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Morphological and biochemical alterations in growing rats induced by etretinate

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Summary. Etretinate is an aromatic retinoid extensively used on Dermatology. Its toxic effects, however, reduce its application from a clinical point of view. In the present paper, we study etretinate intoxication of 48 growing Wistar rats. The intoxication was for 12 weeks using etretinate doses of 0.5 and 6 (mg/kg) / day. The concentrations of etretinate in plasma and liver were determined.

Total seric cholesterol and triglyceride concentrations were analyzed. Structural and ultrastructural histological studies of liver samples were carried out. Continuous etretinate ingestions seem to produce an alteration in the detoxication of enzymatic complexes in the growing rats with both the concentrations, due to the increase in etretinate blood plasma observed during the study. There is a relationship between the etretinate dose and its blood plasma concentration and toxic effect, but there is not with etretinate concentration in the liver. The blood plasma concentration of cholesterol and triglycerides is not related to histological liver lesions.

The histological study confirms hepatotoxicity with both doses. Nevertheless, the anatomopathological lesions observed do not seem to be related to the blood plasma and liver etretinate concentrations.

Key words: Morphology, Liver, Etretinate, Rat

Introduction

Retinoids are drugs related to vitamin A by molecular manipulation. These compounds are pharmacologically active in a number of dermatological diseases and for the treatment of severe psoriasis (Ott and Bollag, 1976; Geiger et al., 1984).

Etretinate is an aromatic retinoid (RO 10-9350) that has a more favourable therapeutic index than Retinol. Therefore it is very useful in severe and extensive psoriasis resistant to other treatments (Paravicini, 1981). Nevertheless, etretinate is associated with severe side effects including a potential teratogenic effect and hepatotoxicity (Committes on drugs and on nutrition, 1971).

The pharmacokinetics of etretinate has been studied previously (Hänni et al., 1977; Ray et al., 1981; Vahlquist et al., 1981). After enteric absorption, etretinate is partially hydrolized to etretin (RO 10-1670) which is less lipophylic than etretinate and more rapidly eliminated
by urine. Etretinate is stored in the liver (90%) and slowly eliminated (Hänni et al., 1977; Lauharanta, 1981; Brazzell, 1982; Paravicini et al., 1985 and Larsen et al., 1987).

The toxic effects of etretinate after successive doses have especially been studied in the liver. Several hepatic lesions have been detected (Glacer et al., 1982; Foged et al., 1984; Van Voorst et al., 1984; Weiss et al., 1984; Zachariae et al., 1985).

In the present paper we analyze the toxic effects of etretinate (RO 10-9350) in growing Wistar rats (I Co: WI-IOPS AF/HAN).

Materials and methods

Animals

The study was carried out with 48 growing Wistar rats. The animals were randomly allocated into four groups (each with 12 rats):

1.– Control group = untreated animals.

2.– Group 0 = animals treated with the ingestion of pure olive oil.

3.– Group 1 = animals treated with ingestion of 0.5 mg of etretinate/kg/day (Dose – 1).

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4.- Group 2 = animals treated with the ingestion of 6.0 mg of etretinate/kg/day (Dose - 2).

Reagents

All the reagents were purchased from Merck. Acetonitrile (CH_3CN) and acetic acid were from FEROSA, HPLC grade.

Analysis of etretinate in blood plasma

The serum sample (0.25 ml) was treated with 1 ml of 1 M phosphate buffer (pH = 6.0). Then the etretinate was extracted from the serum sample by means of 3 × 5 ml of diethylic ether and the solvent was removed. The solid residue was disolved in 1 ml of acetonitrile containing 0.5% of acetic acid.

Analysis of etretinate in liver

The rat liver samples (0.2 to 0.8 g weight) were homogenized by ultrasound (Selecta 100 watt) in a mixture of 5 ml of petroleum spirit, 2 ml of diethylic ether and 2 ml of 1 M phosphate buffer (pH = 6.0). The sonication time was 10 min. Then, the organic solvents were removed and 1 ml of acetonitrile solution (0.5% acetic acid) was added to the sample.

HPLC analysis of the samples

The samples previously obtained were analyzed by means of a series of 2 HPLC PERKIN-ELMER, 10 μg column... = 360 nm. Flux = 1.60 ml/min. Two solutions were used in the eluent Gradient. Solution A consisted of 0.5% (V/V) acetic acid in acetonitrile. Solution B consisted of 0.5% (V/V) acetic acid in water. A linear gradient was used between T_{0min} and T_{2min}. The total run time was 6 min.

