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THE EFFECT OF CHRONIC CARBON TETRACHLORIDE INHALATION ON THE SECRETION OF LIPID BY RAT LIVER*

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Abstract—The effect on hepatic metabolism of chronic daily 8-hr exposures of rats to 68 or 680 ppm CCl_4 vapor was studied. After 6 days of exposure there is an elevation of serum glutamic oxaloacetic transaminase and hepatic triglyceride concentration and a decrease in hepatic glycogen levels. Intravenous administration of palmitate-^{14}C at the end of the fifth daily exposure period to 68 ppm CCl_4 revealed a decrease in the rate of release of labeled lipid from the liver when compared to that in control animals. This was reflected in a decrease in the amount of labeled triglycerides in the serum of the intoxicated animals. After exposure to 680 ppm for 5 daily 8-hr periods, leucine-1-^{14}C and palmitate-9,10-^{3}H were injected i.v. and the serum lipoprotein fraction was isolated. A significant decrease in the radioactivity of both lipid and protein moieties in the low density lipoproteins was noted. It was concluded that, as is the case in acute CCl_4 intoxication, chronic inhalation of CCl_4 leads to an accumulation of hepatic triglycerides and an inhibition of secretion of low density lipoproteins.

A GREAT deal of work has been done on the mechanism of production of fatty liver after carbon tetrachloride administration.[1] It has been demonstrated that the fatty liver resulting from acute intoxication with CCl_4 is secondary to a failure of the liver to secrete triglycerides in the form of low density lipoprotein.[2–4] The latter in turn may be due to an inhibition of protein synthesis[5] following a dislocation of the polyribosomes from the endoplasmic reticulum by CCl_4,[6] either as a result of peroxidation of the lipid membranes[7,8] or a dissociation of the ribosomes due to a release of ribonuclease from lysozomes.[9]

Most of the data leading to theories of the action of CCl_4 have been obtained after acute administration of the toxin either i.p. into the gastrointestinal tract or with slices or subcellular particles. Relatively little work has been done on the effects of chronic CCl_4 intoxication, although industrial exposure to CCl_4 is usually chronic in nature. It has been established that chronic intoxication results in liver cirrhosis[10,11] with the alteration of various enzymes in serum. Thus serum prothrombin levels[12] and esterase activity[13] are decreased. In addition, increases in the rat serum levels of xanthine oxidase and serum glutamic oxaloacetic transaminase (SGOT)[14] and in rabbit serum glutamic pyruvate transaminase (SGPT), isocitric, glutamic, malic and lactic dehydrogenases, aldolase and phosphohexose isomerases were noted after chronic intoxication.[15]

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In contrast to the fatty liver and decreased lipoprotein secretion noted after acute administration of CCl_4, chronic intoxication has been reported to cause a decrease in liver lipids[16] and an increase in serum lipids.[17] It is possible that the latter results are due to prolonged exposure times. In the light of the development of our understanding of the mechanism of fatty liver formation after acute CCl_4 intoxication, a study of the lipid levels and rates of secretion from the liver in rats exposed to CCl_4 daily over periods of 5 days was felt to be of value. The results of these investigations are presented in this communication.

METHODS

Male hooded rats (obtained from Quebec Breeding Farms, St. Eustache, Quebec), weighing between 180 and 200 g and maintained on Purina chow, were used in all experiments. Animals were exposed in groups of four to mixtures of air and CCl_4 vapors for daily 8-hr periods in an exposure chamber consisting of a large desiccator equipped with a central air supply, an air exit and a water supply. The flow of air was measured by flowmeters placed before and after the exposure chamber and regulated with a needle valve. The CCl_4 was injected into the airstream at a steady rate by a dual infusion pump (Harvard Apparatus Inc., Dover, Mass.). Changes in the concentration of CCl_4 (ppm) were achieved by either increasing or decreasing the pump speed and the rate of air flow. The air supply line was constricted in order to increase the rate of air flow, thus insuring complete evaporation of the CCl_4. Control animals were exposed under identical conditions except that no CCl_4 was added to the air supply line. It was found that the CCl_4-exposed animals lost their appetite during exposure. Thus both the control and CCl_4-exposed rats were deprived of food but allowed water *ad libitum* during the exposure periods.

