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The Effect of 3′-Methyl-4-Dimethylaminoazobenzene on Foetal Rat Hepatocytes in Culture*

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Abstract—*In hepatocyte cultures derived from 15-day and 19-day rat foetuses tyrosine aminotransferase accumulates during culture. In 15-day foetal hepatocytes enzyme is not detectable on day 1, whereas enzyme is already present in 19-day foetal hepatocytes at the same time. The effect of the hepatocarcinogen, 3′-methyl-4-dimethylaminoazobenzene (MDAB) on these two populations of differentiating hepatocytes is the subject of this study. This was undertaken to test the proposal that carcinogens may exert their effect on differentiating precursor cells.*

In cultures of 19-day foetal hepatocytes, accumulation of tyrosine aminotransferase is suppressed by MDAB whereas other liver enzyme markers examined, phosphoenolpyruvate carboxykinase and glucose-6-phosphatase, are not depressed. It is inferred from this result that hepatocytes that are about to initiate synthesis of tyrosine aminotransferase are prevented from doing so by the action of the carcinogen. In contrast, the synthesis of phosphoenolpyruvate carboxykinase by these cells which was initiated much earlier in development is unaffected. These findings would be consistent with the concept that carcinogens affect differentiating cells.

In 15-day foetal hepatocytes, contrary to expectation, no effect of the hepatocarcinogen could be demonstrated. It is suggested that this may be due to the inability of the very immature hepatocytes to activate the hepatocarcinogen.

In agreement with the biochemical data, the 19-day foetal hepatocytes but not the 15-day foetal hepatocytes display altered morphology when exposed to MDAB.

INTRODUCTION

THE NOTION that tumourigenesis results from abnormal differentiation is not new [1, 2]. Two recent observations lend support to this proposal. Firstly, it has been shown that the tumour promoter phorbol myristate acetate (PMA) blocks differentiation of precursors of myoblasts [3] and chondroblasts [4] as well as adipocytes [5]. Secondly, in culture, the presence of Rous sarcoma virus prevents myogenesis [6] as well as chondrogenesis [7]. These findings could be interpreted to suggest that as a consequence of the effects of the tumour promoter or viral infection, precursor cells have failed to differentiate normally and have instead generated transformed progeny. Implicit in this hypothesis is that the target cells of tumourigenic agents are the precursor cells of the respective cell types and it should follow that the susceptibility to carcinogens of a given tissue should correlate with the numbers of precursor cells present at the time of exposure.

It has previously been shown that cultured hepatocytes derived from 15-day gestation rat foetuses contain 'precursor hepatocytes' which differentiate in culture with the concomitant acquisition of ability to synthesize tyrosine aminotransferase [8]. In contrast, cultures derived from 19-day gestation foetuses display significant levels of enzyme from the time the culture is initiated. It is concluded that a proportion or all of these cells have begun synthesizing tyrosine aminotransferase [8]. The above hypothesis predicts that cultures of foetal hepatocytes derived from early gestation (15 days) rats should be more susceptible to carcinogen treatment than hepatocytes from late gestation (19 days) rats.

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In this study, the effects of a hepatocarcinogen, 3′-methyl-4-dimethylaminoazobenzene (MDAB), on these two groups of hepatocytes is examined with respect to the accumulation of tyrosine aminotransferase, a marker acquired during culture. This is compared with another liver enzyme marker, phosphoenolpyruvate carboxykinase, which is detectable in both groups of hepatocytes from the onset of culture. This enzyme may also serve to assess the extent of toxicity resulting from carcinogen treatment. A third liver enzyme, glucose-6-phosphatase, which is restricted to the endoplasmic reticulum was also measured in these experiments.

MATERIALS AND METHODS

Chemicals

L[3,5-^{3}H]-tyrosine and U[^{14}C]-glucose-6-phosphate were obtained from the Radiochemical Centre, Amersham, Berks., U.K. L-Tyrosine and sodium diethyldithiocarbamate were from Merck, Darmstadt, Federal Republic of Germany. Pyridoxal 5-phosphate, 2-oxoglutarate, NADH, dexamethasone, ATP, ADP, phosphopyruvate, dibutyryl-cAMP, dithiothreitol, EDTA and diaminobenzoic acid were obtained from Sigma Chemical Co., St. Louis, MO. Oxaloacetate, collagenase type II, lactate dehydrogenase and pyruvate kinase were products of Boehringer, Federal Republic of Germany. Tokyo Kasei Kogyo, Japan supplied 3′-methyl-4-dimethyl aminoazobenzene.

