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An ultrastructural study of spontaneous and phenobarbitone-induced nodules in the mouse liver*

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Summary. Male C3H/He mice were given 0 (control) or 85 mg/kg/day phenobarbitone (PB) in the diet. At 40, 60 and 93 weeks, groups of mice were killed and the ultrastructure of spontaneous and PB-induced liver nodules was examined. Treated mice showed typical centrilobular hypertrophy and eosinophilic nodules which may be considered as an end stage lesion. The nodule cells were similar in appearance to those in areas of centrilobular hypertrophy except for the presence of convoluted membranes which are considered to be indicative of proliferation. The incidence of carcinoma was not increased by PB treatment. The carcinomas from control and treated animals differed in their ultrastructure in that increased levels of smooth endoplasmic reticulum (SER) were seen in the carcinomas of the PB animals. The presence of SER proliferation in the carcinomas of PB animals suggests that carcinoma may respond to the enzyme-inducing effects of PB.

Keywords: hepatocellular carcinoma, simple nodules, phenobarbitone, ultrastructure

Certain strains of mice (C3H, C3B6F1) show a high and variable incidence of spontaneous basophilic hepatic nodules which may be simple nodules or hepatocellular carcinomas (Grasso & Hardy 1975). The incidence of both lesions can be increased by treatment, e.g. a single neonatal injection of a genotoxic carcinogen such as diethylnitrosamine (Vesselinovitch *et al.* 1978; Cunninghame *et al.* 1990). In contrast, feeding phenobarbitone (PB) to the susceptible strains of mice increases the total number of liver nodules, but this is due to the appearance of eosinophilic nodules. The incidence of either simple basophilic nodules or carcinoma is unaffected or even decreased (Evans *et al.* 1986).

Studies of the histogenesis of the basophilic and eosinophilic nodules suggest at least two separate pathways of development (Evans *et al.* 1986). Basophilic nodules arise as small foci of basophilic hepatocytes within the lobule and an increased incidence is associated with an increase of hepatocarcinomas. Eosinophilic nodules arise from centrilobular hypertrophy and hyperplasia. They are essentially benign end stage lesions.

The present study examines the ultrastructural features of spontaneous and PB-induced nodules in order to compare the eosinophilic nodules with basophilic nodules and carcinomas.

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Materials and methods

Four-week-old male C3H/He mice were obtained from OLAC 1976 Ltd (Bicester, Oxon, UK). The animals were housed in plastic cages with wire mesh floors, and maintained in rooms held at 22±2°C with a relatively humidity of 55±10%. The mice were allowed free access to a semi-synthetic diet (Labsure Animal Foods, Poole, Dorset, UK) and to tap water. After a 4-week period of acclimatization animals were either kept on the control diet or given the diet containing PB to provide a daily intake of 85 mg/kg bodyweight. At 40, 60 and 93 weeks, groups of five control and five treated animals were anaesthetized with Sagatal (May & Baker Ltd, Dagenham, England) and fixed by whole body perfusion through the left ventricle with 4% glutaraldehyde in 0.066 M sodium cacodylate buffer (pH 7.4). The livers were left *in situ* for 1 h before being removed, sliced and transferred to fresh fixative for 4 h. After washing in 0.066 M sodium cacodylate buffer containing 0.25 M sucrose, multiple pieces of tissue were post-fixed in 1% osmium tetroxide in Millonig's buffer overnight. The tissues were dehydrated through graded alcohols, followed by propylene oxide before embedding in Epon Araldite resin. Thin sections (1 μm) of the slices were stained with toluidine blue for examination by light microscopy. Ultrathin sections of selected areas were cut and picked up onto uncoated copper grids, stained with uranyl acetate followed by lead citrate (Reynolds 1963) and examined with a Jeol 100CX electron microscope. In addition, pieces of liver were also embedded in paraffin wax, sectioned at 5 μm and stained with haematoxylin and eosin (H&E).