During the study of lipid metabolism the animals were given, under nembutal anesthesia, an injection of 2·1 μmole albumin-bound palmitate-1-^{14}C containing 2×10^6 cpm/100 g body wt. via the jugular vein after the final 8-hr period of exposure. In some experiments, where it was desired to follow the secretion of the protein moiety of lipoproteins, 1·2 μmole of L-leucine-1-^{14}C containing $2{\cdot}6 \times 10^6$ cpm/100 g body wt. was injected via the jugular vein, followed 30 min later by 2·1 μmole albumin-bound palmitate-9,10-^{3}H containing $2{\cdot}6 \times 10^6$ cpm/100 g body wt. We have previously noted that under these conditions the maximum radioactivity levels in the lipid and protein moieties of the serum lipoproteins tend to coincide.[18] The first blood samples were then drawn 30 min after the palmitate injection. It has also been demonstrated that the lipoprotein secretion and turnover pattern in any individual animal is constant from week to week.[18] Therefore, all animals used in experiments in which the effect of chronic CCl_4 intoxication upon lipoprotein secretion was to be studied were first exposed for 5 days in air as controls and their lipoprotein secretion pattern was established. One week after the end of the control experiment, the same animals were subjected to a second exposure using CCl_4.

Blood samples were collected from the tail vein, allowed to clot and the serum was removed quickly. Serum glutamic oxaloacetic transaminase activity was determined according to the method of Reitman and Frankel.[19] Glycogen was isolated by precipitation with alcohol after digestion of the liver tissue in 30% KOH, then dissolved and reprecipitated several times and analyzed colorimetrically using the anthrone reagent.[20] Liver lipids were extracted by the method of Folch *et al.*[21] and free fatty

acids were removed by an alkaline wash. Neutral glycerides and phospholipids were separated on silicic acid columns and the ester bonds determined by the method of Rapport and Alonzo.[22] Serum triglycerides were isolated, counted and measured colorimetrically by the method of van Handel and Zilversmit[23] after removal of the free fatty acids by the Borgstrom technique.[24] Albumin-bound palmitate was prepared according to the procedure of Milstein and Driscoll.[25] Separation of serum lipoprotein fractions was carried out by the method of Havel *et al.*[26] in an International B-60 preparative ultracentrifuge with an SB-405 rotor and an average centrifugal force of 360,000 *g*. Aliquots of the lipoprotein fractions were dried on filter paper strips and the radioactivity was measured in a Tri-Carb liquid scintillation counter at a setting for the simultaneous counting of ^{14}C and ^{3}H with efficiencies of 64 and 10 per cent respectively.

Radioisotopes were obtained from New England Nuclear Corp., Boston, Mass. and SGOT kits from Sigma Chemical Company, St. Louis, Mo.

RESULTS

In order to assess the degree of liver damage being caused by the chronic exposure to CCl_4, the serum transaminase levels of animals exposed to 68 and 680 ppm CCl_4 vapor for daily periods of 8 hr were studied. The results shown in Fig. 1 indicate that,

