The Effect of Carbon Tetrachloride Poisoning on Serum Lipoproteins Associated with Atherosclerosis

By F. T. Pierce, M. D., and J. W. Gofman, M. D., Ph. D.

Carbon tetrachloride was injected into normal rabbits and the levels of $S_f$ 3–12, 12–20, and 20–40 classes of lipoproteins were determined during the course of these injections. A marked increase in concentration of these three classes of lipoproteins occurred during the administration of carbon tetrachloride. After the cessation of this drug, the lipoproteins of largest $S_f$ rate returned to normal first with those of lower $S_f$ rate returning to normal levels in the order of decreasing $S_f$ rates. Rabbits fed cholesterol were also injected with carbon tetrachloride, and their lipoproteins continued to increase after cessation of this drug.

Ultracentrifugal studies reported by Gofman and co-workers have revealed certain lipoproteins in the serum of rabbits and humans that are associated with the development of atherosclerosis. Studies now in progress in this laboratory are directed toward elucidation of the factors responsible for the maintenance of the blood levels of the various lipoproteins. The probable role of the liver as an important organ involved in lipid metabolism prompted the study of the effect of impaired liver function on the levels of the lipoproteins in the blood. Carbon tetrachloride (CCl₄) has long been known for its ability to produce a fatty liver and eventual hepatic cirrhosis. This agent has also been shown to be capable of producing an elevation in the total serum cholesterol levels in the rabbit. It was felt, therefore, that the nature of lipid transport (in terms of the lipoproteins involved) in animals treated with carbon tetrachloride might provide information as to certain factors, at least, involved in maintaining blood lipoprotein levels.

The normal rabbit shows ultracentrifugally the presence of lipoproteins characterized by flotation rates in the range of $S_f$ 3–12.* A few apparently normal rabbits show, in addition, very low concentrations of lipoproteins with flotation rates $S_f$ 12–20. During cholesterol feeding experiments rabbits in general show increases in the $S_f$ 3–12 components, followed by the development of very high levels of other lipoproteins of flotation rates greater than 12 $S_f$ units and up to flotation rates over 100 $S_f$ units. In general the molecules of higher $S_f$ rates develop following a prior increase in those of the lower $S_f$ rates, for example, appreciable concentrations of $S_f$ 20–40 lipoproteins appear only after significant levels of $S_f$ 12–20 components have appeared. These previous studies have indicated that the extent of atherosclerosis developing in such rabbits parallels the level of the $S_f$ 12–30 class of lipoproteins. These studies also indicated that the molecules

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* One $S_f$ unit represents a flotation rate of $1 \times 10^{-13}$ cm. per sec. per dyne per Gm. in a sodium chloride solution of density 1.063 Gm. per cc. at 26 C. The lipoproteins were previously isolated from other serum proteins (and high density lipoproteins) by a preparative ultracentrifugation, Spinco Model L and Model E ultracentrifuges were used.

† In the original report by Gofman and associates, the normally occurring lipoproteins in rabbits migrated with rates less than 10 $S_f$ units. However, since some rabbits are now known to have normal components migrating at rates up to 12 $S_f$ units, the class of “abnormal” lipoproteins is so chosen as always to exclude the normally occurring lipoproteins.
of successively higher flotation rates are of successively lower densities and of lower protein content per molecule. It is entirely possible that the lipoprotein molecules of higher S\textsubscript{f} values than 12 occur normally in the lipid metabolic pathways, but are maintained at such low levels in the blood as to escape resolution ultracentrifugally or to be demonstrable at very low levels. The studies reported below suggest the nature of the interrelationships of several of these lipoprotein classes involved in lipid transport.

Arbitrarily, the ultracentrifugal diagrams have been analyzed into three broad classes of lipoproteins, the S\textsubscript{f} 3–12 group (which include those normally appearing in rabbits), the 12–20 class, and the 20–40 class.

Two general types of experiments were performed. In one group, the effect of carbon tetrachloride injections alone on the blood lipoprotein pattern was determined. In a second group, the combined effect of carbon tetrachloride injection plus cholesterol feeding was studied. Female rabbits, weighing between 2 and 4 Kg., of the New Zealand white strain were used in all experiments. Seven of 14 animals originally started on carbon tetrachloride injections survived the entire 10 week period of study. Three of these were fed cholesterol throughout the entire period and 4 were on a normal diet (Albers family style rabbit pellets). The cholesterol food was prepared by dissolving 1 Gm. of cholesterol in 8 cc. Wesson Oil, which was then thoroughly mixed with 100 Gm. rabbit pellets.

After a control blood specimen was drawn, carbon tetrachloride was injected subcutaneously using a dose of 1 cc. per Kg. These injections were given twice a week for five and one-half weeks (a total of 11 injections). Blood specimens were obtained at weekly intervals for 10 weeks at which time the experiment was terminated and the animals sacrificed.