Eagle's Minimal Essential Medium (MEM) was obtained in powder form from Flow Laboratories, Annandale, N.S.W., Australia, and foetal calf serum from Commonwealth Serum Laboratories, Parkville, Victoria, Australia. Fungizone, penicillin/streptomycin and glutamine were purchased from Grand Island Biological Co., Grand Island, NY, U.S.A.

Animals

Wistar albino rats were used. Gestational age was determined from the time of detection of spermatozoa in the vaginal tract and is accurate to within 7 hr.

Culture medium

The basic culture medium was modified MEM supplemented with 10% foetal calf serum, glutamine (2.4 mM final concentration), Fungizone (28 μg/ml) and penicillin/streptomycin (57 units/ml and 570 μg/ml, respectively). The culture medium was supplemented with 10 μM dexamethasone in experiments in which tyrosine aminotransferase was assayed. This level of the steroid analogue has previously been shown to maximize the yield of enzyme [9]. Similarly, 24 hr prior to harvesting cells for phosphoenolpyruvate carboxykinase assay, cultures were exposed to 0.15 mM dibutyryl-cAMP in order to maximize the level of the enzyme [10].

Hepatocyte isolation

Livers from 19-day foetal rats were chopped and incubated with collagenase in balanced salts solution [11] as described by Yeoh *et al.* [8]. Cells were harvested and washed twice in balanced salts solution by centrifugation at 50 ***g*** for 2 min, then suspended in culture medium.

Culture conditions

The cultures were maintained at 37°C in a water-saturated atmosphere of 5% CO_2/95% air. The hepatocytes formed a monolayer, attached to the collagen substrate. Most of the haemopoietic cells remained in suspension and were discarded when the medium was replaced 24 hr after inoculation. The residual haemopoietic cells were completely removed by subsequent media replacement.

Preparation of hepatocyte cytosol

Cell cultures were washed with balanced salts solution before harvesting. The cells were then removed from the dishes with a Teflon scraper and suspended in balanced salts solution and collected by centrifugation at 1600 ***g*** for 2 min. For phosphoenolpyruvate carboxykinase assay, the pellet was suspended in 0.15 ml of cold 0.05 M KH_2PO_4, pH 7.5, containing 1 mM EDTA, 1 mM dithiothreitol and homogenized in a Teflon–glass homogenizer. A sample of homogenate was taken for DNA assay and the remainder was centrifuged in a Beckman Airfuge at about 210 kPa (165,000***g*** max) for 12 min in the A-100 rotor.

For tyrosine aminotransferase assay, the pellet was suspended in 0.15 ml of 0.05 M KH_2PO_4, pH 6.8, sonicated and a supernatant obtained by centrifugation in the Airfuge.

For glucose-6-phosphatase assay, the pellet was sonicated in isotonic saline, then centrifuged at 200,000***g*** for one hour in an SW 50.1 Beckman rotor fitted with adapters. A 1:5 (w:v) suspension in 0.9% NaCl was prepared from the high speed pellet.

Enzyme assays

Tyrosine aminotransferase was assayed by a radiochemical method described previously [8]. The enzyme was assayed in fractions eluted from a CM-Sephadex column to which extracts from cultured hepatocytes were applied. Phosphoenolpyruvate carboxykinase was assayed spectrofluorometrically as previously described [11].

Glucose-6-phosphatase was assayed by the following method: the assay medium consisted of 0.35 ml of 0.05 M Tris, 2 mM EDTA adjusted to pH 6.8 and 0.01 ml of purified U[^{14}C] glucose-6-phosphate. Following preincubation for 5 min at 37°C, the reaction was started by the addition of an aliquot of cell preparation. Routine incubation was carried out for 0, 15 and 30 min for each of two volumes of stock enzyme preparation, i.e., 50 and 100 µl. This provided a range of two protein concentrations for the assay. Dilutions were made with 0.9% NaCl to give a constant incubation volume of 0.5 ml. The reaction was stopped by transferring the thin-walled tubes into a boiling water-bath for 5 min. Marbles were used to cover the mouths of the tubes to minimize loss of solution by evaporation. The assay tubes were allowed to cool before the incubation medium was applied to Dowex 1-*x*2 (formate form) anion exchange columns.