Results

Basophilic nodules were seen in three of the control mice while carcinoma were found in another two control mice. In PB-treated mice, 11 nodule bearing mice were examined, 10 of which had eosinophilic nodules. Of these 10 mice bearing eosinophilic nodules, four had eosinophilic nodules only, two had both eosinophilic and basophilic nodules, and three had developed carcinoma. One mouse had a basophilic and an eosinophilic nodule, and in addition bore a carcinoma. The remaining mouse had only a basophilic nodule. Centrilobular hypertrophy was seen in all treated mice.

In control livers the nodules appeared at 40 weeks. Simple hepatic nodules were recognized by the presence of single cell plates, whereas hepatocellular carcinoma were recognized by the presence of thickened irregular trabeculae. In toluidine blue sections, hepatocytes from simple nodules were paler staining than normal cells and in some cases contained greatly increased amounts of fat and glycogen (Fig. 1). Ultrastructurally, the cells generally contained large single nuclei, some showing indentations, and were densely packed with mitochondria and increased amounts of rough endoplasmic reticulum (RER) which was often dilated (Fig. 2) and accounted for basophilic appearance of these nodules in H&E sections.

The carcinoma found in control animals generally formed part of a larger nodule mass. In toluidine blue sections they were composed of solid masses of cells or papilliform trabeculae separated by dilated sinusoids (Fig. 3). Some increase in cell size was seen but many small cells with irregular nuclei were also visible, particularly in areas where the carcinoma formed a solid mass. In places, bile canaliculi were greatly dilated and formed adenomatous areas. Electron microscopic examination showed the cells to contain large amounts of RER and variable numbers of mitochondria. The fat content of carcinoma cells was low. Tight junctions were generally small except in the adenomatous areas where they were extensive. Hepatocyte membranes facing the space of Disse were simplified and lacked microvilli but highly convoluted membranes were often seen between adjacent hepatocytes (Fig. 4). Areas of necrosis with hepatocytes containing apoptotic bodies appeared within the well developed carcinomas.

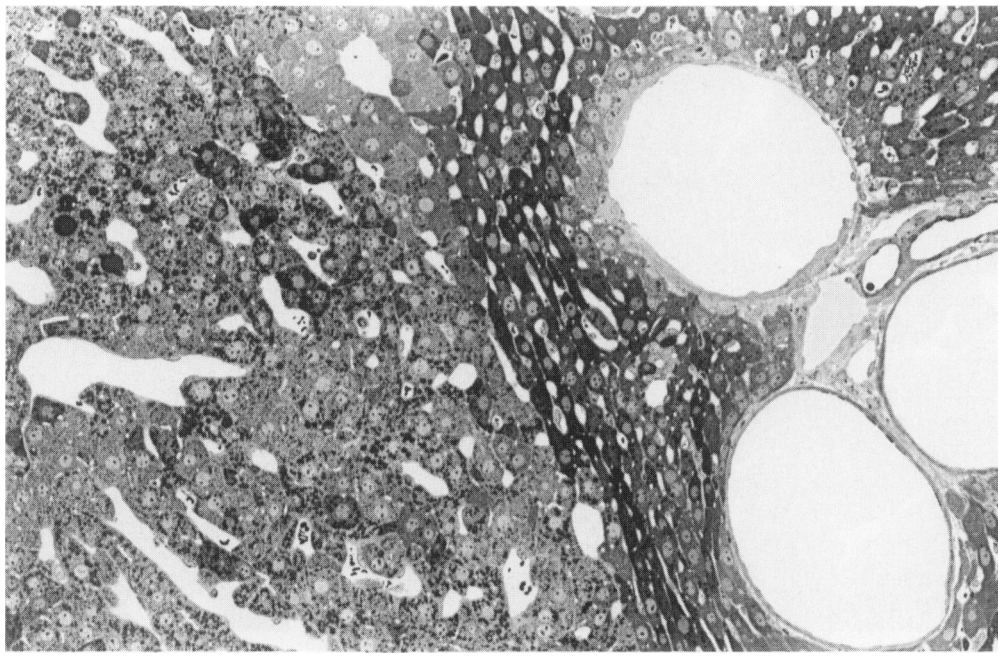

Fig. 1. A plastic embedded section stained with toluidine blue showing a simple nodule from a control mouse. × 100.

