Mechanism of fatty liver development and hyperlipemia in rats treated with allylisopropylacetamide

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ABSTRACT Treatment of rats with allylisopropylacetamide results in two related effects that occur sequentially. After one injection, serum FFA concentration increases and fatty liver develops without any decrease in lipoprotein synthesis. With repeated administration of the drug, fatty acid mobilization continues and acetate incorporation into lipids increases. However, fatty liver disappears with a concomitant increase in lipoprotein synthesis, resulting in hyperlipemia. It is postulated that accumulation of the liver lipid might be a regulating factor in the synthesis and transport of lipoproteins.

SUPPLEMENTARY KEY WORDS serum triglyceride · serum free fatty acids · plasma lipoprotein synthesis · acetate incorporation · porphyria

Allylisopropylacetamide has been widely used experimentally for induction of increased synthesis of liver porphyrins (1). It has been reported that treatment with AIA produces fatty livers in rats (2, 3). Derangements in lipoprotein metabolism appear to play a role in the development of fatty livers. Thus, animals treated with puromycin, which inhibits the synthesis of the protein portion of the lipoproteins, develop fatty livers (4). When ethionine (5, 6) or carbon tetrachloride (7–9) is administered, fatty livers develop and there is also inhibition of protein synthesis and decreased lipoprotein synthesis. However, in the latter two cases, change in ATP levels (6) in the liver and lipid peroxidation (9) have been suggested as causes of the reduced lipoprotein formation. The feeding of orotic acid to rats results in the development of a fatty liver without inhibiting protein synthesis. It interferes either with lipoprotein transport from the liver or the coupling of lipid to protein (10).

The present study is concerned with lipoprotein metabolism as related to alterations in liver lipids, using AIA-treated rats as experimental models.

Abbreviations: AIA, allylisopropylacetamide; VLDL, very low density lipoproteins; LDL, low density lipoproteins; HDL, high density lipoproteins; FFA, free fatty acids; TG, triglyceride.

MATERIALS AND METHODS

Female Sprague–Dawley rats weighing 150–170 g were used. Since the treatment of animals resulted in a decreased food intake, the control animals received the same amount of food as that consumed by the rats in the experimental group. The animals were fed Rockland Farms mouse pellets and had free access to water until they were killed. AIA (supplied by the courtesy of Hoffmann-La Roche, Inc., Nutley, New Jersey) was injected subcutaneously in doses of 400 mg/kg at 26-hr intervals. This interval was chosen to allow the animals to wake up and feed. Animals injected with AIA were in a deep sleep for 20–24 hr after the first and second injections. Unless otherwise noted the animals were anesthetized with ether and bled by cardiac puncture 16–20 hr after the last injection of AIA.

Lipid Determination

Tissue and serum lipids were extracted by the method of Folch, Lees, and Sloane Stanley (11). Triglycerides were measured according to van Handel's modification (12) of the method of van Handel and Zilversmit (13). Cholesterol was determined by the method of Abell, Levy, Brodie, and Kendall (14). Phospholipids were determined according to Beveridge and Johnson (15), and

free fatty acids were determined by the method of Dole (16) as modified by Trout, Estes, and Friedberg (17).

Incorporation of ^{14}C-Labeled Acetate into Liver Lipids

Animals were killed by decapitation. The livers were removed, chilled, and sliced with the Stadie-Riggs tissue slicer. Liver slices weighing 500 mg were placed in flasks containing 5 ml of Krebs-Ringer phosphate (18) buffer and 5 mg of glucose. To each vessel 3.15 μCi of sodium acetate-1-^{14}C (SA 2 mCi/mmole) was added and the slices were incubated in a Dubnoff shaker for 120 min at 37°C. At the end of the incubation period 10 ml of a solution containing 1% sodium acetate at 0°C was added to each flask. The liver slices were then homogenized, extracted, and washed according to the method of Folch et al. (11). The washing was repeated until no radioactivity could be detected in the upper phase. Radioactivity of the total lipids in the chloroform extract was measured by liquid scintillation counting. Free cholesterol and triglycerides were separated on thin-layer plates coated with Silica Gel H developed in hexane–ether–acetic acid 80:19:1. The separated bands were scraped from the plates and rechromatographed. The bands were again scraped and extracted with chloroform. Aliquots were taken for measurement of radioactivity and determination of cholesterol and triglyceride.

