined in apparent carbon tetrachloride concentration for 7 days. Reference solutions prepared by diffusion equilibrium were stable

for 4 weeks (the longest period tested). The reference solutions were stored in the dark at 20° C. The concentration of carbon tetrachloride at this temperature was taken as 0.8 mg. per ml. of aqueous phase. The solubility of carbon tetrachloride changes approximately 1 per cent per degree C. in the range 0° to 37°; therefore, small changes in the temperature of the carbon tetrachloride reference solution can be tolerated.

Colorimetric Procedure

The color reaction was carried out in 50 ml. glass-stoppered Pyrex Erlenmeyer flasks. Additions were made in the following order: 10 ml. of pyridine; 1 ml. of water*; 3 ml. of 35 per cent potassium hydroxide w/w prepared immediately before each analysis; 0.5 ml. of toluene; 0.15 ml. of acetone; 0.05 to 0.5 ml. of aqueous carbon tetrachloride standard, corresponding to 0.04 to 0.4 mg. of carbon tetrachloride. For unknowns, the carbon tetrachloride enters the reaction in the 0.5 ml. of toluene addition. The toluene, acetone, and carbon tetrachloride standard were added well beneath the surface of the mixture in the flasks, which were kept stoppered between additions. The Fujiwara chromogen was developed by heating at 70° C. for 40 minutes in a water bath equipped

TABLE I

STABILITY OF FUJIWARA CHROMOGEN

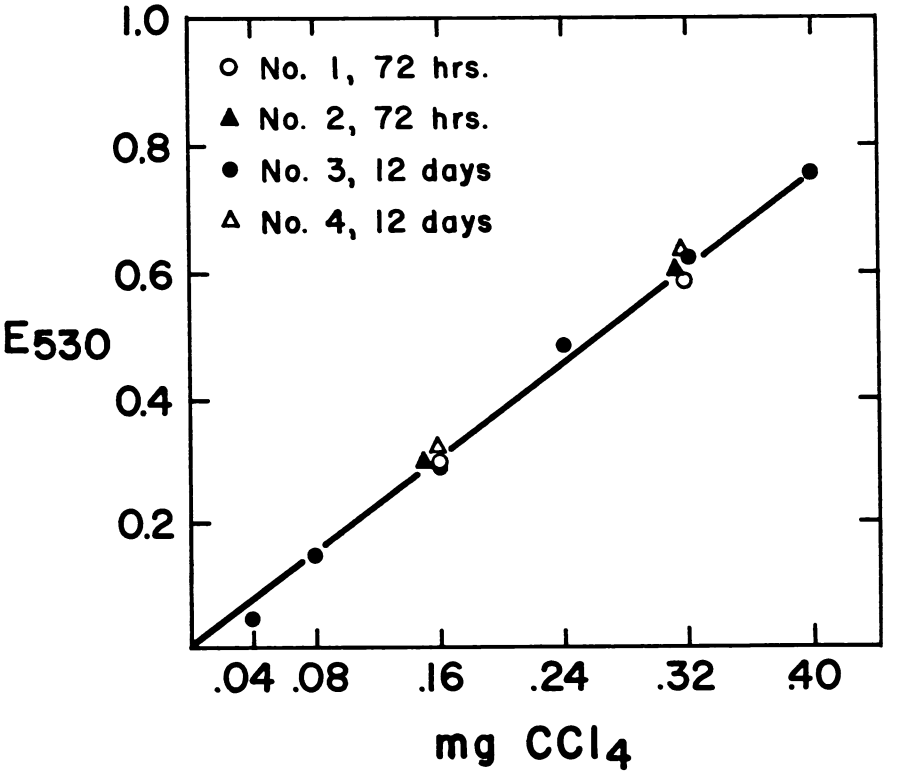
	Optical density at 530 mμ		
CCl_4 mg.	Initial reading	1 hour	3 hours
Blank	0.010	0.000	0.000
0.08	0.180	0.170	0.155
0.08	0.190	0.190	0.180
0.16	0.360	0.360	0.333
0.16	0.395	0.390	0.360
0.32	0.720	0.750	0.700
0.32	0.770	0.700	0.640
0.40	0.875	0.860	0.800
0.40	0.900	0.860	0.800

Conditions: Color developed as in Colorimetric Procedure. Initial readings were taken immediately after mixing 2 ml. aliquots of the pyridine phase, containing the chromogen, with 0.5 ml. of 0.01 N NaOH in the colorimeter tubes.