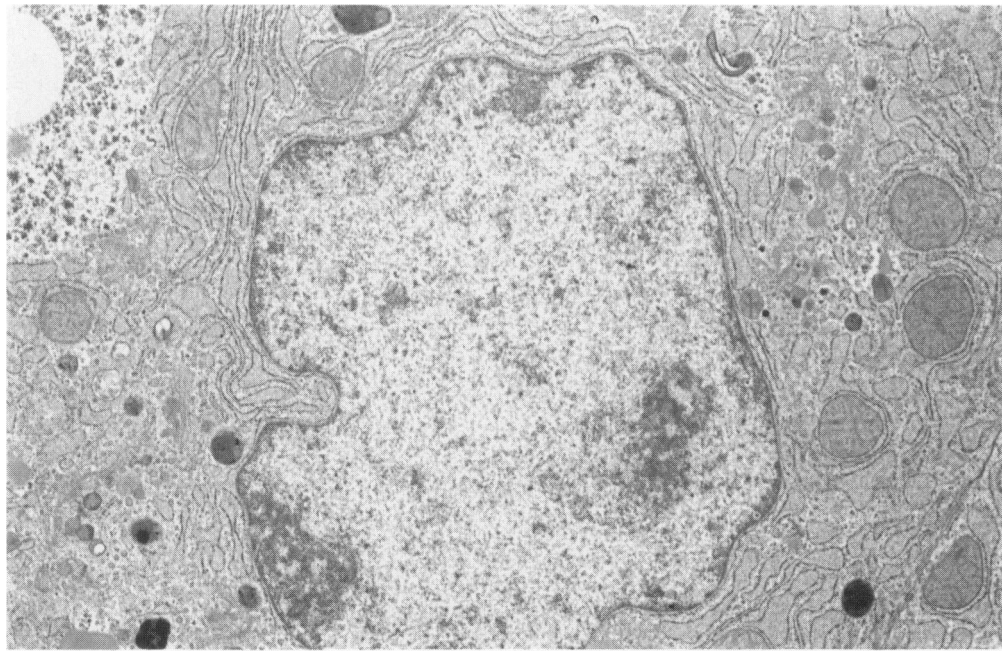

Fig. 2. Electron micrograph of a hepatocyte from a control nodule containing increased amounts of dilated RER and showing an indented nucleus. × 10 500.

