Histol Histopath (1989) 4: 31-37

Histology and Histopathology

Effects of 4-aminopyrazolo (3,4-d) pyrimidine on rat hepatocytes: an ultrastructural morphometric study

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Summary. The prolonged administration of the hypolipidemic drug 4-aminopyrazolo (3,4-d) pyrimidine (4APP) induced conspicuous morphological changes in rat hepatocytes, which are clearly demonstrated by stereology. We observed a significant decrease in the rough endoplamic reticulum, and an accumulation of lipid droplets, which was coupled with a comparable rise in the hepatic concentrations of total cholesterol and triglycerides. These changes were interpreted as the expression of the 4APP-provoked impairment of the synthesis of the polypeptide chains of lipoproteins and of the consequent suppression of the assembly of lipid molecules in exportable lipoproteins. We also noted a neto lowering in smooth endoplasmic reticulum and perxisomes, a decrease which was hypothesized to be the morphological counterpart of a reduced *de novo* cholesterol synthesis, due to the 4APP-induced rise in the intracellular cholesterol concentration.

Key words: Liver, 4-Aminopyrazolo-pyrimidine, Cholesterol metabolism, Rat, Stereology

Introduction

4-Aminopyrazolo(3,4-d)pyrimidine (4APP) is an adenine analog (Brockman and Anderson, 1963), which suppresses lipoprotein synthesis in the liver, thus decreasing the level of circulating cholesterol (Shiff et al., 1971). Among the various hypocholesterolemic drugs so far available 4APP is the one most commonly employed in the biochemical studies on lipoprotein and cholesterol metabolism (Balasubramaniam et al., 1977; Andersen and Dietschy, 1978; Feingold et al., 1981; van't Hooft and van Tol, 1986; Mazzocchi et al., 1987). However, in contrast to other hypolipidemic drugs (such as aryloxyacetic acid derivatives) (for references, see Stäubli and Hess, 1975), data concerning the ultrastructural alterations induced by 4APP in hepatocytes are very sparse. Murakoshi et al. (1985) described lipid-droplet accumulation, as well as dilation and degranulation of the cisternae of rough endoplasmic reticulum. Almeida et al. (1987) focused their attention on nucleolar fragmentation. It, therefore, seemed wortwhile to carry out a complete morphometric description of the effects of a prolonged 4APP administration on rat hepatocytes.

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Materials and methods

Male rats of the Wistar strain, about 200 g body weight, were employed. They were divided into six equal groups, five of which received, at 10 am, daily intraperitoneal injections of 4 mg/kg 4APP (Sigma, St. Louis, U.S.A.), dissolved in 0.5 ml 10 mM sodium phosphate (pH 3.5), for 1, 2, 3, 4 or 5 days. The sixth group was injected for five consecutive days with 0.5 ml of vehicle and served as a control. The rats were killed by decapitation at 10 am, 24 h after the last intraperitoneal injection.

The trunk blood was collected from each rat and the plasma concentration of free and esterified cholesterol was determined by HPLC, according to Duncan et al. (1979).

From 3-day and 5-day 4APP-treated rats, as well as from control animals, the liver was removed, weighed and, with the exception of a small fragment of the left lobe (see below), homogenized in 0.25 M buffered sucrose. Total liver cholesterol concentration was measured by the enzymatic spectrophotometric method of Deacon and Dawson (1979), following the procedure described by Civen et al., (1984). Liver triglyceride concentration was assayed according to Foster and Dunn (1973).

Small pieces of the left lobe of the liver were fixed in 3% glutaraldehyde in 0.1M cacodylate buffer, postfixed in 1% osmium tetroxide in 0.1M phosphate buffer and embedded in an epoxy resin (Dow: Serva, Heidelberg, W. Germany). Thick sections were cut with LKB III ultramicrotome and observed with the light microscope

to select the middle portion of the lobules. Adjacent thin sections were examined in a Hitachi H-300 electron microscope at a direct magnification of × 7000. A preliminary pilot experiment showed that 4APP did not differentially affect either various hepatic lobes or hepatocytes located in different portions of the lobules.