Mobile phase	$T_{0.min}$	80% A	20% B
	$T_{2.min}$	90% A	10% B
	$T_{4.min}$	90% A	10% B
	$T_{6.min}$	80% A	20% B

Anthrazene was used as the internal standard.

Analysis of cholesterol

Seric cholesterol was determined by the Liebermann-Burchard method.

Analysis of triglycerides

Seric triglycerides were determined by the 14.341 Mercktest colorimetric method.

Histological analysis

The samples were carried out on a weekly basis. One animal from each group was sacrified. The liver was immediately embedded in 5% glutaraldehyde in 0.1 M phosphate buffer and fixed in 2% OsO_4.

Samples of hepatic parenchyma were treated with propylene oxide, after washing and dehydration in increasing concentrations of acetone, were finally embedded in Araldite. For ultrastructural study, 40 nm sections were cut and stained with uranyl acetate and lead citrate. Then they were examined under a 200 CX Jeol electron microscope. For structural study, the samples were fixed in 10% formaldehyde and embedded in paraffin. Afterwards, they were cut in 4 μm sections, stained with hematoxylin-eosin and examined under a light photomicroscope Dialux 20 model, Leitz.

Results

The studies of subacute etretinate toxicity found in this work were carried out for 12 weeks using growing Wistar rats (ICO: WI-IOPS AF/HAN) because little work has been done on the toxicity on retinoids in growing animals.

The variations in seric etretinate concentration with the two different doses is shown in Fig. 1. We can observe that with Dose - 1 (0.5 mg/kg/day), etretinate was only observed after the 5th week. Therefore, we could say that the etretinate was stored in silent compartments. On the other hand, with the second higher dose (Dose-2;6 mg/kg/day), we detected a very high amount, of free etretinate in plasma at the end of the 1st week. Then this concentration diminished during the second week and later increased in the next weeks. As could be expected, the etretinate concentration from the animals treated with Dose - 2 was greater than that found with Dose - 1.

The presence of etretinate in the liver was observed during the fifth week in both cases as is also found in the papers of Roenigk (1985). Etretinate doses do not significantly influence the variation in etretinate concentration in the liver. Similar conclusions were obtained from Fig. 2 where we show the variation of total cholesterol and triglyceride concentration in serum.

Histopathological results

The histopathological results showed alteration in the hepatic parenchyma even during the first weeks of treatment and with the smaller Dose - 1. In all cases we observed congestion of central veins and hepatocyte tumefaction (Figs. 3, 4). Thus we can deduce that the hepatic tissues undergoe a toxic process. These lesions increased throughout the weeks of treatment with etretinate.

In the case of Dose - 2, we observed greater lesions than with Dose - 1. We observed congestion and portal leucocitary infiltration, large amounts of glycogen in the hepatocytes and hypertrophy of Kupffer cells (Fig. 5) that showed lipofuscin pigment fagocitosis with electron microscopy.

During the fifth week of treatment with Dose - 2 the lesions were very stron. We observed that the hepatocytes were very tumified and degenerated. On the other hand we observed narrowing of the sinusoids, and

Table 1. Etretinate in liver concentration (mg/g of liver).

Weeks	Dose - 1	Dose - 2
1	0	0
2	0	0
3	0	0
4	0	0
5	0	0
6	$5.7.10^{-3}$	$6.6.10^{-3}$
7	$0.9.10^{-3}$	$1.6.10^{-3}$
8	2.10^{-3}	$6.3.10^{-3}$
9	$1.3.10^{-3}$	2.10^{-3}
10	$1.2.10^{-3}$	$4.2.10^{-3}$
11	$1.3.10^{-3}$	$1.3.10^{-3}$
12	$1.4.10^{-3}$	$1.9.10^{-3}$