Serum was analyzed ultracentrifugally for lipoproteins and by the Schoenheimer-Sperry method for free and total cholesterol.*

**RESULTS AND DISCUSSION**

A summary of the results obtained is given in tables 1 and 2. The data of table 1, which summarize the effect of carbon tetrachloride alone on the serum lipoproteins, show a consistent trend of events in all 4 animals. The serum level of the normally occurring S\textsubscript{f} 3–12 class of lipoprotein molecules is invariably increased in concentration during the course of carbon tetrachloride injections. Coincident with this increase is the appearance of considerable levels of both the S\textsubscript{f} 12–20 and 20–40 classes of molecules. This sequence of changes is the same as that found in a rabbit fed cholesterol.

In other words, carbon tetrachloride in rabbits not fed cholesterol is capable of mimicking the lipid and lipoprotein alterations of the serum found when rabbits are fed cholesterol. This represents the synthesis of these “abnormal” giant cholesterol-bearing molecules (of the S\textsubscript{f} 3–12 group).
12–20 and 20–40 classes) from endogenous sources alone. Parallel with this rise in these three classes of lipoproteins during carbon tetrachloride administration is a concomitant rise in serum cholesterol levels and an increase in the ratio of free to total cholesterol.

When the carbon tetrachloride injections were stopped, an interesting sequence of events occurred during the recovery of the animals from carbon tetrachloride poisoning. As can be seen from table 1, the S1 20–40 class of molecules returned to relatively low levels within two weeks of the last injection of carbon tetrachloride. At this time the concentration of the S1 3–12 and 12–20 classes was still elevated, and, in some instances, the S1 12–20 class showed a moderate increase in concentration. By four weeks after the last injection of carbon tetrachloride, however, the S1 12–20 class of molecules returned to approximately control levels, although the S1 3–12 class was still considerably above the original levels before carbon tetrachloride treatment was begun. At the end of the experiment the concentration of the S1 3–12 class had begun to decrease toward control levels.

During the recovery phase, the ultracentrifugal photographs portray the disappearance of molecules of high S1 value first followed by those of progressively lower S1 value. (See fig. 1.) For example, within the S1 12–20 class, the species at the higher S1 range decrease before those of the lower S1 range do.

None of these non-cholesterol fed animals showed any atherosclerosis in spite of the transient elevation of the S1 12–20 and 20–40 classes of molecules during carbon tetrachloride administration. However, the concentration of the S1 12–40 molecules in rabbits fed cholesterol and developing atherosclerosis is considerably higher than was observed in this experiment. Hence macroscopic atheroma would not be expected in the carbon tetrachloride injected rabbits in the short experimental period studied.

Table 2 shows the sequence of events in a carbon tetrachloride injected rabbit being fed cholesterol. The increase in the three classes of molecules analyzed occurs actually more slowly than in a normal rabbit fed cholesterol at the dosage used. However, these animals were rapidly losing weight during the injections and ate very little of the food presented to them. After the last injection of carbon tetrachloride, large quantities of the S1 12–20 and 20–40 classes of lipoproteins developed very rapidly.

<table>
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<tr>
<th>Rabbit</th>
<th>Day from Start of Experiment</th>
<th>Lipoproteins, mg. %</th>
<th>Cholesterol, mg. %</th>
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<tr>
<td></td>
<td></td>
<td>S-10</td>
<td>12-20</td>
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<td>11</td>
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* Last injection given on day 39.
† Given no CCl₄ from the second to the third week because of extreme jaundice and caecalitis. This was the only animal which became jaundiced.

while the S1 3–12 class remained the same or diminished. The serum cholesterol of this group of animals similarly increased during the carbon tetrachloride injections. After the last injection of carbon tetrachloride, the cholesterol levels increased rapidly to very high levels as the animals began to eat more food and consequently increased their intake of cholesterol. All these animals fed cholesterol as well as injected with carbon tetrachloride and which survived the 10 week period showed atherosclerosis.

It should be pointed out that normal untreated rabbits not fed cholesterol consistently show the distribution of lipoproteins as illu-
Fig. 1. A. Ultracentrifugal photographs showing the flotation of low density lipoproteins of a normal rabbit. Each frame is ruled for calculation of the $S_f$ rate of any peak appearing in that frame. In this and all the other series of photographs, successive frames are at 0, 6, 12, 22, 30, and 38 minutes after the ultracentrifuge rotor has reached full speed (52,640 revolutions per minute). Consequently, these $S_f$ markings can be used on corresponding frames in all the series of photographs below. In this pattern, the low density lipoproteins from 5 cc. of serum were concentrated into 1 cc. by preparative ultracentrifugation and then analyzed in the ultracentrifuge as described above.