From each rat, three tissue blocks were examined. At low magnification, two technically perfect series of thick and adjacent thin sections for each block were selected. Three light micrographs were recorded for each series of thick sections at a magnification of × 1000 (18 light micrographs, i.e. about 140000 μm^2 of liver, for each rat). For each series of thin sections, six electron micrographs at a final magnification of × 21000 (36 electron micrographs, i.e. about 3000 μm^2 of hepatocytes, for each animal) and four electron micrographs at a final magnification of × 42000 (24 electron micrographs, i.e. about 800 μm^2 of hepatocytes, for each rat) were recorded for morphometric analysis.

The volume densities (V_v) of liver stroma and of nuclei and cytoplasm of hepatocytes, as well as the numerical density (N_a) of parenchymal nuclear profiles were estimated on the light micrographs by differential point counting, using a square lattice test system of type A (400 test points/dm^2) (Weibel, 1979). The number of nuclei of hepatocytes per mm^3 of liver parenchyma (N_v) was estimated by the formula of Weibel and Gómez (Weibel, 1979):

$$N_V = \frac{K}{ß} \times \frac{N_a^{3/2}}{V_v^{1/2}}$$

The shape coefficient ß, which depends on the axial ratio of estimated nuclear profiles, was calculated from the curve for ellipsoids of Weibel (1979) and found to range between 1.350 and 1.370. The size-distribution coefficient K ranged between 1.2 and 1.3. No significant differences in ß and K values were observed among the various experimental groups. Subsequently, by dividing the μm^3 of parenchyma per mm^3 of liver by N_v, the average volume of hepatocytes was calculated. Since the relative number of binucleated cells was not computed, according to Weibel et al. (1969), the term «hepatocyte» is used to mean the «cell portion related to one nucleus» or «mononuclear hepatocyte». Multiplying V_v of nuclei per the average volume of hepatocytes gave the mean nuclear volume.

On the electron micrographs at final magnification of × 21000, the V_v of mitochondria, lysosomes, peroxisomes, lipid droplets and «membrane space» (i.e. the cell space containing the membranes of endoplasmic reticulum, including Golgi apparatus, and glycogen rosettes) were estimated by differential point counting, using a test system of type A (100 test points/dm^2) (Weibel, 1979). On the electron micrographs at a final magnification of × 42000, the surface densities (S_v) of the membranes of rough (RER) and smooth (SER) endoplasmic reticulum were evaluated according to Loud (1962), employing a parallel-line test system (100 cm of test line/dm^2).

The data obtained from each rat were averaged for experimental group and the standard deviation (S.D.) was calculated. As revealed by the χ^2test, the data were not different from normal distribution ($P<0.05$). After testing the equality of variances, the statistical comparison of the data was performed by the multiple range test of Duncan.

Results

4APP treatment significantly lowered plasma concentrations of total and esterified cholesterol. The decreases were already apparent on the first day of treatment (-34% and -44%, respectively), and reached values of about -46% and -60% on the third and fifth days. The blood level of free cholesterol was not significantly affected (Fig. 1). The hepatic concentrations of total cholesterol and triglycerides showed significant increases after 3 days (22% and 140% respectively) and 5 days (22% and 248%, respectively) of 4APP administration (Fig. 2).

Morphometry showed that 4APP treatment did not induce significant changes in the volume of either hepatocytes or their nuclei (Table 1). No striking disruption of the ultrastructure of hepatocytes was seen in 4APP-treated rats. However, a moderate increase in the number of lipid droplets (which, as a rule, are very few in normal rat hepatocytes) was observed after 3 days of treatment, and a conspicuous one was noted after 5 days (Figs. 3, 5). Stereology (Table 2) did not evidence significant 4APP-induced modifications in V_v of mitochondrial and lysosomal compartments. The V_v of peroxisome compartment and membrane space were notably lowered (-42% and -13% after 3 days; -70% and -14% after 5 days), and that of the lipid-droplet compartment showed a tremendous rise (5-fold after 3 days, and 9-fold after 5 days). The S_v of both RER and SER were significantly reduced (-35% and -32% after 3 days; -52% and -36% after 5 days).