Incorporation of ^{14}C-Labeled Amino Acids into Serum Lipoproteins

Animals were injected intravenously with a ^{14}C-labeled amino acid mixture prepared from protein hydrolysate (Schwarz BioResearch Inc., Orangeburg, N.Y.) (7.5 μCi/100 g body weight), and were bled 100 min later.

Lipoprotein Separation and Analysis

Since the animals were small, sera of 3 or 4 animals were pooled for each lipoprotein separation. The lipoproteins were separated by ultracentrifugation according to the method of Havel, Eder, and Bragdon (19). Ultracentrifugation was performed in the Spinco Model L using 40.3 rotors. Lipoproteins were separated into three classes, VLDL (d < 1.006), LDL (d 1.006–1.063), and HDL (d 1.063–1.21). Separation of VLDL and LDL was carried out by centrifugation for 20 hr at 114,000 *g;* for separation of the HDL ultracentrifugation was continued for at least 40 hr at 114,000 *g*. The separated lipoproteins in the upper third of the tube were then removed. The lipoprotein fractions were washed once by resuspending them in a salt solution of appropriate density and then recentrifuged. Proteins were prepared for determination of radioactivity and chemical analysis as previously described by Roheim, Miller, and Eder (20). Protein determination was performed according to the method of Lowry, Rosebrough, Farr, and Randall (21). For the determination of radioactivity aliquots were suspended in Cab-O-Sil, obtained from Cabot Corp., Boston, Mass., and counted in a Tri-Carb scintillation counter (22).

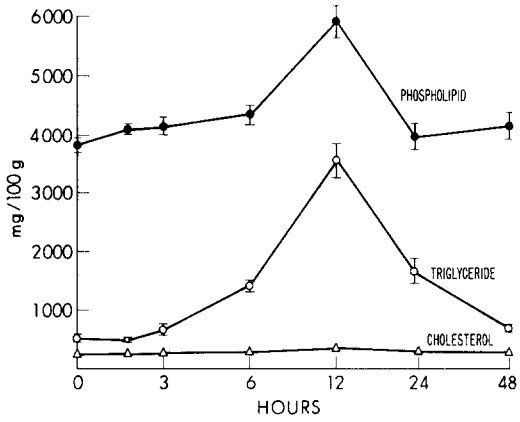

FIG. 1. Concentration of liver lipids after a single injection of AIA. Rats received 400 mg/kg of AIA by subcutaneous injection and were killed at various time intervals thereafter. Each group consisted of four animals and the points represent the mean value and standard error.

RESULTS

Concentrations of Liver and Serum Lipids

Effects of a Single AIA Injection. The time course of the development of fatty liver after one injection of AIA (400 mg/kg) is shown in Fig. 1. An increase in liver TG concentration was observed after 6 hr, and the peak concentration was reached at 12 hr (an eightfold increase). After 48 hr, the liver TG concentration returned to normal. The phospholipid concentration increased but not as much as did the triglyceride concentration, and it returned to normal by 24 hr; there was no change in liver cholesterol. In the same experiment, considerable fluctuation of serum TG, cholesterol, and phospholipid was observed with a trend towards slightly higher values at 24 hr. The most marked change in serum lipid was a 75% increase in FFA concentration after 20 hr (Table 1).

Effects of Repeated AIA Injections. With repeated daily injections of AIA an increase in liver weight was observed. After the third injection the liver weight increased by 35%, and after the sixth injection, by 60%.