with a shaker (3.8 cm. excursion, 90 cycles per minute). The red color develops in the upper pyridine phase. After incubation, the tubes were placed in an ice bath, the contents transferred to 22 by 150 mm. glass-

* For analysis of reference solutions, the total volume of water per aqueous carbon tetrachloride reference solution was 1 ml.

stoppered test tubes, and the phases allowed to separate. Two ml. of the upper phase were added to 0.5 ml. of 0.01 N sodium hydroxide in 13 by 100 mm. culture tubes calibrated to serve as colorimeter tubes. The contents of the colorimeter tubes were mixed and the tubes stoppered with corks. Optical density was read at 530 mμ in a Coleman Jr. spectrophotometer, Model 6A.



TEXT-FIGURE 1. Beer-Lambert plots for 4 carbon tetrachloride standard solutions. The elapsed times for preparation to analysis are indicated. Each point represents the mean of duplicate samples.

Stability of the Fujiwara Chromogen. In the absence of added alkali, samples of the final chromogen dissolved in pyridine frequently faded and turned yellow. The fading reaction occurred much more rapidly with stirring or if air was blown through the solution. Stabilizing conditions for the Fujiwara chromogen were achieved by mixing the pyridine phase containing the chromogen with a small amount of alkali, and by keeping the tubes stoppered. The data of Table I indicate the complete absence of color in the blank when read against water, and the reproducibility of the method on duplicate samples from the same carbon tetrachloride reference solution.

Comments on Analytic Procedure

Color development was incomplete when the concentration of potassium hydroxide in the 3 ml. addition of alkali was below 30 per cent or greater than 40 per cent. The observation[12] that addition of a small amount of acetone greatly enhances color development has been confirmed. A concentration of acetone higher than that recommended will depress color development. Since toluene enters the final reaction mixture from the Conway microdiffusion employed for the analysis of carbon tetrachloride in biologic material (see below), the effect of this additive was investigated. It is a fortunate circumstance that 0.5 ml. of toluene, which is a convenient aliquot in the Conway microdiffusion method, proved to be optimum for development of the Fujiwara chromogen under conditions recommended. Heating above 70° or for less than 40 minutes was suboptimal.

Reproducibility and Range of Analysis of the Method. Linearity between color density and carbon tetrachloride content was observed when 0.04 to 0.4 mg. of carbon tetrachloride are present during the chromogenic reaction. Analysis of 4 separate aqueous carbon tetrachloride reference solutions is given in Text-figure 1. It is evident that the preparation and analysis of standard aqueous solutions of carbon tetrachloride by the method described is an adequately reproducible procedure for study of the carbon tetrachloride content of tissues.

TABLE II

RECOVERY OF CARBON TETRACHLORIDE FROM LIVER HOMOGENATES BY CONWAY MICRODIFFUSION METHOD

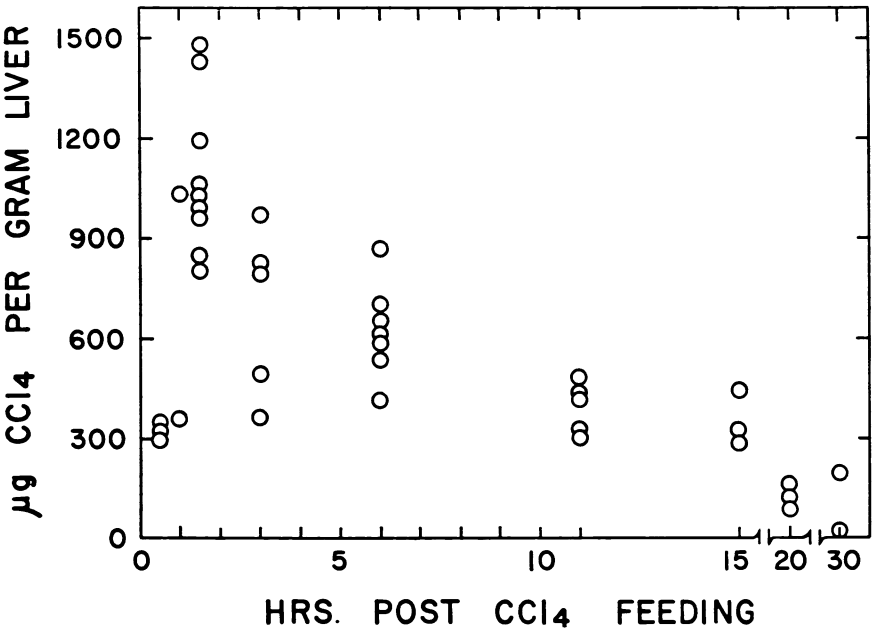
CCl_4 added (μg.)	Number of determinations	CCl_4 recovered (μg.)	Per cent recovery
160	4	66	41
320	15	156	49
640	15	358	56
800	13	438	55
		Weighted mean recovery:	52%

Conditions: Known amounts of carbon tetrachloride were added as aqueous reference solution to liver samples from normal rats, homogenized, and carried through the analysis.