Table 1. Morphometric parameters of hepatocytes of 4APP-treated rats.

Experimental groups (8)	Volume of cells (μm^3)	Volume of nuclei (μm^3)
Controls	5263.7 ± 720.1	258.8 ±37.2
3 days 4APP	5020.8 ± 751.6	260.2 ±41.3
	P NS	P NS
5 days 4APP	5311.0 ±812.4	277.4 ± 34.7
	P NS	P NS

Values are group means ±S.D. The number of rats in each experimental group is indicated in parentheses. P, level of significance of the difference from the control group; NS, not significant.

Table 2. Stereological parameters of hepatocytes of 4APP - treated rats.

Experimental groups (8)	V_v of mitochondrial compartment ($\mu m^3/100\ \mu m^3$)	V_v of lysosomal compartment ($\mu m^3/100\ \mu m^3$)	V_v of peroxisome compartment ($\mu m^3/100\ \mu m^3$)	V_v of lipid droplet compartment ($\mu m^3/100\ \mu m^3$)
Controls	20.4 ± 3.7	0.2 ± 0.04	1.2 ± 0.21	1.2 ± 0.25
3 days 4APP	22.6 ± 4.1 P NS	0.2 ± 0.05 P NS	0.7 ± 0.11 P < 0.01	6.2 ± 1.16 P < 0.01
5 days 4APP	21.0 ± 3.4 P NS	0.2 ± 0.05 P NS	0.3 ± 0.05 P < 0.01	11.5 ± 3.15 P < 0.01

	V_v of membrane space ($\mu m^3/100\ \mu m^3$)	S_v of rough endoplasmic reticulum ($\mu m^2/\mu m^3$)	S_v of smooth endoplasmic reticulum ($\mu m^2/\mu m^3$)
Controls	72.1 ± 10.9	7.9 ± 1.22	4.4 ± 1.07
3 days 4APP	62.2 ± 9.5 P < 0.05	5.1 ± 0.92 P < 0.01	3.0 ± 0.64 P < 0.05
5 days 4APP	51.8 ± 11.1 P < 0.05	3.8 ± 0.68 P < 0.01	2.8 ± 0.72 P < 0.05

Explanations as in Table 1.

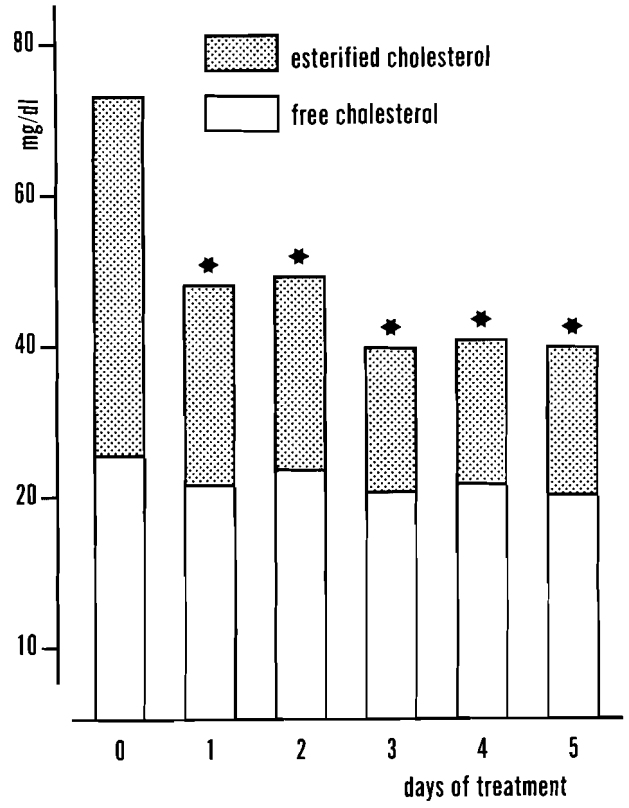

Fig. 1. Effect of 4APP administration on the plasma concentration of free, esterified and total cholesterol. *, P<0.01.