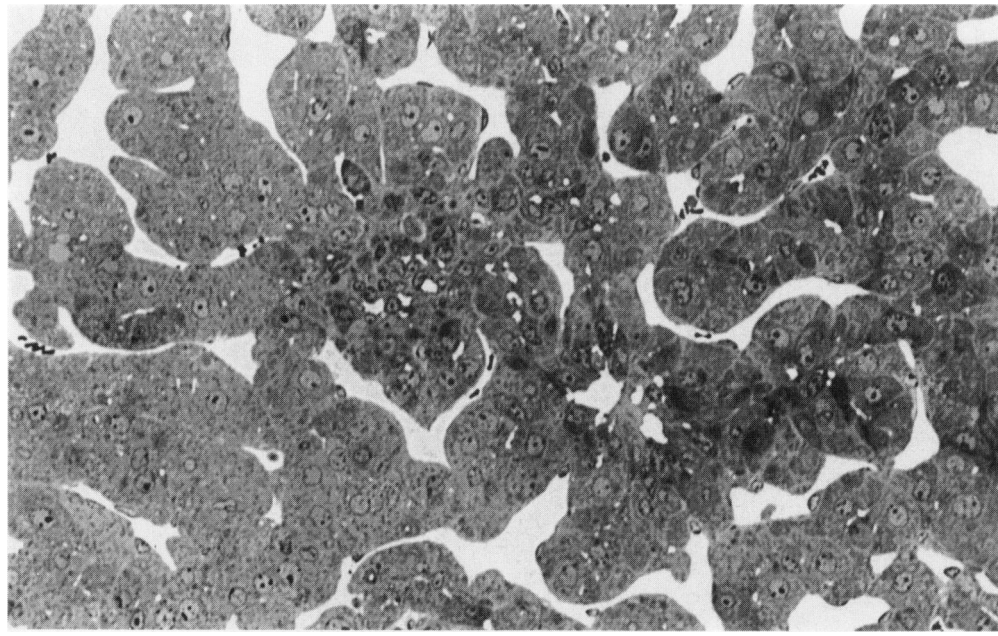

Fig. 3. A plastic embedded section stained with toluidine blue showing a carcinoma from a control mouse. × 100.

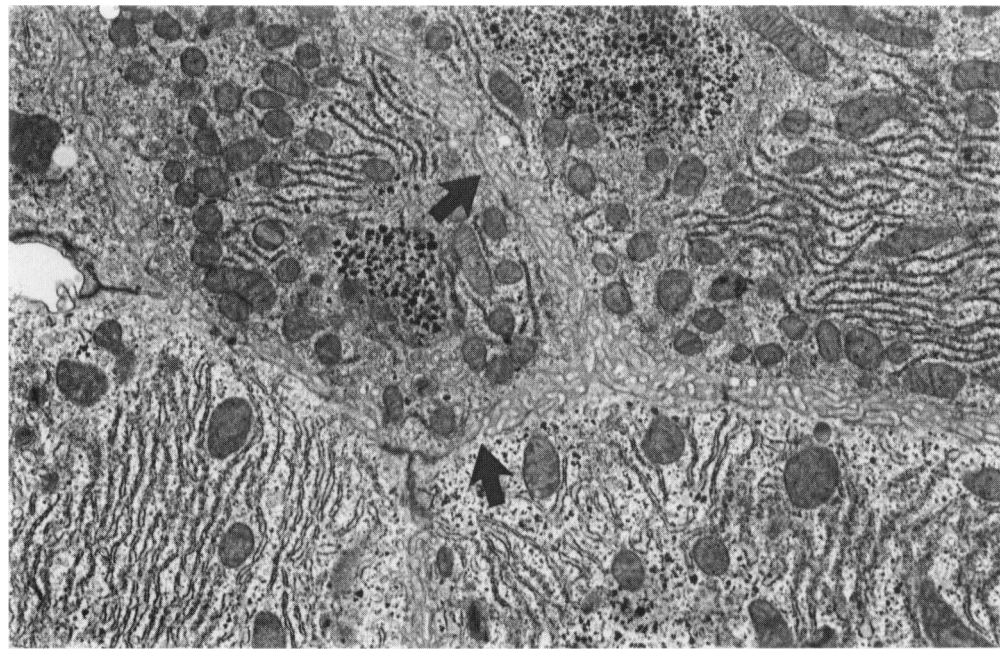

Fig. 4. Electron micrograph of an area of carcinoma from a control animal showing convoluted membranes between the hepatocytes (arrows). × 10 500.