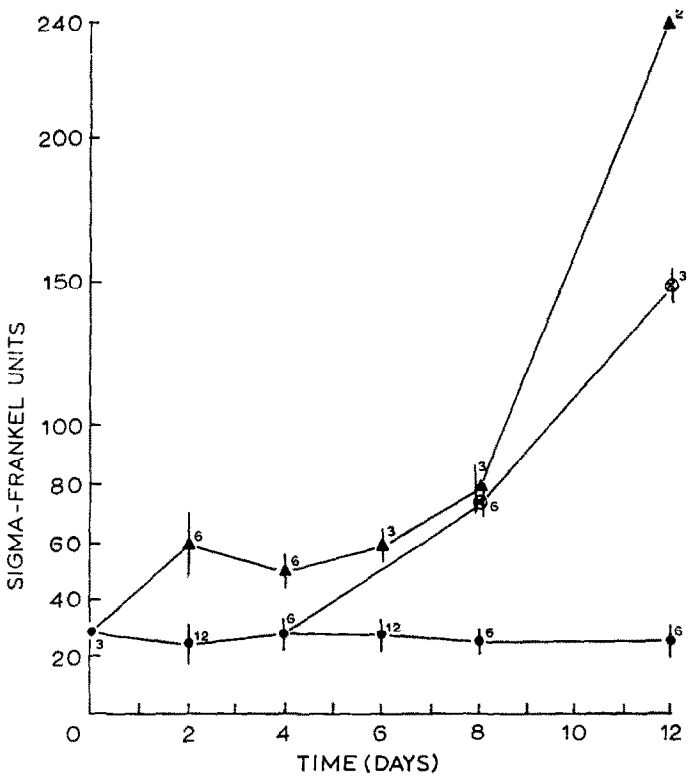
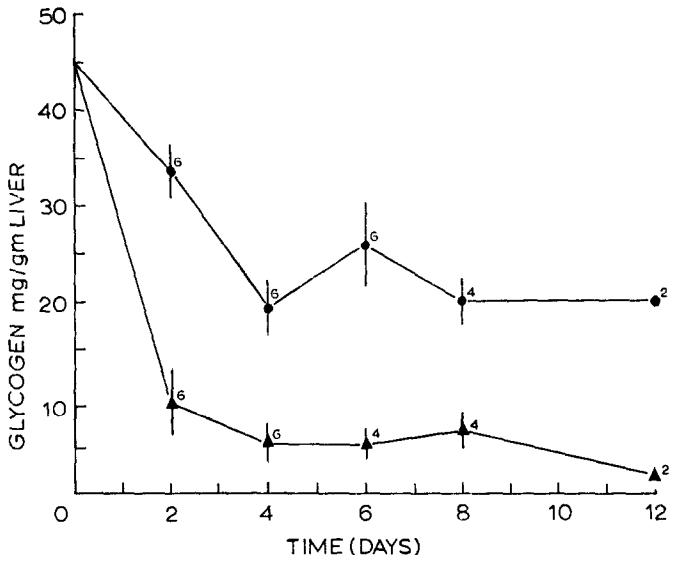
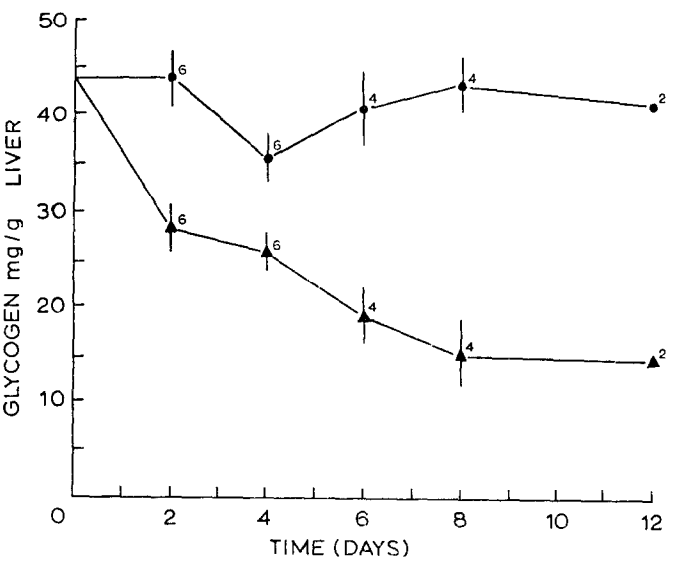


FIG. 1. Rat serum glutamic oxaloacetic transaminase levels during 12 days of daily exposure for 8 hr to CCl_4 vapors. The numbers on the curves refer to the number of animals; the vertical bars represent one S.E.M. ● = Control; ⊗ = exposed to 68 ppm CCl_4; ▲ = exposed to 680 ppm CCl_4.

after exposure to 680 ppm CCl_4, a rise in SGOT was noted after 2 days and that a further significant rise did not occur until the twelfth day of intoxication. With one-tenth the dose, no change in SGOT activity was noted for 4 days after which a rise in activity occurred.



(a)



(b)

FIG. 2. Liver glycogen levels after daily exposure for 8 hr to CCl_4 vapors: (A) immediately after the last exposure; (B) 16 hr after the last exposure. The numbers on the curves refer to the number of animals; the vertical bars represent one S.E.M. ● = Control; ▲ = exposed to 680 ppm CCl_4.