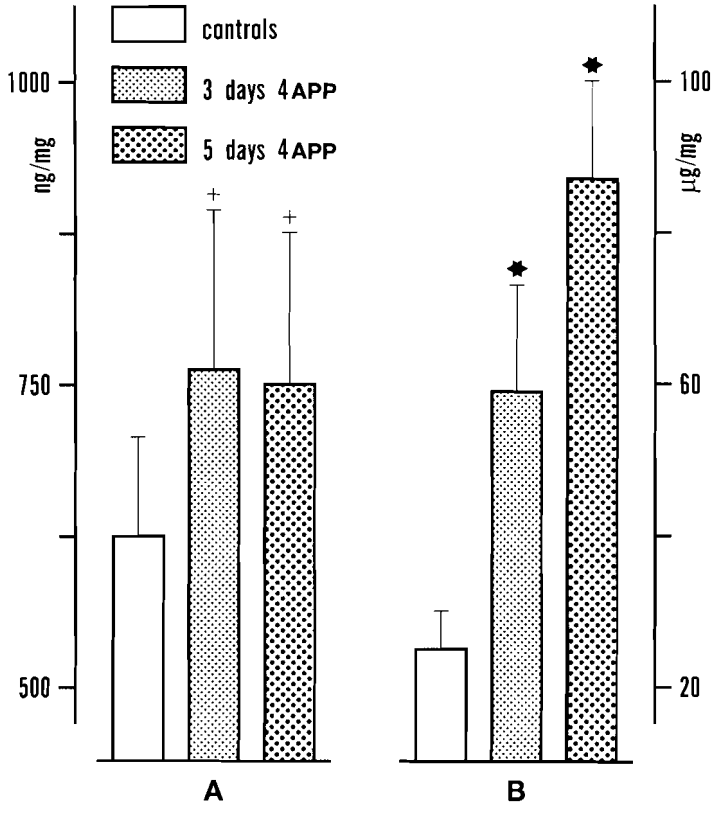

Fig. 2. Effect of 4APP administration on the hepatic concentration of total cholesterol (A) and triglycerides (B). +, P<0.05; *, P<0.01.