Extraction of Carbon Tetrachloride from Liver and Blood

Food was removed from male rats (The Holtzman Company, Madison, Wisconsin) 10 to 12 hours before carbon tetrachloride feeding. A 1:1 mixture of carbon tetrachloride in mineral oil was administered via stomach tube at a dose of 0.5 ml. of the mixture per hundred gm. of body

weight. Three weighed samples of liver (0.5 to 1 gm.) were taken from each rat. Each sample of liver was homogenized for 30 seconds in 3 ml.



TEXT-FIGURE 2. Concentrations of carbon tetrachloride in rat liver. The values found at each time period were compared with the values found at 1.5 hours in the ranking test (T test) of C. White as given in Snedecor.[28] The corresponding probabilities were: 0.5 hours, $p = 0.01$; 1 hour, $p > 0.05$; 3 hours, $p < 0.05$; all subsequent times, $p \leq 0.05$. Each point represents the mean of 3 determinations on a single rat.

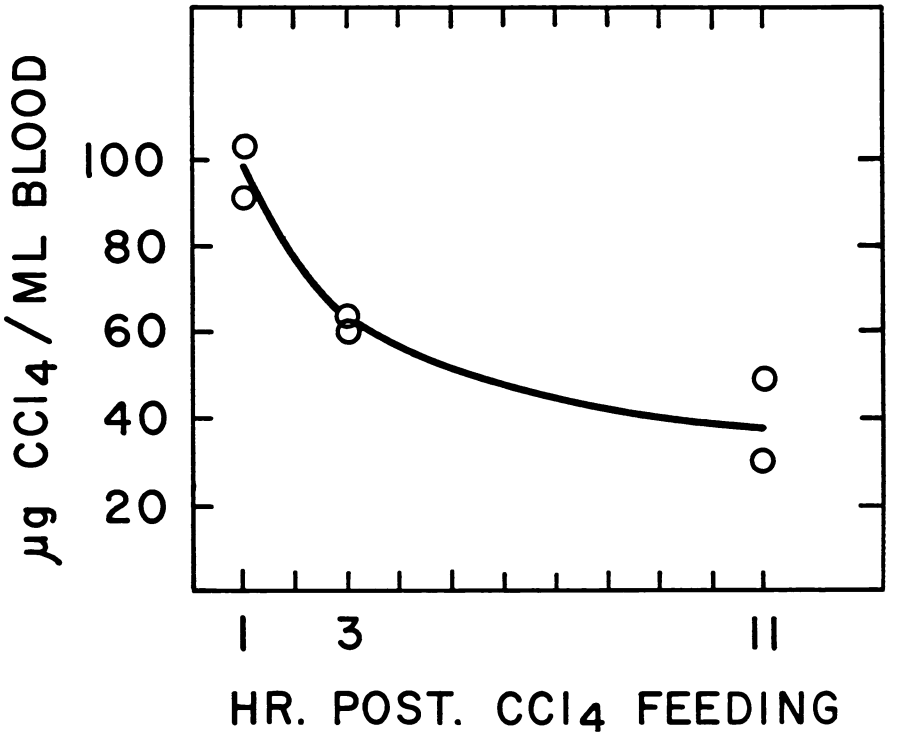
of water in a Virtis 45 homogenizer. The resulting homogenate was transferred to the outer well of a Conway microdiffusion cell[15] which contained 1 ml. of toluene in the center well. The cells were sealed immediately with gum tragacanth paste[16] and gently shaken at the lowest possible speed of an Eberbach shaker (62 cycles per minute, 3.8 cm. excursion). Diffusion equilibrium was reached in 2 hours. A 0.5 ml. aliquot of the toluene phase was analyzed for carbon tetrachloride. The data of Table II show that there was an overall mean loss of 48 per cent when known amounts of carbon tetrachloride, added as aqueous carbon tetrachloride reference solution, were carried through the entire homogenization and microdiffusion procedure. The data of Text-figure 2 have been corrected for this loss, most of which occurs during the homogenization step. For analysis of carbon tetrachloride in blood, 2 ml. of blood were removed from the dorsal aorta in a heparinized syringe and added to 2 ml. of water in the outer well of a microdiffusion cell containing 1 ml. of toluene in the center well. Further procedure was as for liver. The mean recovery of known amounts of carbon tetrachloride

from blood in 18 tests was 81.9 per cent (standard deviation 8.96). The data of Text-figure 3 have been corrected for this loss.