The alterations in liver and serum TG are shown in Fig. 2: the highest liver TG concentration was obtained after the first injection. The concentration decreased with repeated injections and returned to normal after the fourth day. Plasma triglyceride concentration increased progressively during the first three days of treatment although the liver triglyceride concentration had fallen almost to control levels. The plasma triglyceride concen-

TABLE 1 SERUM FFA CONCENTRATIONS 20 HR AFTER LAST AIA ADMINISTRATION

Number of Injections	Number of Rats	Concentration
		meq/l
None	5	0.536 (±0.051)*
1	5	0.953 (±0.047)
		$P < 0.001$
4	6	0.916 (±0.053)
		$P < 0.001$

* ± Standard error.

tration then fell but remained slightly elevated while liver triglyceride concentration returned to normal. The hypertriglyceridemia may reflect the discharge of triglyceride from the liver.

An increase in serum cholesterol and a small increase in phospholipid concentration were seen after the fourth day of treatment (Table 2). There was no change in liver cholesterol and phospholipid concentrations. A sustained increase in serum FFA concentration (70%) was seen (Table 1), indicating that FFA mobilization was still increased at a time when liver TG had returned to normal.

Incorporation of Acetate-1-^{14}C into Liver Lipids

Incorporation of acetate-1-^{14}C into total lipids during in vitro incubation of liver slices (Table 3) showed no change after one AIA injection. However, after 4 days of treatment, the total incorporation of acetate into liver lipids almost doubled. After one AIA injection, the percentage incorporation into the liver triglycerides was unchanged, while the specific activity of the liver triglyceride was drastically reduced, reflecting the increase of the triglyceride pool size (Table 4). After repeated injections, there was a small increase of incorporation of

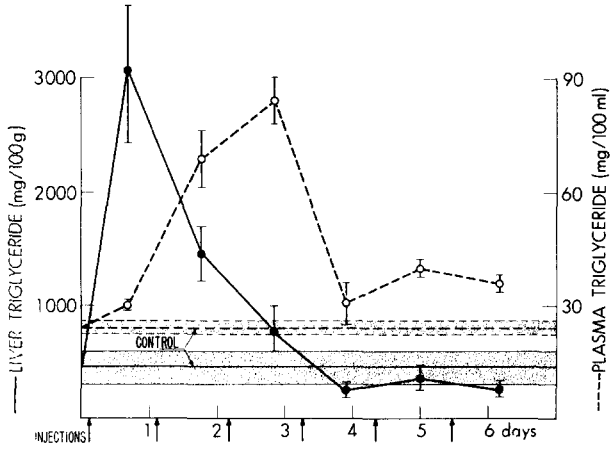

FIG. 2. Changes of liver and plasma triglyceride concentration after repeated AIA injections (400 mg/kg).

TABLE 2 CHOLESTEROL AND PHOSPHOLIPID CONCENTRATIONS AFTER AIA INJECTIONS

Number of Injections	Cholesterol		Phospholipid	
	Plasma	Liver	Plasma	Liver
	mg/100 ml	*mg/100 mg*	*mg/100 ml*	*mg/100 mg*
0	79	301	152	3810
(6)*	(±15)†	(±14)	(±4)	(±105)
1	112	315	159	3977
(4)*	(±27)	(±5)	(±10)	(±219)
	NS‡	NS	NS	NS
4	137	292	167	4170
(6)*	(±14)	(±9)	(±5)	(±120)
	$P < 0.025$	NS	$P < 0.05$	NS

Animals were killed 20 hr after AIA injections.
* Number of animals in group.
† ± Standard error of the mean.
‡ Not significantly different from the control.

TABLE 3 INCORPORATION OF ACETATE-1-^{14}C INTO LIPIDS DURING INCUBATION WITH LIVER SLICES

Number of Injections	Total dpm	% Incorporation of Acetate into Lipids*
0	29.0×10^4	4.14
(6)†	($\pm 3.3 \times 10^4$)‡	(±0.48)
1	22.7×10^4	3.23
(4)†	($\pm 2.7 \times 10^4$)	(±0.39)
	NS§	NS
4	52.9×10^4	7.54
(6)†	($\pm 3.2 \times 10^4$)	(±0.73)
	$P < 0.001$	$P < 0.005$

* (Total dpm in lipids/total dpm incubated) × 100.
† Number of animals.
‡ ± Standard error.
§ Not significant.