B. Flotation pattern of low density lipoproteins of a rabbit after two weeks of carbon tetrachloride injections, showing markedly increased levels of lipoproteins. The low density lipoproteins from 3 cc. of serum were concentrated into 1 cc. in this pattern. Consequently, the increase in lipoproteins is 67 per cent greater than represented by these photographs when compared with the pattern above.

C. Flotation pattern of low density lipoproteins from a rabbit one week after the cessation of carbon tetrachloride injection. This shows the return toward normal levels of the lipoproteins of $S_f$ 20—40 class, with those lipoproteins of higher $S_f$ value disappearing first. The lipoproteins of 3 cc. serum were concentrated into 1 cc. in this pattern.

D. Flotation pattern of low density lipoproteins from a rabbit two weeks after the cessation of carbon tetrachloride injections. The molecules of the $S_f$ 20—40 class are greatly reduced in concentration, and those of the $S_f$ 12—20 class are disappearing with those of higher $S_f$ value disappearing first. The lipoproteins of 5 cc. of serum were concentrated into 1 cc. in this pattern.

E. Flotation pattern of low density lipoproteins from a rabbit four weeks after the cessation of carbon tetrachloride. Molecules of the $S_f$ 20—40 and 12—20 classes have returned almost to normal levels and the 3—12 class is beginning its return to normal levels. The lipoproteins of 5 cc. of serum were concentrated into 1 cc. in this pattern.
stromed in day 0 (control levels) in the rabbits shown in tables 1 and 2. Repeat samples drawn
from a normal rabbit show very little variation from week to week. Consequently the experi-
mental changes reported are in marked con-
trast to these consistent low levels.

Rabbits injected with alloxan also show a transitory rise of serum cholesterol¹ and of
the $S_t$ 3–12, 12–20 and 20–40 and even higher
classes of lipoproteins.² This substance pro-
duces acute liver damage with the development
of a fatty liver. In rabbits fed cholesterol, fatty livers are also produced. The increase,
often to very high level, in these various classes
of lipoproteins which may be present normally
in very small quantities may be a reflection of
the inability of the damaged liver to handle
endogenous or exogenous cholesterol (and other
lipids) in the synthesis of the “normal” protein-
lipid complexes and in their degradation, by
whatever means it occurs. However, the more
widespread toxic effects of carbon tetrachloride
render it impossible to exclude involvement of
other organ systems in the lipoprotein meta-
bolic abnormality.

The changes in free:total cholesterol ratios
which occur parallel with the rise and fall in
level of the $S_t$ 12–20 and $S_t$ 20–40 classes
of lipoproteins provide some information relative
to internal structural features of the various
lipoproteins. In the animals receiving carbon
tetrachloride injections only, the free:total ra-
tio is less than 0.25 at the outset when the
lipoproteins present are predominantly in the
$S_t$ 3–12 class. As the lipoproteins of the $S_t$
12–20 and 20–40 classes appear in progressively
increasing concentration, the free:total ratio
rises significantly. This shows that these classes
of lipoproteins differ structurally from the $S_t$
3–12 class in containing a higher proportion of
the cholesterol of the molecule in the nonesteri-
fied state as fractionated by the Schoenheimer-
Sperry method. These data are in harmony
with the data on human lipoproteins which
show progressively higher free:total ratios with
increasing $S_t$ rate of the lipoprotein species.³

SUMMARY

1. Normal and cholesterol fed rabbits were
injected with carbon tetrachloride and their
serum cholesterol levels and lipoproteins of the
$S_t$ 3–12, 12–20, and 20–40 classes measured
during and after the injections.

2. Carbon tetrachloride produced a marked
increase above control levels in all classes of
lipoproteins and in cholesterol in the non-cho-
lesterol-fed rabbit. These substances gradually
decreased to control levels after the cessation
of carbon tetrachloride injections. No macro-
scopic atherosclerosis developed in this non-
cholesterol fed group of animals as was pre-
picted by the relatively low concentration of
the $S_t$ 12–40 class of molecules present for
this limited amount of time.

3. In the cholesterol fed rabbit, cholesterol
and all classes of lipoproteins increased during
carbon tetrachloride injections but continued
to increase after the cessation of carbon tetra-
chloride injections. Very large quantities of
serum lipoproteins and cholesterol developed
by the end of 10 weeks, and at this time all
animals had developed atherosclerosis.

4. The data suggest that the increase in the
normally occurring $S_t$ 3–12 class and the
appearance of high concentrations of $S_t$ 12–20
and 20–40 classes of lipoproteins may occur
as a result of impaired function of the degrada-
tion and synthetic system (possibly in the liver)
involved in the metabolism of these molecules.

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