Columns were prepared by packing 4 ml of Dowex 1-*x*2 resin into 4 ml syringes (Terumo Corp., Japan) fitted with 13 mm filter paper discs and 21 gauge needles. This provided columns with 80 times the required anion exchange capacity. Following equilibration of the samples with the resin, U[^{14}C] glucose was eluted from the columns with 10 × 1 ml aliquots of decarbonated distilled water and collected into counting vials. Elution was by downward flow at a rate of 3 ml/min. The 10 ml volume of eluate was evaporated to dryness by heating the vials at 95°C in an aluminium block under a stream of N_2 gas over the liquid to prevent oxidation of U[^{14}C] glucose. After cooling, 1 ml of distilled water and 10 ml of dioxane scintillant were added to the vials which were left to dark-adapt overnight before counting.

Protein and DNA assay

Protein content of samples was determined using the dye-binding method of Bradford [12] incorporating the modification of Bearden [13] using bovine serum albumin as a standard. DNA was assayed by a fluorometric procedure [14] using highly polymerized calf thymus DNA (Sigma Chemical Co.) as a standard.

RESULTS

When hepatocytes derived from 19-day foetuses were exposed to MDAB in culture each of the enzymes studied gave a different response (Fig. 1). The activity of glucose-6-phosphatase increased gradually in controls during culture and was elevated by nearly two-fold as a result of exposure to MDAB. The activity of phosphoenolpyruvate carboxykinase decreased during culture beyond three days. It diminishes to very low levels by day 9, and although enzyme activity was demonstrable, it could not be estimated reproducibly and was therefore not shown in Fig. 1. There was no significant effect of MDAB

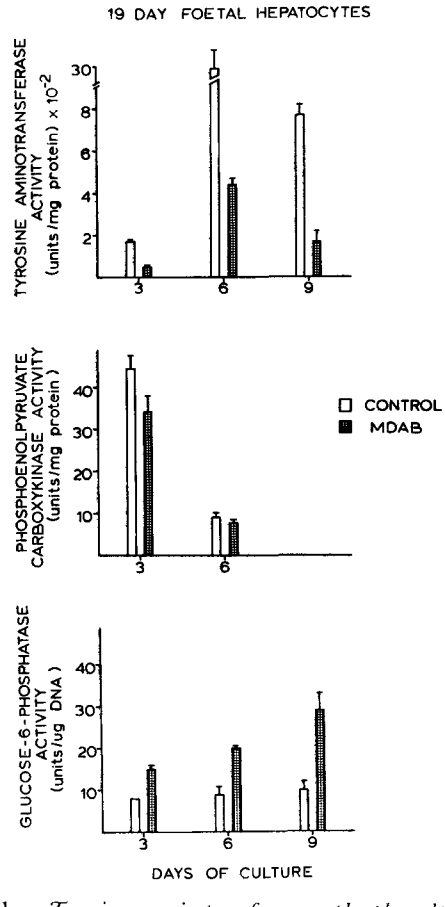

Fig. 1. Tyrosine aminotransferase, phosphoenolpyruvate carboxykinase and glucose-6-phosphatase activity in 19-day *gestation hepatocytes exposed to MDAB in culture. Control cultures received* 50 µl *of propylene glycol/*10 ml *culture medium (open bars) and MDAB-treated cultures (hatched bars) received* 25 µg *MDAB in* 50 µl *of propylene glycol/*10 ml *culture medium. The cultures were harvested on the days indicated and prepared for enzyme assay as described in Materials and Methods. Nine cultures were assayed for tyrosine aminotransferase and six each for phosphoenolpyruvate carboxykinase and glucose-6-phosphatase. Error bars indicate S. E. M.*

on this enzyme activity on days 3 and 6 of culture. In contrast, tyrosine aminotransferase activity increased between day 3 and day 6 and was markedly suppressed as a result of carcinogen exposure. After three days a 70% reduction in enzyme activity was observed. This reduced level of activity was maintained after 6 and 9 days of exposure.