Results

Content of Carbon Tetrachloride in Rat Liver

The time course of the rise and fall of the liver content of carbon tetrachloride following intubation into rats is shown in Text-figure 2. The data indicate that the content of carbon tetrachloride in the liver reached a maximum 1.5 hours following intubation. In control experiments it was shown that the recovery of carbon tetrachloride from homogenates



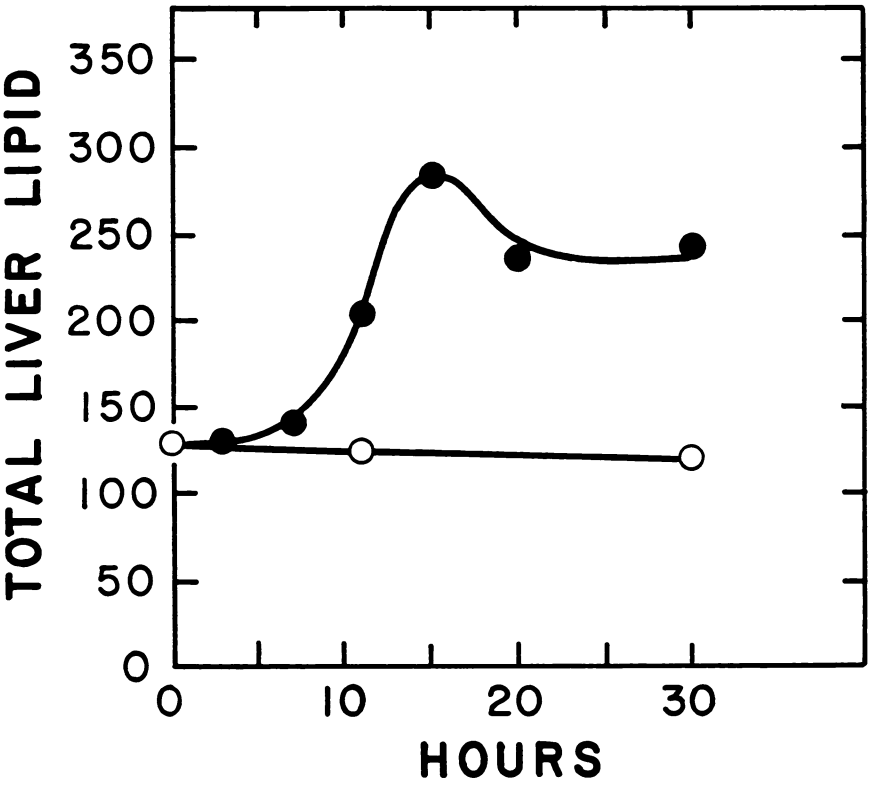
Text-figure 3. Concentration of carbon tetrachloride in rat blood. Each point represents the mean of duplicate determinations on a single rat.

of fatty livers was equal to that from normal livers; therefore, the decline after 1.5 hours is real and not due to failure to recover the carbon tetrachloride because of the increasing fat content. Furthermore, the drop in carbon tetrachloride concentration is too abrupt in comparison to the gradual rise in the liver content of fat.

Content of Carbon Tetrachloride in Rat Blood.

Data on the content of carbon tetrachloride in rat blood are shown in Text-figure 3. Comparison of the data in Text-figures 2 and 3 reveals

that at 1.5 hours carbon tetrachloride is concentrated in the liver approximately 13-fold with respect to arterial blood.



TEXT-FIGURE 4. Rise in liver lipid following carbon tetrachloride feeding. The ordinate represents mg. of total liver lipid per 100 gm. of rat. For the rats fed CCl_4 (upper curve), each point represents the mean of data from 3 rats. For the rats not fed carbon tetrachloride, the number of rats used were: zero, 4 rats; 11 hours, 2 rats; 30 hours, 1 rat. All lipid determinations were done in duplicate or triplicate by the method of Folch, Lees and Sloane-Stanley.[24] Food was withdrawn from the rats receiving no CCl_4 10 to 12 hours before zero time, as in the CCl_4 fed rats.