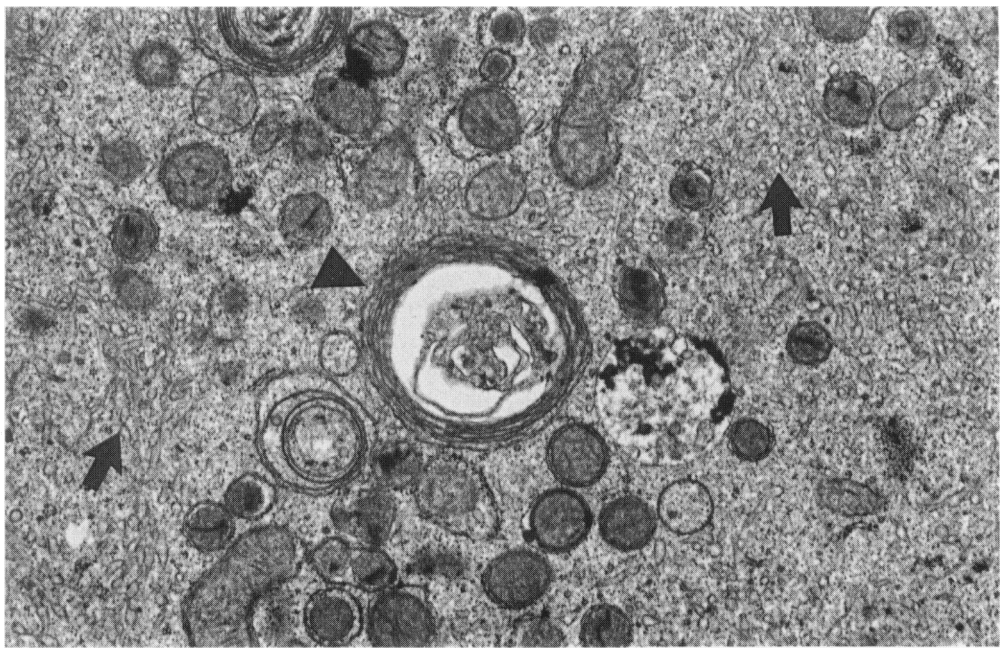

Fig. 5. Electron micrograph of a hepatocyte from an area of centrilobular hypertrophy showing increased amounts of SER (arrows) and myelin whorls (arrow heads). × 16 500.

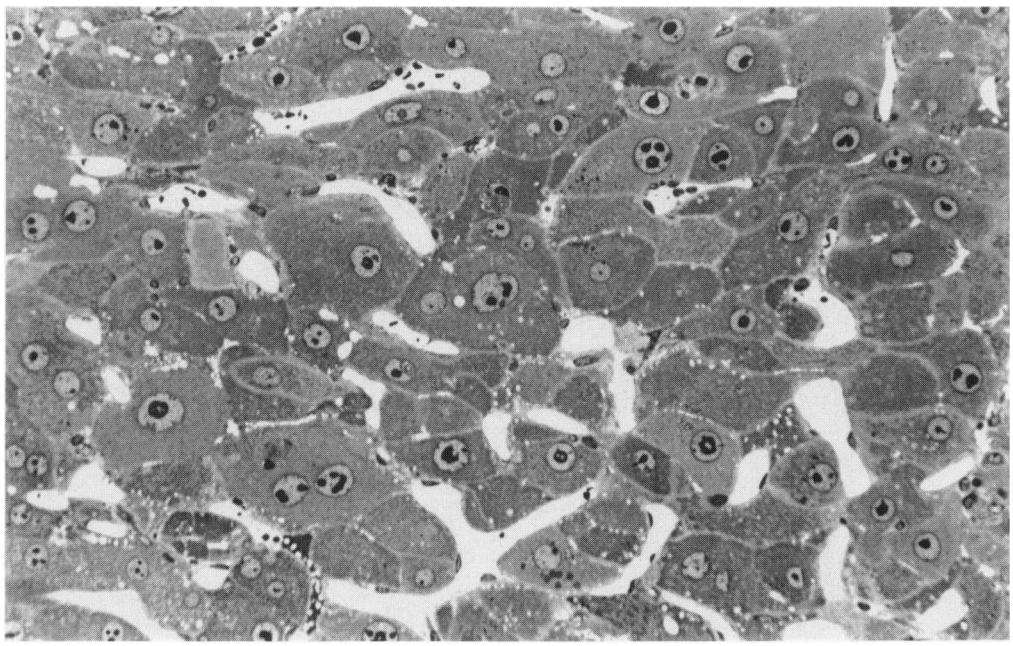

Fig. 6. A plastic embedded section stained with toluidine blue showing part of an eosinophilic nodule found in a PB treated mouse. × 100.