The cells harvested after MDAB treatment were yellow and this was clearly seen after the cells were harvested prior to the preparation of cell extracts. They show enlarged distorted oval nuclei compared to controls which were regular, smaller and round (Figs. 2a and 2b). Consequently, there was an increase in the nuclear:cytoplasmic ratio. The phase-contrast photomicrographs (Fig. 2) also show the nucleoplasm to be less phase dense in controls than in MDAB-treated cells.

Electron micrographs of the respective cultures (Figs. 3a and b) reveal the presence of electron-dense granules of MDAB in the test cultures. The altered nuclear shape and size is again evident. An increase in number of nucleoli is more clearly seen under the electron microscope than in the phase contrast photomicrographs. Electron microscopy of the cells suggest that carcinogen treatment has also increased the rough endoplasmic reticulum content of the cells.

When hepatocytes derived from 15-day foetuses were treated in culture with MDAB, tyrosine aminotransferase levels were unaltered until after 9 days of exposure (Fig. 4). At this stage a 55% reduction in enzyme activity was observed. No significant difference in phosphoenolpyruvate carboxykinase levels were observed with hepatocytes exposed to MDAB on days 3 and 6. The activity of the enzyme was too low to measure reliably after 9 days of culture (data not shown). Glucose-6-phosphatase activity in these cells was not detectable with the assay used. Phase microscope observation of these cells on days 3 and 6 (Figs. 5a and b) revealed no evidence of the types of changes which were seen with 19-day gestation hepatocytes.

DISCUSSION

Exposure of hepatocytes derived from 19-day foetal rats to MDAB in culture results in a marked reduction in tyrosine aminotransferase activity. The lack of an effect on phosphoenolpyruvate carboxykinase and the increase in glucose-6-phosphatase suggests that there were no severe generalized toxic effects of the carcinogen at the dose level used. Hepatocytes obtained from younger foetuses were unaffected by the carcinogen when analysed on days 3 and 6 of exposure. On day 9, the mean activities of tyrosine aminotransferase were reduced by 45% However, it cannot be concluded that this is due to a specific effect of the hepatocarcinogen since it was not possible to reliably measure glucose-6-phosphatase or phosphenolpyruvate carboxykinase and thus assess the possibility of a generalized toxic effect resulting from treatment over 9 days.

The measurements on days 3 and 6, which reveal no alteration in either tyrosine aminotransferase or phosphoenolpyruvate carboxykinase activities, suggest that the 15-day foetal hepatocytes are less susceptible to the hepatocarcinogen than their more mature counterparts. This is contrary to the prediction based on the hypothesis that the carcinogen acts on less mature hepatocytes. However, the lack of an effect on day 3 in 15-day foetal hepatocytes may possibly be explained by the absence of mixed function oxidase in these cells. Since this is required for the activation of carcinogens, the enzyme(s) would have to be acquired during culture before a response could be elicited in 15-day foetal hepatocytes. This explanation is supported by evidence in the literature which indicates that the smooth endoplasmic reticulum of early foetal liver is poorly developed [15]. More recently, it has been shown that the mixed function oxidases are not detected in 15-day gestation rat liver [16]. The fact that glucose-6-phosphatase was not detected in hepatocytes from 15-day foetal liver in this study is consistent with the above observations.

Morphological data (Figs. 2 and 3) support biochemical evidence which indicates that the hepatocytes derived from 19-day foetuses are either undergoing transformation or have been transformed by MDAB. The observed increase in nuclear:cytoplasmic ratio has been cited as being indicative of cell transformation [17, 18]. Morphological and biochemical criteria are in agreement in suggesting that the hepatocarcinogen is without effect on 15-day gestation hepatocytes.