We had earlier noted that one of the first changes following acute CCl_4 intoxication was a decrease in the level of liver glycogen.[27] The effect of the chronic intoxication upon the liver glycogen levels is shown in Fig. 2(a) and 2(b). Fig. 2(b) illustrates the fall in liver glycogen after successive days of exposure. It will be noted that the level of hepatic glycogen fell in both the intoxicated and control animals, stabilizing in the latter after 4 days. The glycogen level at the start of the experiment was obtained by fasting the animals for 8 hr in their cages. We assume that the stabilization of the glycogen at a lower, but constant, level represented an adaptation to the regular 8-hr periods of food deprivation. In the case of the animal exposed to 680 ppm CCl_4, there was a marked fall in the level of glycogen with stabilization after 4 days at one-tenth the initial level. The curves of glycogen levels obtained with 68 ppm, not shown in the figure, were similar to that shown with 680 ppm. The influence of the 8-hr daily period of food deprivation compared to that of the CCl_4-intoxication was further studied by measuring the glycogen levels of both control and exposed animals the following morning, after the animals had had 16 hr during the night for food consumption. The food intake of both the control and CCl_4-exposed animals was similar during the nightly 16-hr periods. The results shown in Fig. 2(b) indicate that the control animals readily restored their glycogen levels to normal after each period of exposure. Under these conditions, the capacity of the intoxicated animals to deposit glycogen was diminished and the level was never restored to normal, although some glycogen was laid down.

Fatty livers are one of the prime results of acute CCl_4-intoxication. Hence the level of liver lipids found after the chronic inhalation used in our experiments was studied. The results shown in Fig. 3 indicate that there is a rapid rise in the level of liver fatty acid esters in the liver, the level of lipid being related to the dose. However, with both

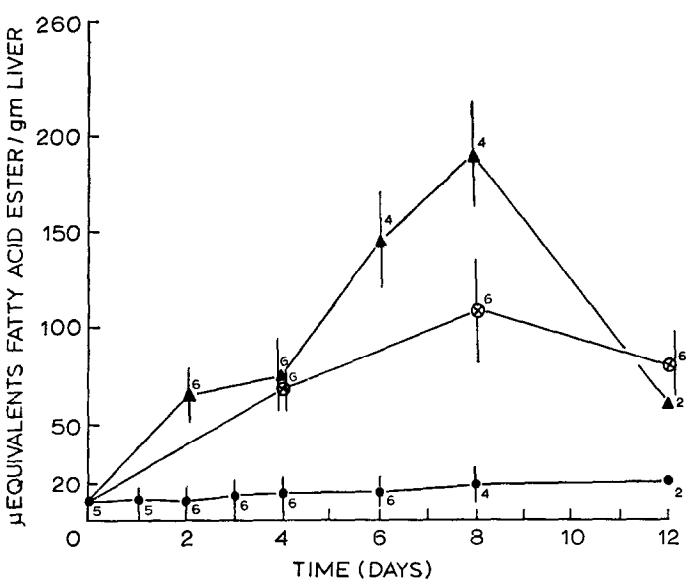


FIG. 3. Hepatic neutral glyceride ester levels after daily exposure for 8 hr to CCl_4 vapors. The numbers on the curves refer to the number of animals; the vertical bars represent one S.E.M. ● = Control; ⊗ = exposed to 68 ppm CCl_4; ▲ = exposed to 680 ppm CCl_4.

doses the level of lipid in the liver reached a maximum after 8 days; additional exposure resulted in a decrease. No significant change in the control liver lipid level was noted. Although not shown in the figure, the levels of lipid after the nightly 16-hr recovery period with food intake were somewhat higher than that seen immediately after exposure.

In order to study the handling of lipid by the livers from intoxicated animals, palmitate-1-^{14}C was administered i.v. to rats after 5 days of daily 8-hr exposure to 68 ppm CCl_4. The accumulation of the i.v. administered palmitate-1-^{14}C in the neutral lipids of the liver at various time intervals is shown in Table 1. It will be noted that the radioactivity is greater in the livers from the CCl_4-exposed animals at all the time intervals investigated. Between 30 and 60 min after the injection of