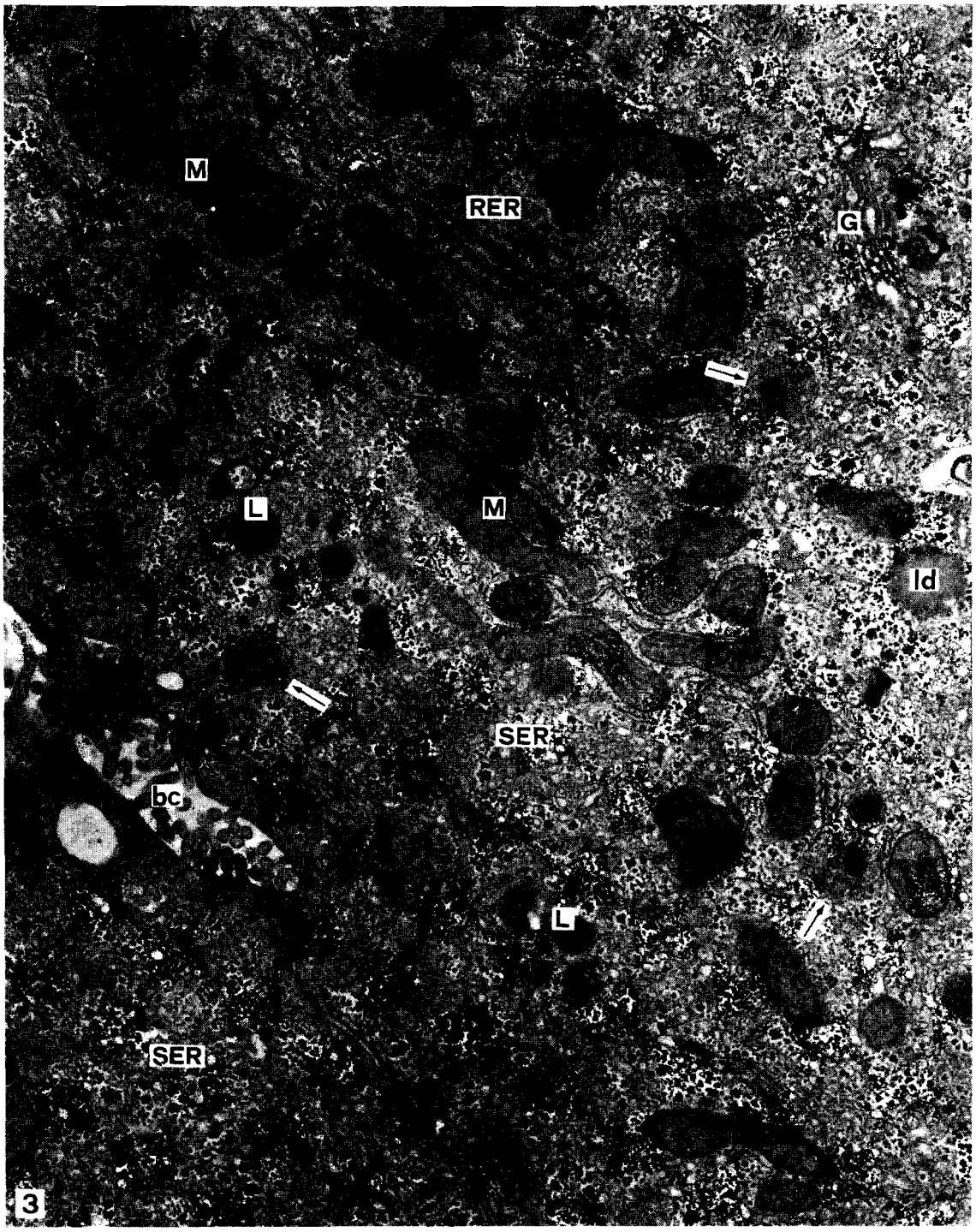

Fig. 3. Mid-lobular hepatocytes of a control rat. **M,** mitochondria; **RER,** rough endoplasmic reticulum; **SER,** smooth endoplasmic reticulum profiles intermingled with abundant glycogen deposits; **G,** Golgi apparatus; **ld,** lipid droplet; **L,** lysosomes; **bc,** bile canaliculus; arrows, peroxisomes. × 15,000