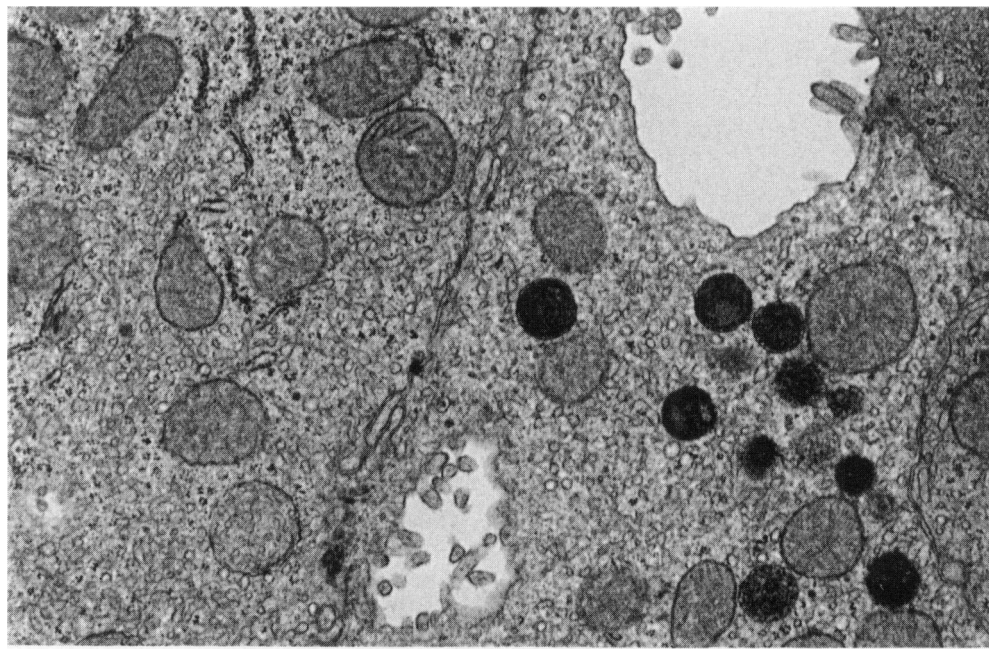

Fig. 7. Electron micrograph of hepatocytes from an eosinophilic nodule showing increased amounts of SER. × 20 000.

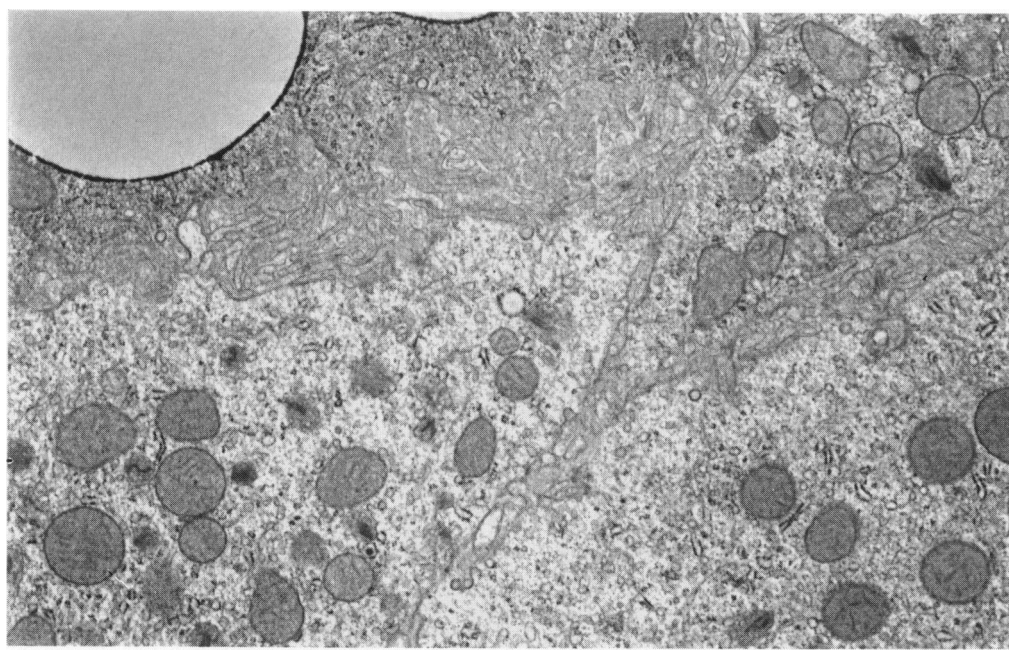

Fig. 8. Electron micrograph of hepatocytes from an eosinophilic nodule separated by convoluted membranes. × 13 860.