TABLE 4 INCORPORATION OF ACETATE-1-^{14}C INTO TRIGLYCERIDE DURING INCUBATION WITH LIVER SLICES

Number of Injections	Specific Activity	% Incorporation of Acetate into TG*	Liver Triglyceride
	dpm/mg		*mg/100 g*
0	16,305	0.68	782
(6)†	(±3,200)‡	(±0.11)	(±48)
1	2,582	0.76	5086
(4)†	(±699)	(±0.12)	(±706)
	$P < 0.005$	NS§	$P < 0.001$
4	18,043	1.24	1013
(6)†	(±2,874)	(±0.31)	(±219)
	NS	NS	NS

* (Total dpm in triglyceride/total dpm incubated) × 100.
† Number of animals.
‡ ± Standard error.
§ Not significant.

acetate into the liver TG. By the fourth day, the specific activity of liver TG was not significantly different from control levels, despite the higher TG concentration (Table 4). One injection of AIA resulted in no change in the specific activity nor in the percentage incorpora-

TABLE 5 INCORPORATION OF ACETATE-1-^{14}C INTO FREE CHOLESTEROL DURING INCUBATION OF LIVER SLICES

Number of Injections	Specific Activity	% Incorporation of Acetate into Cholesterol*
	dpm/mg	
0	18.5×10^4	1.12
(6)†	$(\pm 1.3 \times 10^4)$‡	(±0.09)
1	17.9×10^4	1.04
(4)†	$(\pm 2.4 \times 10^4)$	(±0.32)
	NS§	NS
4	48.3×10^4	2.50
(4)†	$(\pm 2.9 \times 10^4)$	(±0.23)
	$P < 0.001$	$P < 0.001$

* (Total dpm in cholesterol/total dpm incubated) × 100.
† Number of animals.
‡ ± Standard error.
§ Not significant.

tion of acetate into free cholesterol (Table 5), while repeated injections resulted in more than a doubling of liver free cholesterol, specific activity, and percentage incorporation. Therefore, increased lipid synthesis does not coincide with the development of fatty liver, but is observed in a period when liver TG is returning to normal and serum TG concentrations are increased.

Serum Lipoprotein Concentrations and Incorporation of Labeled Amino Acids

Since the development of fatty liver could be the result of decreased plasma VLDL synthesis, the incorporation of labeled amino acids into VLDL protein was measured at 12, 24, and 48 hr after one injection of AIA (Table 6). There was no decrease observed in the incorporation of amino acids into VLDL. It is apparent that at a time when maximal lipid accumulation is observed in the liver (after one AIA injection), there is no inhibition of VLDL synthesis or its transfer from the liver into the circulation.

With repeated administration of AIA, the concentration of VLDL increased and remained elevated through the sixth day of treatment (Table 7). A transient elevation was observed in LDL concentration; no significant change in HDL concentration was observed (Table 7). The specific activity and total incorporation of each lipoprotein fraction are shown in Figs. 3 and 4. The most striking increases in specific activity and total incorporation were found in the VLDL fraction. These correlate with elevations in serum triglyceride concentrations observed after repeated injections of AIA. These data suggest that there is an increased synthesis of lipoproteins, especially VLDL, after AIA treatment in rats. There was no definite change observed in the incorporation of amino acids into plasma proteins (d > 1.21), but an increased specific activity of liver proteins was found after repeated injections (Fig. 5).

TABLE 6 EFFECT OF SINGLE INJECTION OF AIA ON THE INCORPORATION OF AMINO ACIDS INTO VLDL (d < 1.006)

Hr after AIA Injection	Specific Activity	Protein Concentration
	dpm/mg	*mg/100 ml*
Control	11,500	1.18
(5)*	(±711)†	(±0.08)
12	18,600	1.07
(2)*		
24	11,700	2.6
(2)*		
48	11,450	1.75
(2)*		

* Number of pooled samples.
† ± Standard error of the mean.