TABLE 1. INCORPORATION OF PALMITATE-1-^{14}C INTO HEPATIC TRIGLYCERIDES*

Time after palminate injection (min)	Total incorporation (cpm/mg liver ± S.E.M.)			Sp. act. (cpm/μg triglyceride ± S.E.M.)		
	Control	CCl_4-exposed	P	Control	CCl_4-exposed	P
30	163 ± 5 (8)	240 ± 2 (10)	<0·001	58·1 ± 3·1	8·1 ± 1·1	<0·001
60	26 ± 2 (6)	190 ± 38 (6)	<0·005	14·0 ± 1·5	19·4 ± 2·1	n.s.
180	32 ± 6 (4)	101 ± 11 (4)	<0·01	11·5 ± 2·2	35·0 ± 3·0	<0·001

* Figures in parentheses represent the number of experiments; n.s. = not significant. Albumin-bound palmitate-1-^{14}C injected i.v. immediately after the last of 5 periods of 8 hr of exposure to 68 ppm CCl_4 each.

palmitate, the isotope in the control animals had fallen by approximately 85 per cent while only a slight (25 per cent) decrease had occurred in the livers of the intoxicated animals. The lower sp. act. of the lipid in the livers of the CCl_4-exposed animals compared to the controls 30 min after administration of the palmitate-1-^{14}C was due to the large amount of lipid which had accumulated in the intoxicated livers during the 5 days of exposure to CCl_4. After 60 min, sufficient labeled lipid had disappeared from the normal liver so that the sp. act. was not significantly different in the two groups of animals, and by 3 hr it was significantly higher in the CCl_4-intoxicated animals. The incorporation into phospholipids was measured simultaneously but is not shown here, since there was no significant difference between the control and CCl_4-exposed animals in either the incorporation or sp. act. of this fraction. The relationship between the accumulation and apparent retention of fatty acids as neutral lipids in the intoxicated liver and the secretion of radioactive triglycerides into the serum can be followed with the data in Fig. 4. It will be noted that there is a much lower rate of appearance of labeled triglycerides in the serum of the CCl_4-intoxicated animals, the largest difference occurring within 30 min. However, due to the variation in rate of secretion among different animals it was not possible to obtain statistically significant differences. To overcome this, the experiment cited in Table 1 and Fig. 4 was repeated, each animal first undergoing 5 days of exposure to air alone as a control, then 1 week later being exposed to 680 ppm CCl_4. At the end of each exposure period each animal received palmitate-9,10-^{3}H and leucine-1-^{14}C so that the rates of secretion of both the lipid and protein moieties of lipoproteins

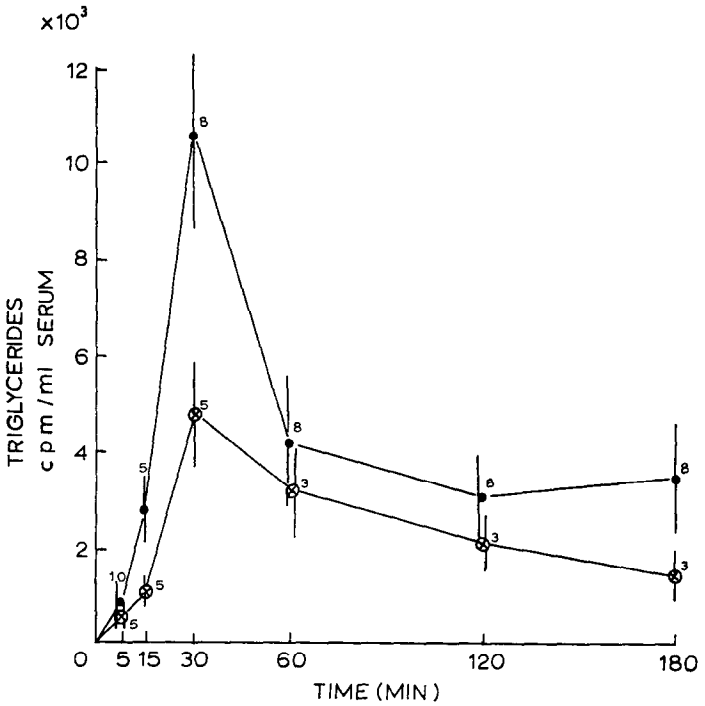


FIG. 4. Appearance of ^{14}C in serum triglycerides after injection of palmitate-1-^{14}C *in vivo* immediately after 8 hr of exposure to CCl_4 vapors for 5 days. The numbers on the curves refer to the number of animals; the vertical bars represent one S.E.M. ● = Control; ⊗ = exposed to 68 ppm CCl_4.