The centrilobular hypertrophy found in PB-treated mice was characterized by an increase in cell size and the appearance of multinucleate cells containing pleomorphic nuclei with multiple nucleoli as previously described (Jones & Butler 1975). Ultrastructurally, these cells contained increased amounts of SER and occasional whorls of myelin and RER which largely accounted for the increase in cell size and the eosinophilic nature of hypertrophied cells in H&E sections (Fig. 5). There was an increase in the number of macrophages and lymphocytes in the areas of centrilobular hypertrophy, and many necrotic cells were present.

In toluidine blue sections, the eosinophilic hepatic nodules showed a change in trabecular pattern with an increase in pale staining cytoplasm and compression of surrounding hepatocytes. These cells were characterized by the large pleomorphic nuclei observed in the areas of centrilobular hypertrophy (Fig. 6). Ultrastructurally, hepatocytes from the eosinophilic nodules were seen to contain large amounts of SER which caused displacement and in places an apparent reduction in RER and mitochondria (Fig. 7). The ultrastructure of these hepatocytes closely resembled that seen in areas of centrilobular hypertrophy. In addition, areas of basophilic cells were often seen within large pleomorphic nodules and in some of the nodules, hepatocytes were separated by convoluted membranes (Fig. 8). Macrophages were occasionally seen at the periphery of nodules, usually at sites of necrosis.

Also seen in the toluidine blue sections of treated animals, were simple nodules composed of small, densely staining cells with single nuclei which corresponded to the basophilic nodules in H&E sections. On electron microscopic examination, no significant increase in SER was observed and in general the cells showed features similar to those described for the basophilic nodules from control animals (Cunninghame *et al.* 1990).

The first observed carcinoma in PB-treated mice was at 60 weeks and was recognized by the presence of thickened abnormal trabeculae. As with control livers, the incidence of

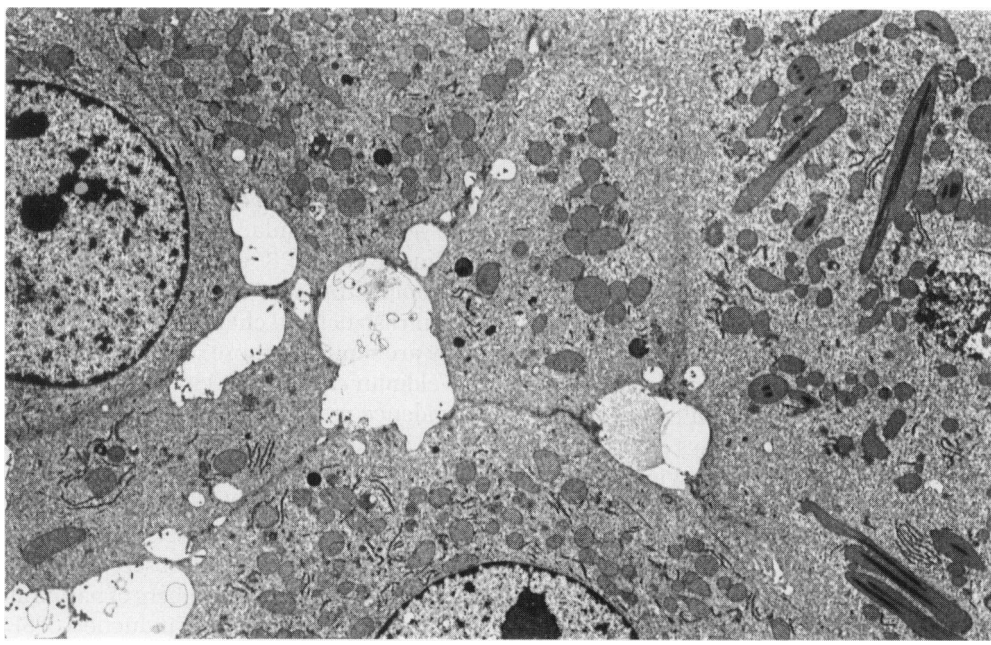

Fig. 9. Electron micrograph of hepatocytes from a carcinoma found in a PB treated mouse. ×4200.