TABLE 7 EFFECT OF REPEATED AIA INJECTIONS ON THE LIPOPROTEIN PROTEIN CONCENTRATION

Number of Injections	VLDL	LDL	HDL
	mg/100 ml of serum		
0	1.03	5.69	44.5
(10)*	(±0.13)†	(±1.12)	(±3.96)
1	2.6	6.9	41.9
(2)*			
2	2.01	7.8	35.7
(3)*	(±0.3)	(±1.65)	(±2.06)
	$P < 0.025$	NS‡	NS
3	2.4	7.6	28.5
(1)*			
6	5.07	4.08	64.1§
(4)*	(±0.62)	(±0.61)	
	$P < 0.001$	NS	

* Number of pooled samples.
† ± Standard error of the mean.
‡ Not significant.
§ Two determinations.

Cytochemical and Ultrastructural Changes

Detailed morphologic studies of the sequential changes in the hepatocytes after administration of AIA have been reported elsewhere (3, and footnote 1). Coincident with the increase in triglyceride content of the liver, numerous large fat droplets appear scattered throughout the cytoplasm and their number decreases markedly after the second injection of AIA. Activity of the Golgi apparatus, shown by staining for thiamine pyrophosphatase, is increased after the first injection of AIA and remains so throughout the experiment. As seen in Fig. 6, the Golgi apparatus is prominent. Numerous electron-dense spherules are present in the Golgi saccules and numerous Golgi vacuoles packed with these spherules are seen. These spherules correspond to the particles shown to be VLDL by Mahley, Hamilton, and LeQuire (23). Coincident with the drop in the triglyceride content of the

[1] Biempica, L., N. S. Kosower, and P. S. Roheim, unpublished data.

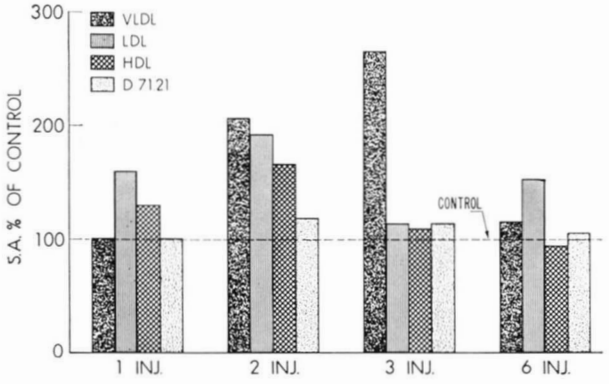

FIG. 3. Effect of AIA treatment on the specific activity of serum lipoproteins. The specific activity of the lipoproteins in the treated animals is expressed as % of control values. The number of determinations in each group was as follows: control, 5; 1 injection, 2; 2 injections, 3; 3 injections, 1; and 6 injections, 4.

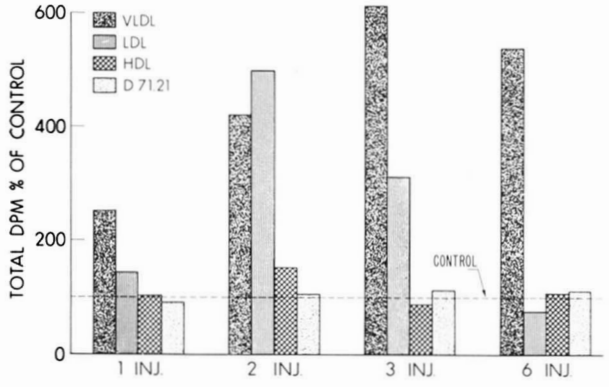

FIG. 4. Effect of AIA treatment on the total incorporation of ^{14}C-labeled amino acids into serum lipoproteins. The total incorporation into serum lipoprotein classes of the treated animals is expressed as % of the controls. The number of determinations in each group is the same as in Fig. 3.

liver, numerous vacuoles filled with these spherules are seen near the sinusoidal surface of the hepatocytes and free spherules are seen in the space of Disse,[1] suggesting that there has been increased transport of the lipoproteins in the vacuoles into the circulation. Hyperplasia of the smooth endoplasmic reticulum is prominent in the central lobular zone after the third injection. At this time the liver triglyceride concentration has returned to normal and stainable lipid is no longer found in the liver.