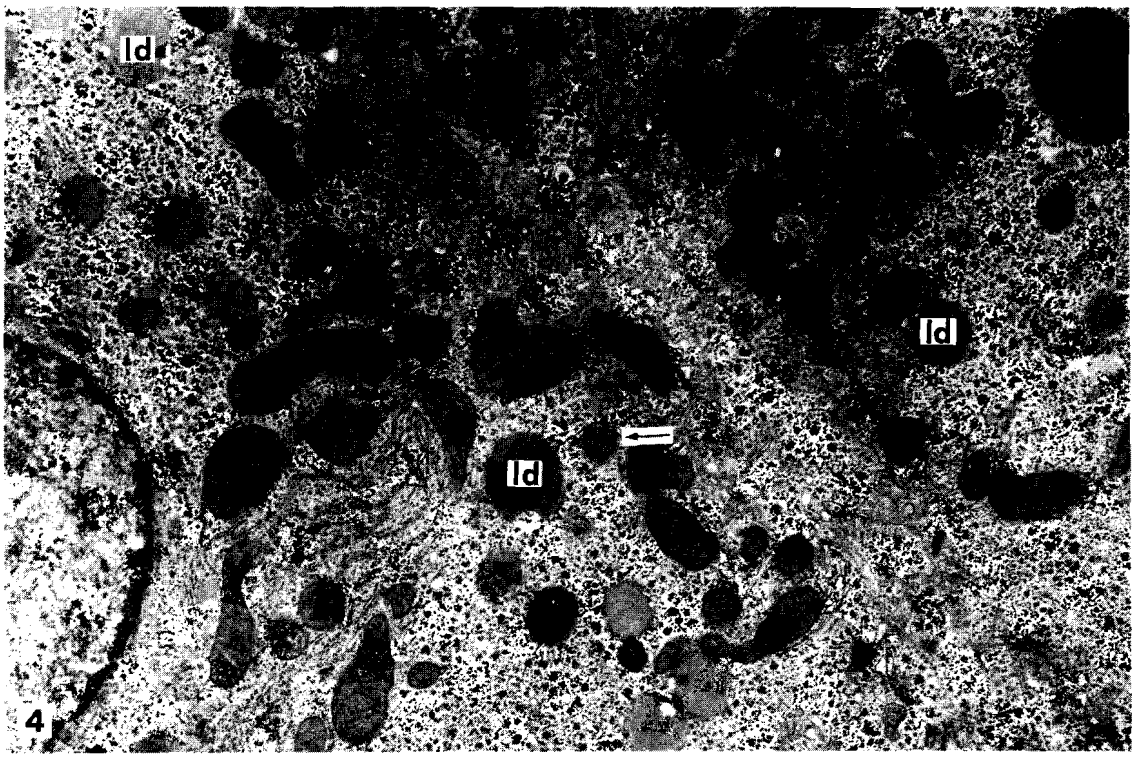

Fig. 4. After 3 days of 4APP administration, many small lipid droplets **(ld)** are present in the cytoplasm of hepatocytes. Arrow, peroxisome. × 10,000

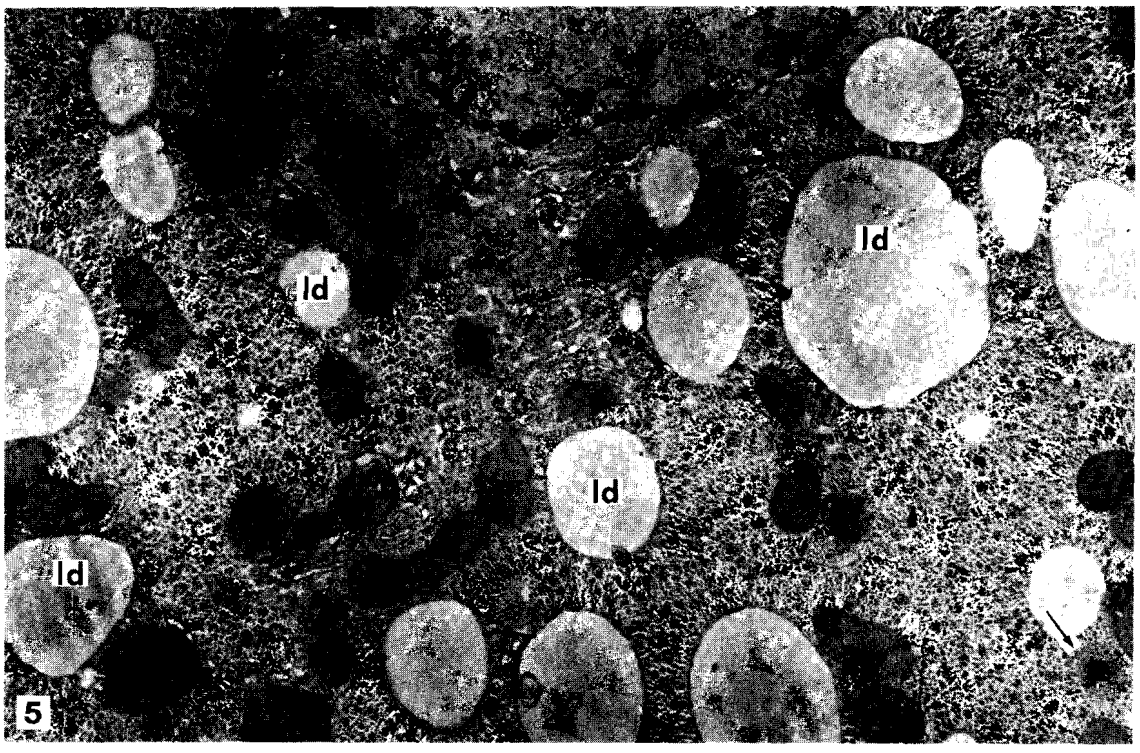

Fig. 5. Hepatocyte from a 5-day 4APP-treated rat. The cytoplasm is crowded with lipid droplets **(ld),** but other ulfrastructural features are well preserved. Arrow, peroxisome. × 12,000