carcinoma was low and often formed part of a larger mass. The cells of the carcinomas were irregular in size and shape and contained large single nuclei with occasional multiple nucleoli. The RER content was variable and generally less than that seen in control tissue. However, many of the cells contained abnormally high levels of SER. Mitochondria were numerous and some abnormally elongated mitochondria with an electron-dense core were present (Fig. 9). In places, the hepatocyte membranes appeared to be convoluted as seen in the carcinomas found in control tissues (Fig. 4).

Discussion

The C3H/He strain of mice shows a naturally high incidence of hepatic nodules (Andervont 1950; Heston & Vlahakis 1961). Administration of the xenobiotic drug PB increases the incidence of nodules in these mice (Peraino *et al.* 1973; Evans *et al.* 1986), the increased nodule burden being due to the appearance of a type of nodule not usually observed in controls composed of large eosinophilic cells (Evans *et al.* 1986; Jones & Butler 1975). The incidence of basophilic nodules and carcinoma seen in control animals does not increase following PB treatment (Evans *et al.* 1986).

Following treatment with PB, the cells of the large eosinophilic nodules were ultrastructurally similar to the cells seen in the areas of centrilobular hypertrophy. Biochemical studies have shown a marked increase in mixed function oxidase enzyme activities in both large nodules and surrounding host liver tissue following treatment with PB (Collins *et al.* 1984). Histological studies have indicated that centrilobular hypertrophy/hyperplasia grows directly into the eosinophilic nodules. In previous experiments (Evans *et al.* 1986) we have shown that PB increased the incidence of eosinophilic nodules but did not increase the number of carcinomas within the life span of the mouse. This suggests that the eosinophilic nodules, having the ultrastructural characteristics of the enzyme-induced centrilobular cells, are end stage lesions. Previously we have reported (Cunninghame *et al.* 1990) that carcinomas in diethylnitrosamine-treated mice have a markedly convoluted plasma membrane and also have abnormal membrane glycoproteins (Pritchard *et al.* 1989). In the present study we have observed convoluted plasma membranes in simple eosinophilic nodules which may correspond to changes recorded in membrane glycoproteins (Pritchard *et al.* 1989). Changes of this nature in the cell surface have been associated with neoplasia (Abercrombie & Ambrose 1962) and may simply indicate an increased cell proliferation within a population of hepatocytes and may not be specific for neoplasia.

The incidence of carcinoma was not increased following PB treatment. However, we have found ultrastructural differences between carcinomas derived from control and PB-treated mice. The increased amounts of SER in the carcinomas of PB-treated livers is a feature normally associated with enzyme induction and is observed in centrilobular cells and the eosinophilic nodules. Therefore in addition to these populations of inducible cells, there appears to be a portion of cells within carcinomas which also show evidence of enzyme induction. The appearance of increased levels of SER in carcinomas arising in PB-treated mice may be indicative of an adaptive cellular response to long-term treatment with PB within a spontaneous developing tumour (Feldman *et al.* 1981). The ultrastructural changes observed in this study are similar to those reported previously by Feldman *et al.* (1981) in the rat.

Evidence previously reported (Evans *et al.* 1986) and the present results indicate that in the main, basophilic nodules arising in PB-treated mice are not able to respond to the enzyme induction of PB as recognized by the proliferation of SER. The observation of a slight reduction in the numbers of basophilic nodules and the evidence of induction of SER

in carcinomas might suggest that a few of the basophilic nodules and carcinomas retain the capacity to respond to PB.

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