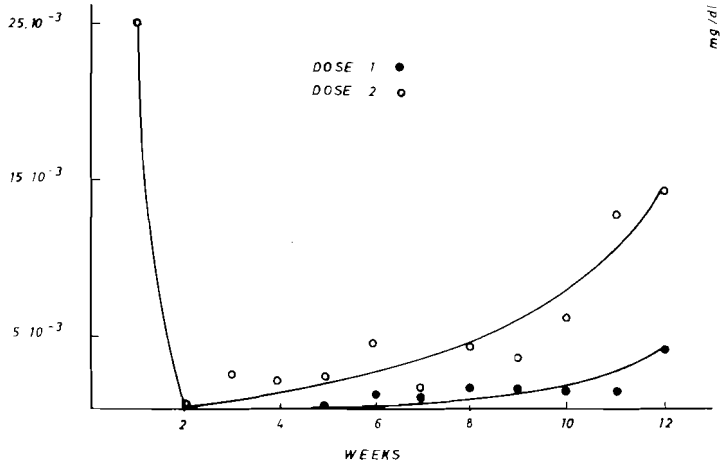

Fig. 1. Variation of the blood plasma etretinate (mg/ml) in growing rats.

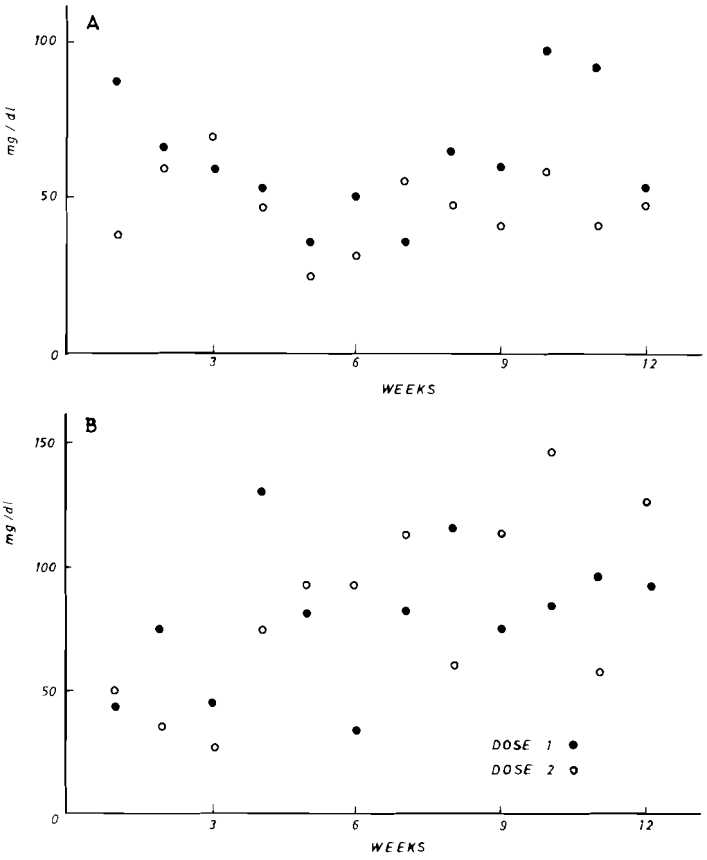

Fig. 2. Total cholesterol, and triglycerides (mg/ml) in plasma in the growing rats studied.

parenchymatosa hepatitis with lymphocytes and macrophage infiltration (Fig. 6). During the last weeks, these lesions got larger and we observed very strong perivascular edema in the portal veins (Fig. 7). The hepatocytes showed a large number of glycogen spots (Fig. 8). These glycogen spots were wrapped in membranes in some cases (Fig. 9). We have not yet found the explanation for this.

Furthermore, in all cases, we observed the presence of abundant lipid droplets in the hepatocytes (Fig. 10). This process is related to the toxic fatty change.

Discussion

From the results in Fig. 1, we can say that growing rats can accept the ingestion of etretinate at low doses (Dose - 1) during short periods of time because free etretinate were not detected during the first 5 weeks. Nevertheless, higher doses (Dose - 2) produced a very high concentration of seric etretinate during the first week which could be explained by the low activity level of microsomal detoxication liver enzymes in very young animals (Foye, 1984). This enzymatic complex would be completely developed by the second week of life, therefore the blood plasma concentration diminishes drastically. This fact could explain the data observed in the first and second weeks (Fig. 1). On the other hand, this diminishing in the etretinate level can only be explained by assuming a very low hepatotoxicity for etretinate at short ingestion periods.