DISCUSSION

One experimental approach to the study of the regulation of lipoprotein metabolism is through exploration of mechanisms of fatty liver production. Measurements of changes in lipoprotein synthesis during the development of fatty livers have contributed to our understanding of lipoprotein metabolism (4–7, 9, 10). AIA appears to be

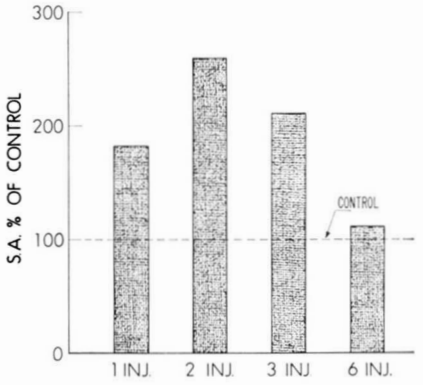

FIG. 5. Effect of AIA treatment on the specific activity of liver proteins. The specific activity of the liver proteins of the treated animals is expressed as % of the controls. The number of determinations in each group was at least six. Liver tissue was homogenized with H_2O and precipitated with trichloroacetic acid at a final concentration of 5%. After washing, the precipitate was dissolved in 1 N NaOH and aliquots were taken for measurement of radioactivity and protein determination.

a peculiarly interesting compound with respect to its effects on lipoprotein metabolism and causation of fatty livers.

It has been reported previously that one injection of AIA results in a marked increase in lipid staining of the liver (3, 24). We have shown by chemical determinations that one injection of AIA results in an eightfold increase in concentration of liver triglyceride and that a maximal level is reached at 12–18 hr. Serum lipid values do not change markedly after one injection with the exception of serum FFA concentration which increases by 75%.

One possible mechanism for the development of fatty liver is decreased lipoprotein synthesis, such as observed after administration of puromycin, ethionine, or carbon tetrachloride (4, 5, 9). There was, however, no demonstrable decrease in lipoprotein synthesis at the time of marked lipid accumulation. The finding of a significant increase of serum FFA concentration, but no increase of acetate incorporation into liver lipids, suggests that increased fatty acid mobilization is an important factor in the development of fatty liver after one AIA injection. That elevation of serum FFA levels can result in development of fatty livers was demonstrated in the studies of Feigelson, Pfaff, Karmen, and Steinberg (25), who produced increased fatty acid mobilization by administration of norepinephrine to dogs. Machado, Lozzio, and Royer (24) demonstrated that administration of reserpine, which inhibits mobilization of FFA (26), prevented the development of fatty livers after AIA injections.

The fatty liver produced by AIA is transient. The liver triglyceride concentration returns to normal within 3–4 days in spite of continuous daily injections. Coinciding with the return of liver triglyceride content to normal is a marked rise in serum triglyceride concen-