Rise in Lipid Content of the Liver

In Text-figure 4 are shown data on the rise of the lipid content of the liver in the rats used in these studies. In a subsequent study in which a minimum of 6 rats was analyzed at each time interval, the decline at 20 hours was not observed.

DISCUSSION

The significance of the data presented in this communication is that they form a basic frame of reference, in terms of which studies relevant to the underlying biochemical mechanisms involved in carbon tetra-

chloride fatty liver may be evaluated. For example, biochemical tests for mitochondrial damage based on failure of pyridine nucleotide-dependent oxidations, uncoupling of oxidative phosphorylation (measured indirectly), and a transformation in adenosine triphosphatase properties, have shown [1,9,17] that gross loss of mitochondrial function does not occur until about 20 hours after carbon tetrachloride feeding. A physiologic test system, viz., the ability of potassium depleted mitochondria to reaccumulate potassium,[10] has also indicated that mitochondrial degeneration does not set in until many hours after the peak level of carbon tetrachloride in the liver has been reached. If carbon tetrachloride *per se* is the toxic agent, then the data of Text-figure 2 suggest that the primary hepatic lesion occurs very early following carbon tetrachloride feeding. On the basis of these considerations, the theory [2,3] that loss of mitochondrial function is the key lesion must be seriously doubted.

A variety of studies do indeed indicate that biochemical and physiologic changes occur early following carbon tetrachloride administration. India ink fails to enter the hepatic sinuses and centrilobular veins within the first 2 to 4 hours [18] following subcutaneous injection of carbon tetrachloride into rats. This observation, along with other considerations, led to the theory [18] that vascular involvement and centrilobular ischemia were the key changes in carbon tetrachloride hepatotoxicity. However, evidence against the vascular theory has come from more recent studies of hepatic blood flow measured in unanesthetized, carbon tetrachloride-poisoned rats by the use of a heated thermocouple.[19] These investigations showed that there was no reduction in hepatic blood flow during the period when carbon tetrachloride was producing necrosis. Other evidence for and against the vascular theory has been reviewed.[20] Rosin and Doljanski [21] reported that as early as one hour there was evidence that the centrilobular parenchymal cells were free of pyroninophilic granules, suggesting a loss of cytoplasmic ribonucleic acid. A statistically significant increase in liver fat was observed in rats 3 hours following carbon tetrachloride feeding.[9] An increase in liver weight was evident at 4.5 hours. Further support for the view that the primary biochemical lesion occurs very early following carbon tetrachloride administration comes from the work of Oberling and Rouiller,[22] who examined hepatic cytologic changes following carbon tetrachloride poisoning with the electron microscope. Although some alterations in the mitochondria were noted, these authors emphasized that during the early stages of the lesion, the action of carbon tetrachloride on the mitochondria was much less marked than on the ergastoplasm. The experiments of Neubert and Maibauer,[7] which showed a marked decline in a microsomal enzyme in carbon tetrachloride-poisoned rat liver at a time when mitochondrial

function appeared to be normal, complement at the enzymic level the morphologic observations of Oberling and Rouiller. These observations strongly suggest that the endoplasmic reticulum is one of the earliest subcellular structures to be pathologically altered in carbon tetrachloride poisoning. By comparison of the midpoints in the curves for carbon tetrachloride content as compared to lipid content, the rise in lipid lags about 9 hours behind the rise in carbon tetrachloride. Whatever the primary biochemical lesion proves to be, it appears evident to us that studies of possible derangements in the normal pattern of hepatic physiologic and biochemical functions, suspected of being decisive with respect to the increase in fat, should be carried out during and immediately following the period when the peak concentration of carbon tetrachloride in the liver is reached.

Summary

The concentration of carbon tetrachloride in the liver and blood of the rat following oral administration has been determined. The concentration of carbon tetrachloride in the liver rises rapidly to a maximum level at 1.5 hours, then falls continuously. The maximum concentration in the liver at 1.5 hours is 10 times the concentration at 20 hours. At its maximum concentration in the liver, carbon tetrachloride is concentrated 13-fold with respect to the amount present in arterial blood. The significance of these findings for the relationship of mitochondrial damage to carbon tetrachloride fatty liver is discussed.

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