The progressive increase in seric etretinate levels in serum might be related to the presence of storing tissues that slowly liberate the stored etretinate. It could also be due to a possible lessening in glucoronyl transferase activity which would eliminate free etretinate by conjugation with glucoronic acid, sending the glucoronic derivative from the bile to faeces.

Higher hepatical concentrations of etretinate were obtained with Dose - 2 than with Dose - 1 (Table 1). Nevertheless, there was no relationship (in these growing rats) between the concentration of etretinate in the liver during the experiment and the concentration of etretinate in the doses and hepatical lesions observed. These data agree with previous reports that showed the relationships between etretinate doses and blood plasma concentration and toxicity (Ganguly et al., 1953; Loerch, 1979; Ishizaki, 1983; Brandt, 1983;), but not with the hepatic concentrations (Almquist, 1952; Wright et al., 1979).

Similar conclusions are obtained from Fig. 2. In

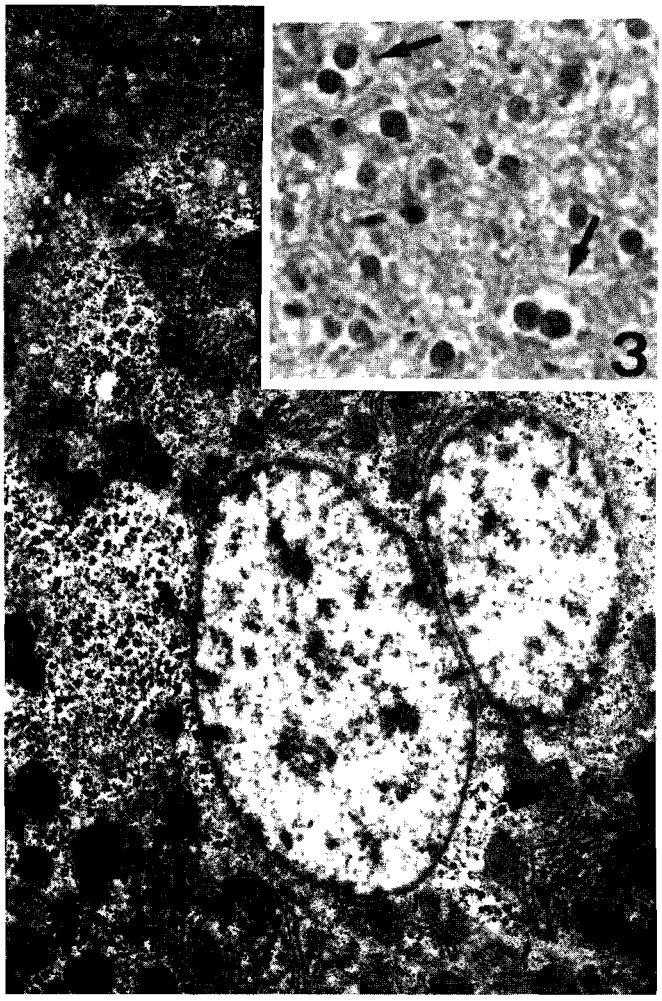

Fig. 3. Rat liver with Dose - 1, treated and sacrificed at 4 weeks showing tumefacts, binucleated, and light cytoplasm-hepatocytes. H-E. × 400

Fig. 4. Electron micrograph of rat liver with Dose - 1, sacrificed at 4 weeks showing binucleated hepatocytes, and degenerated mitochondria. × 4,000