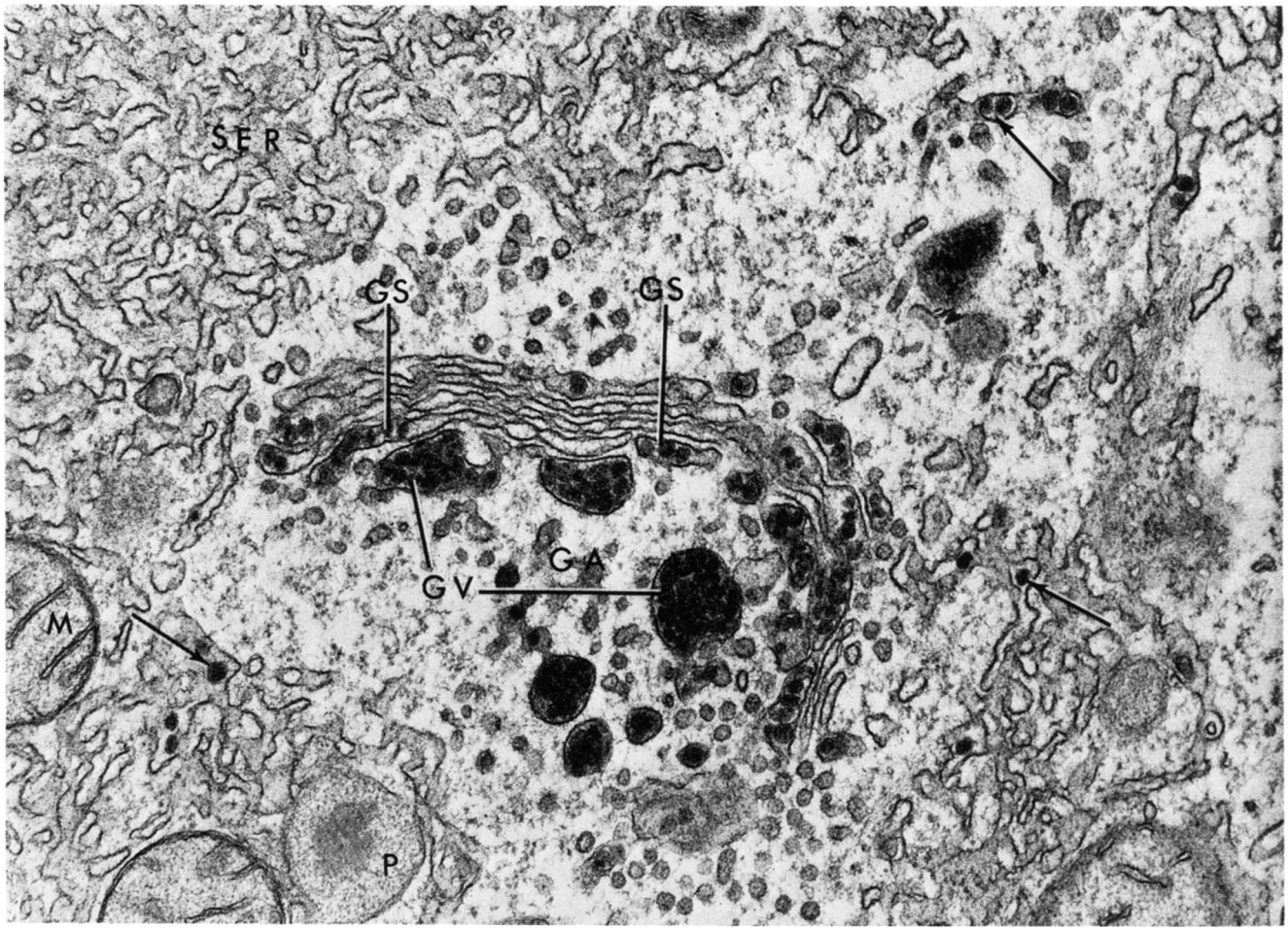

FIG. 6. Electron micrograph from a section of liver approximately 600 A thick from a rat which received 3 doses of AIA. The tissue was fixed in 1% phosphate-buffered osmic acid (40) for 2 hr at room temperature, and was treated with 0.5% uranyl acetate in Veronal–acetate buffer pH 7.4 (41) for 1 hr at room temperature. It was embedded in Epon (42). The section was stained with lead (43) for 15 min. A portion of a hepatocyte is shown with a prominent Golgi apparatus (*GA*). VLDL appear as osmiophilic droplets distributed in a row within Golgi saccules (*GS*) and clustered inside Golgi vacuoles (*GV*). Other electron-dense droplets (arrows) are seen in the lumina of endoplasmic reticulum cisternae. An area of hyperplasia of the smooth endoplasmic reticulum (*SER*) is at upper left. *M*, mitochondria; *P*, peroxisome (microbody). Magnification, 39,000 ×.

tration apparently due to discharge of liver triglycerides into the circulation.