could be followed simultaneously. It was found that a difference between the control and CCl_4-exposure periods occurred only in the low density lipoproteins ($d < 1{\cdot}064$), although the high density lipoproteins and $d > 1{\cdot}21$ proteins were also studied. The results are shown in Table 2 and indicate that there was a significant decrease in the radioactivity of the low density lipoproteins in the serum of the CCl_4-exposed

TABLE 2. SECRETION OF THE LIPID AND PROTEIN MOIETIES OF THE LOW DENSITY LIPOPROTEINS*

Time after palminate injection (min)	Lipid incorporation (cpm/ml serum ± S.E.M.)			Protein incorporation (cpm/ml serum ± S.E.M.)		
	Control	CCl_4-exposed	P	Control	CCl_4-exposed	P
30	11,400 ± 1800	2400 ± 1300	<0·005	570 ± 47	270 ± 54	<0·005
60	3200 ± 1400	1600 ± 800	n.s.	360 ± 44	211 ± 50	<0·05
90	1200 ± 300	1100 ± 300	n.s.	320 ± 54	180 ± 19	<0·05
120	520 ± 75	450 ± 140	n.s.	260 ± 46	180 ± 30	n.s.

* Each figure is the average of 5 experiments. Leucine-1-^{14}C was injected i.v. immediately after the last of 5 daily 8-hr period of exposure to 680 ppm CCl_4 and albumin-bound palmitate-9,10-^{3}H was injected 30 min later. n.s. = Not significant.

animals 30 min after the injection of the palmitate-9,10-^{3}H. A decrease was also seen after 60 min, but this difference was no longer significant. The protein moiety of the serum lipoproteins of the poisoned animals was significantly decreased both at the 30- and 60-min interval. These data are therefore consistent with the view that a decrease in the secretion of low density lipoprotein has occurred.

DISCUSSION

The results reported in this communication indicate that the hepatic changes following periodic carbon tetrachloride intoxication by inhalation over a 5-day period resemble those seen in acute CCl_4-intoxication. Thus, as has previously been reported, after acute intoxication there is a decrease in the liver glycogen level. It is interesting that the loss in glycogen is not due solely to increased glycogenolysis but also involves an inability to resynthesize liver glycogen. The failure to restore the glycogen level to normal during the 16 hr after CCl_4 exposure is not due to a loss of appetite, since it was observed that food consumption was equal in the control and intoxicated rats. It is possible that glycogen synthetase is inhibited, as has previously been observed in liver slices exposed to CCl_4 vapors.[28] After chronic exposure to CCl_4, the liver is characterized by an accumulation of neutral lipids which is not dose dependent until about 8 days. Subsequently there is a disappearance of lipid. The sharp rises in SGOT activity occur at this point. It is possible that these phenomena represent an alteration in the pathology from lipid deposition to necrosis. The separation of the lipid deposition and necrotic phases of CCl_4 intoxication has previously been suggested after acute intoxication.[29,30] Similar conclusions can be drawn from the experiments of Calvert and Brody,[31] who demonstrated that antiadrenergic drugs inhibit necrosis but not accumulation of lipid after acute CCl_4 intoxication.

The data obtained with palmitate-1-^{14}C suggest that, as is the case with acute intoxication, there is a failure of lipoprotein secretion. The accumulation of the labeled fatty acid esters in the liver is reflected by a decreased radioactivity in the serum triglycerides 30 and 60 min after exposure to CCl_4. This corresponds to the significant diminution in the radioactivity of the lipid moiety of the lipoproteins after 30 min. Interpretation of the results is complicated by the observation that the specific activity of the triglycerides in the intoxicated liver is considerably reduced. Thus it is possible that the decrease in counts noted in the low density lipoproteins is simply due to a dilution of the isotope in the lipid moiety of the lipoprotein. However, this seems unlikely since there is also a significant decrease in the amount of leucine-^{14}C incorporated into the protein moiety of the lipoproteins. These results lead to the same conclusion that has been suggested after acute intoxication with CCl_4, namely that after chronic CCl_4 exposure there is a decrease in secretion of low density lipoproteins. The reason for this failure of lipoprotein secretion still remains to be determined.

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