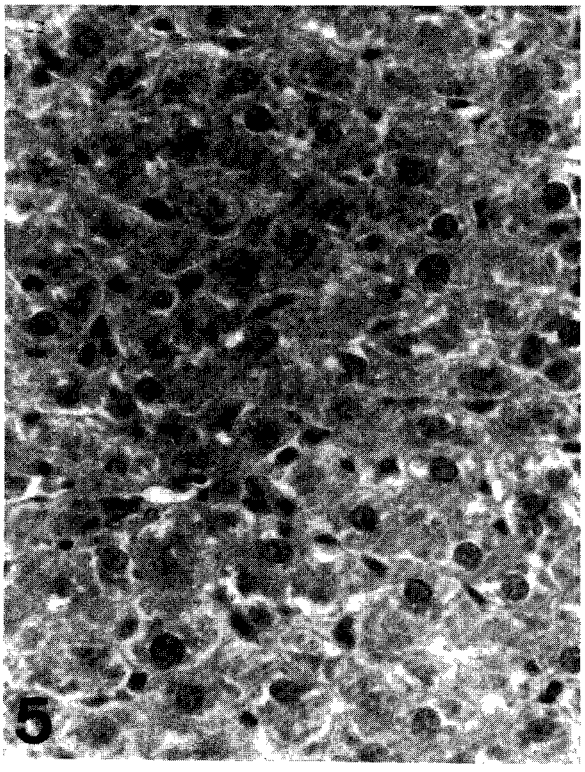

Fig. 5. Hepatic parenchyma with Dose - 2 sacrificed after 5 weeks with hypertrofic Kupffer cells and narrowing of sinusoids. H-E. × 400

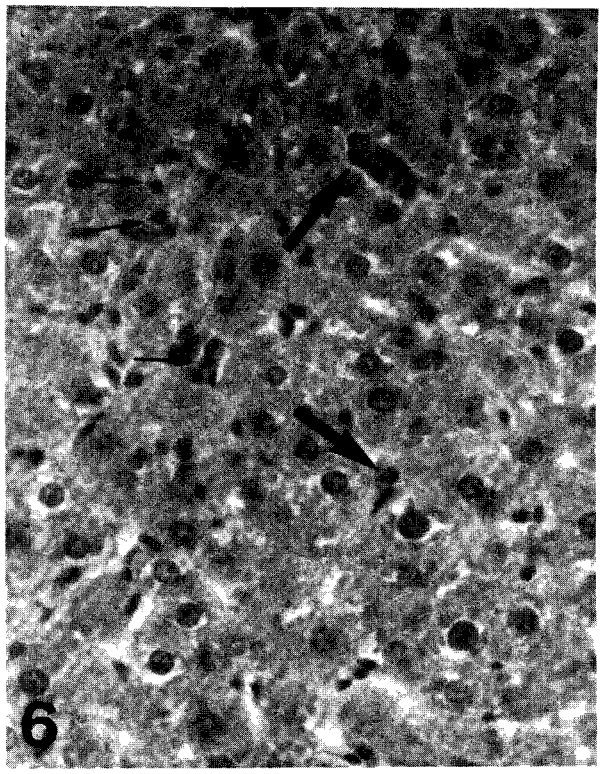

Fig. 6. Detail of liver with Dose - 2 at 7 weeks showing parenchymatous hepatitis with lymphocytes (→) and macrophages (◗). H-E. × 400

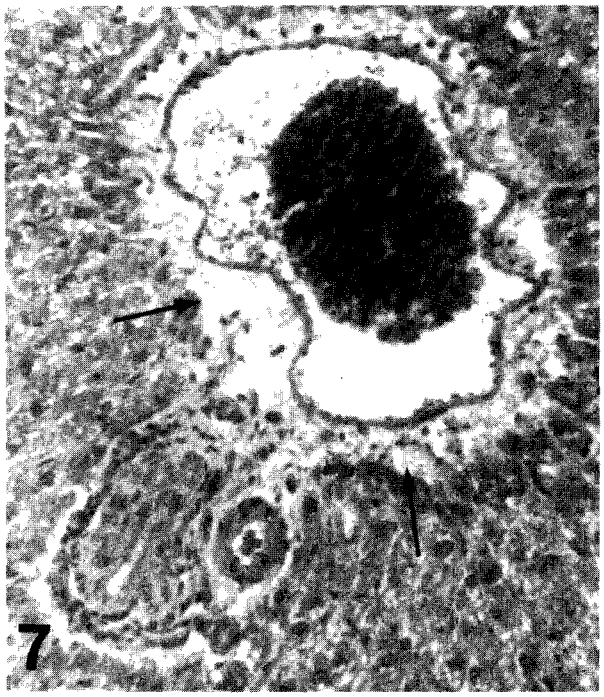

Fig. 7. Rat at 11 weeks. Dose - 2. Portal space that shows abundant perivascular edema. H-E. × 200

growing Wistar rats, the total seric cholesterol and triglycerides are not altered by the ingestion of etretinate. These results do not agree with previous papers that describe an increase in the total cholesterol concentrations due to the ingestion of etretinate (Michäelsson et al., 1981; Ellis et al., 1982). Nevertheless, the origin of the pathology is not described by these authors. This different behaviour could be related to the fact that our