Decreased de novo lipid synthesis cannot be a factor in the return of liver triglycerides to normal levels. Indeed, we have found increased lipid synthesis after the fourth day of AIA treatment, and this has also been found by Labbe, Hanawa, and Lottsfeldt (2). Furthermore, the increased levels of serum FFA persist after repeated injections of AIA so that the return of liver triglyceride content to normal must be the result of increased transfer of triglyceride from the liver into the circulation. The increase in amino acid incorporation into lipoproteins which occurs at this time suggests that this transfer of triglyceride is accompanied by increased lipoprotein synthesis, which is most marked in the VLDL fraction. Since the accumulation of triglyceride in the liver precedes increased lipoprotein synthesis, we suggest that the increase in lipoprotein synthesis is due to the high lipid concentration in the liver. This is consistent with our findings[2] that in rats fed high sucrose diets liver triglyceride concentration increases along with an increase in the rate of synthesis of VLDL. In addition, we and others have found that infusion of FFA into livers results in elevated liver triglyceride content and increased formation of VLDL (27, 28).

It is known that the bulk of the hepatic lipids are present in the liver as storage lipids which are metabolically inactive (29–31). It is possible that in certain situations a well-defined subcellular site might have increased lipid concentration without demonstrable overall increase in liver concentration. This would result in the stimulation of lipoprotein synthesis and hyperlipemia. This

[2] Shiff, T. S., P. S. Roheim, and H. A. Eder, unpublished data.

might be the case after the fifth day of treatment of AIA when liver lipids are normal and plasma triglyceride is elevated, although serum FFA concentration remains elevated. The changes observed at this time in the ultrastructure of the liver are consistent with this hypothesis (Fig. 6). We believe that increase of the liver lipid concentration at a particular subcellular site might have an important influence in increasing lipoprotein synthesis. This should be contrasted with the situation in which fatty liver develops as a result of the defect of lipoprotein production (4, 5, 7).

We believe that the following sequence of events takes place after repeated AIA injections. Increased FFA mobilization results in increased TG synthesis (27, 32). Since lipoprotein synthesis is not yet increased, a fatty liver develops. The increase in liver lipid concentration then induces an increase in lipoprotein synthesis, especially VLDL. This in turn leads to an increased release of lipoproteins from the liver, resulting in hyperlipemia and lowering of liver TG concentration. When liver TG concentration returns to normal, the serum triglyceride levels decrease but remain elevated because of increased lipid and lipoprotein synthesis. Therefore, a new steady state develops as a result of AIA treatment.

Electron microscopic findings support these biochemical observations. The increased staining for thiamine pyrophosphatase of the Golgi apparatus reflects its increased activity. It has been shown that the Golgi apparatus participates in intracellular lipid transport (33) and that particles can be isolated from the Golgi vesicles which are chemically and immunologically similar to the serum VLDL (23). Perfusion of isolated livers with FFA results in an increased activity of the Golgi apparatus (34) and in increased incorporation of the labeled amino acids into VLDL (28).

Administration of orotic acid or AIA produces increased activity of the Golgi apparatus and an increased number of lipid-containing vacuoles (35). However, orotic acid feeding results in decreased release of lipoproteins from the liver (10), while AIA leads to increased lipoprotein synthesis. This suggests that the block in lipoprotein transport in the orotic acid-treated rats might take place between the appearance of lipoproteins in the Golgi apparatus and their actual discharge into the circulation. This possibility is also supported by finding in the orotic acid-treated animals that the synthesis of the apoprotein of the lipoproteins is intact (10). It has therefore been postulated that the defect lies in the transport of lipoproteins (10).

It is also possible that AIA influences lipoprotein synthesis directly and not through the increased lipid concentration in the liver. Administration of AIA is known to produce porphyria (36) and to increase liver phospholipid (37) and serum cholesterol concentrations (38). Increased cholesterol and β-lipoprotein concentrations have been reported in patients with acute intermittent porphyria (39). This finding suggests that porphyria and increased lipoprotein synthesis might be causally related. However, no mechanisms are known at present to explain any such relationship either in naturally occurring or in experimental porphyria.

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