Discussion

Our morphometric and stereological data of control rat hepatocytes are in good agreement with those reported by Loud (1968) and Weibel et al. (1969). The only notable differences concern V_v of membrane space (21-37%) and S_v of SER (16-100%), but it must be noted that the presence of the abundant glycogen renders these estimates rather difficult and not easily comparable to those obtained in experiments carried out in different laboratories. Moreover, in Weibel's experiment rats were fasted for 24 h before the sacrifice.

At variance with previous investigations (Murakoshi et al., 1985; Almeida et al., 1987), in our experiments prolonged 4APP administration, though producing a net hypocholesterolemic effect, does not cause a notable disruption of the morphology of rat hepatocytes. This fact can be easily explained by taking into account that the doses of 4APP employed by previous investigators were 3- to 10-fold higher than ours (12 mg/kg or 50 mg/kg versus 4 mg/kg).

One of the most striking 4APP-induced stereological change is the notable reduction of RER, which, according to Murakoshi et al. (1985) could be ascribed to the loss of attached ribosomes (degranulation). This finding appears to be in keeping with the current views concerning 4APP metabolism in liver: 4APP interferes with purine synthesis (Iwamoto & Martin, 1974), and might affect nucleolar rRNA synthesis and processing (Almeida et al. 1987). This effect may also underly the well-known 4APP-induced impairment of the synthesis of lipoproteins (Shiff et al. 1971), which occurs in RER of hepatocytes (for review, see Stäubli and Hess, 1975).

Cholesterol and triglycerides are the main products that are transported in the bloodstream in the form of lipoproteins (for references, see Stäubli and Hess, 1975). Thus, the 4APP-evoked impairment of the synthesis of the polypeptide chains of lipoproteins may explain the conspicuous rise in the hepatic concentration of both cholesterol and triglycerides. This contention accords well with the second major morphological effect of 4APP on rat hepatocytes: the striking accumulation of lipid droplets, in which are stored cholesterol and triglycerides which cannot be assembled in exportable lipoproteins.

Some comment is required on the finding that 4APP causes a significant decrease in SER and peroxisomes. Parenthetically the lowering of SER may explain the net decrease in the V_v of membrane space, of which this organelle is one of the major components. It is commonly agreed that cholesterol synthesis occurs in SER (Chesterton, 1968), in which 3-hydroxy-3methyl-glutaryl coenzyme A (HMG-CoA) reductase, the rate-limiting enzyme of this process (Brown and Goldstein, 1980), is located (Liscum et al., 1983; Chin et al., 1984; Reinhart et al., 1987). This enzyme seems to be contained also in liver peroxisomes (Keller et al., 1985), which, therefore, might somehow be involved in cholesterol synthesis.

A large body of data suggests that the activity of HMG-CoA reductase is suppressed by an increased level of the intracellular pool of free cholesterol, through a mechanism involving the inhibition of the transcription of HMG-CoA reductase gene (Luskey et al., 1983; Osborne et al., 1985; Brown and Goldstein, 1986). We have not actually measured free-cholesterol pool in the hepatocytes of 4APP-treated rats, but judging from the behaviour of the liver concentration of total cholesterol, it seems reasonable to conceive that it can be raised. In light of these considerations, we propose that the decrease in SER and peroxisomes can be considered the morphological counterpart of an impaired synthesis of new cholesterol, which in turn would be a side-effect of the prolonged 4APP treatment.

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Accepted July 1, 1988