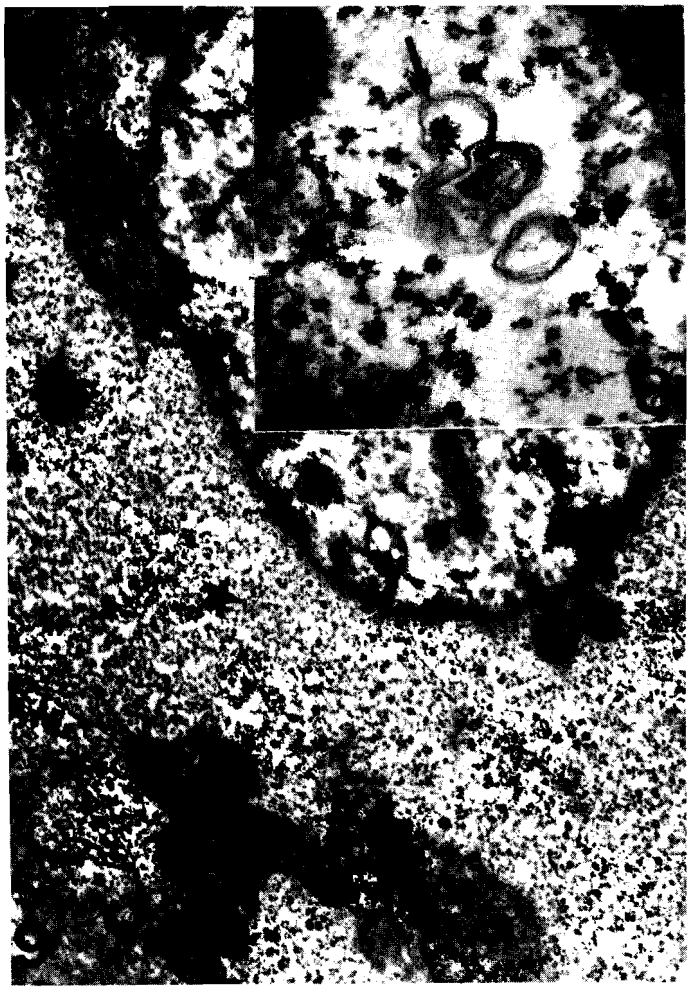

Fig. 8. Detail of rat liver with Dose - 2 at 8 weeks; hepatocytes showing glycogen (*). × 6,000

Fig. 9. Detail of hepatocyte of rat with Dose - 2 at 8 weeks with isolated glycogen spots (◗) and glycogen spots surrounded by irregular membranes. × 12,000

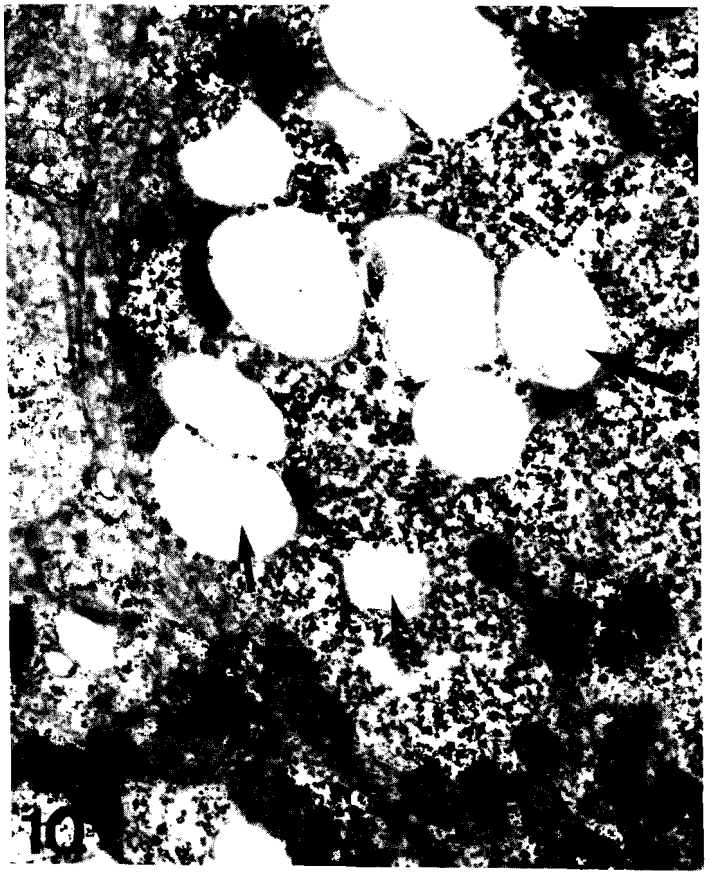

Fig. 10. Electron micrograph of rat with Dose - 2 showing a detail of hepatocyte infiltrated with lipid droplets initiating a fatty change (◗). × 6,000

data were obtained from growing rats.

On the other hand, our results agree with those of Paravicini (1981) and suggest the presence of storing tissue. This tissue could be liver as well adipous tissue (Roenigk, 1985), and not only that of the liver as has been reported by other workers (Olson, 1983). This is because in our experimental conditions the hepatic tissue was completely destroyed by ultrasound.

From the histological results we can affirm that etretinate soon produced hepatical lesions, even with Dose - 1 (Fig. 3). This fact can be explained by the lack of development of the hepatic detoxifying enzymatic complexes in the first weeks of life in the growing rats (Foye, 1984).

There is controversy with respect to the relationship between the ingestion of etretinate and the presence of significative anatomopathological hepatic lesions. This fact has not been reported by some workers (Glazer et al., 1982; Zachariae et al., 1985), although it was observed by others (Van Voorst et al., 1984; Weiss et al., 1984; Zacharie et al., 1985; Roenigk, 1985). This could be related to different genetic resistances of the experimental animals used in these studies.

Unfortunately, there are no studies on growing animals whose results could be compared to those we have obtained.

Our results agree with those of Weiss et al. (1984). We have observed loss in the cord situation and tumefaction of the hepatocytes (Fig. 3). Furthermore, in Fig. 7, we can see the perivascular edema, infiltration of eosinophils and lymphocytes, and hypertrophy of the Kupffer cells (Fig. 5). On the other hand we have observed a great deal of mitosis as the hepatic response to the lesions described in Fig. 3.

By electron microscopy, we have observed alteration and degeneration of the mitochondria in the sacrificed growing rats during the first weeks of life (Fig. 4). The growing rats sacrificed in the last two weeks also showed an accumulation of lipids in the hepatocytes (Fig. 10). These lesions have been much more extensively observed in the rats that received greater etretinate doses (Dose - 2). The results agree with those reported by Forouhar (1984) and Roenigk (1985).

Geubel (1983) has reported hypertrophy of the agranular endoplasmic reticulum. Nevertheless, we have not observed this fact in the animals tested. This could be

explained by assuming that the growing organism of the rat favours the generation of the P_{450} enzymatic complex, placed in the agranular endoplasmic reticulum which would biotransform etretinate in more hydrosoluble products, in order to be eliminated by the kidney as etretin which has been shown to be non-binding with respect to fat-storing tissues (Paravicini et al., 1983; Glazer et al., 1984; Brazzel, 1982). Nevertheless, we have not observed hypertrophy of the agranular endoplasmic reticulum in the hepatocytes, so we must admit that there are biochemical lesions at the level of the citochrome P_{450} complex or more probably in the glucoronyl transferase that eliminates the products from biotransformation reactions (phase-1) as glucoronyl derivates that are eliminated from the bile to the faeces.

The presence of glycogen in the hepatic cells - to a greater or lesser degree - in appreciable amounts in all the animals tested, although with very different locations in the cytoplasm, does not seem to have pathological significance (Figs. 8, 9).

The presence of the edema and tumefaction of the hepatocytes has been described in several cases (Abuirmeileh, 1986) as the response of the hepatic cell to the presence of external toxic substances in order to eliminate them without alteration of the cellular structure. Nevertheless, in other cases, the presence of an edema in the hepatocyte has been related to the alteration of the cellular membrane by the absorption of lipids as could be the case of etretinate. Nevertheless, no results have been reported regarding the behaviour of hepatocytes in growing animals in similar experimental conditions.

We can conclude that biochemical and morphological lesions produced by extended ingestion of etretinate are small and therefore confirm the high CI (Chemical Terapeutical Index) of etretinate (Paravicini, 1981) even for growing rats.

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