These results show that an enzyme marker, tyrosine aminotransferase, acquired by cells during culture is depressed very soon after exposure to a hepatocarcinogen. In contrast, the activity of phosphoenolpyruvate carboxykinase, which the foetal liver is competent to synthesize very early in development, i.e., by day 12, remains unaltered [11]. It could be interpreted from the data obtained that all

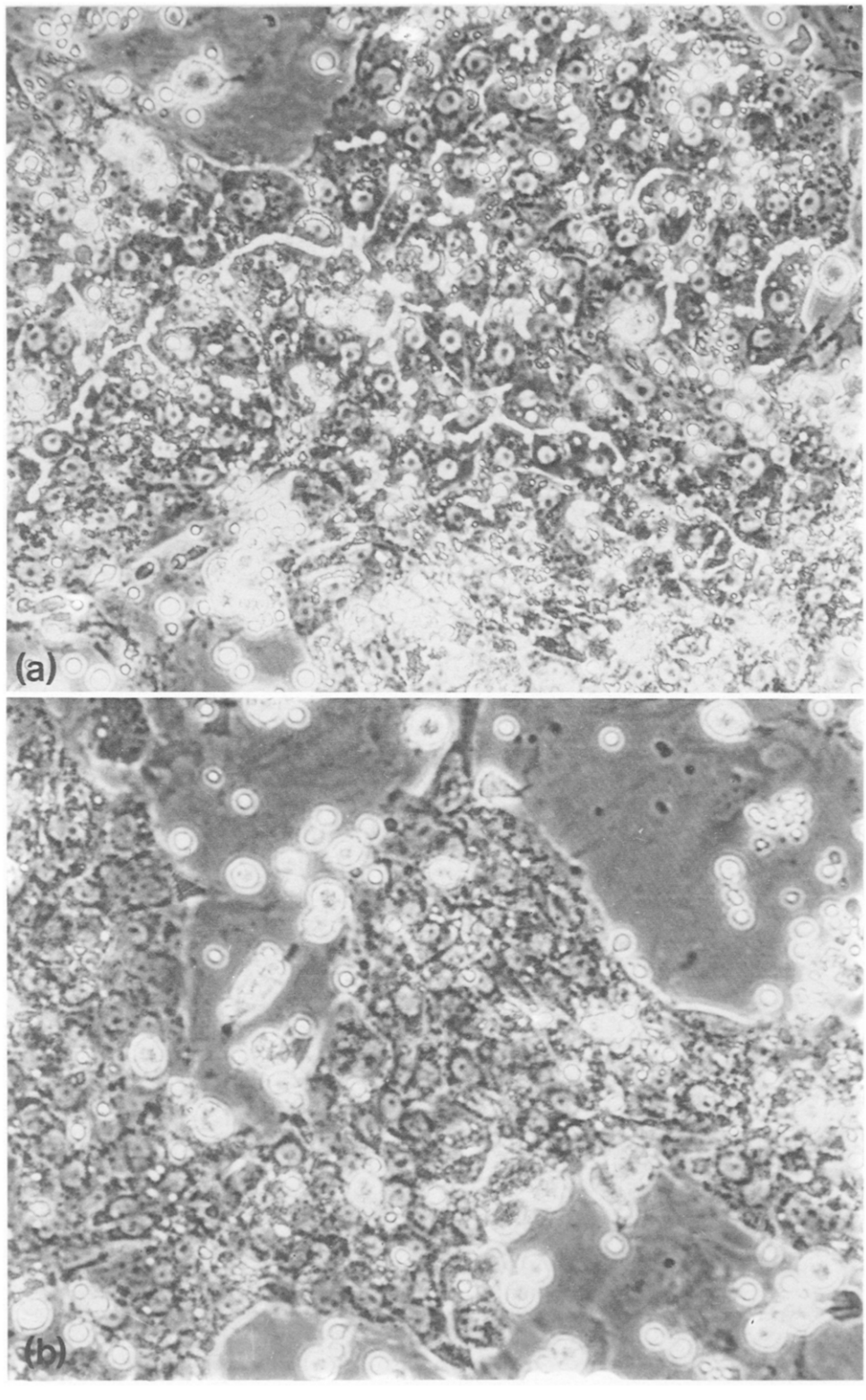

Fig. 2. Phase-contrast photomicrographs of control and MDAB-treated hepatocytes in culture. Control cultures (a) received 50 µl *of propylene glycol and MDAB cultures (b)* 25 µg *of carcinogen. The cultures were photographed on* day 3. *Magnification* × 125.

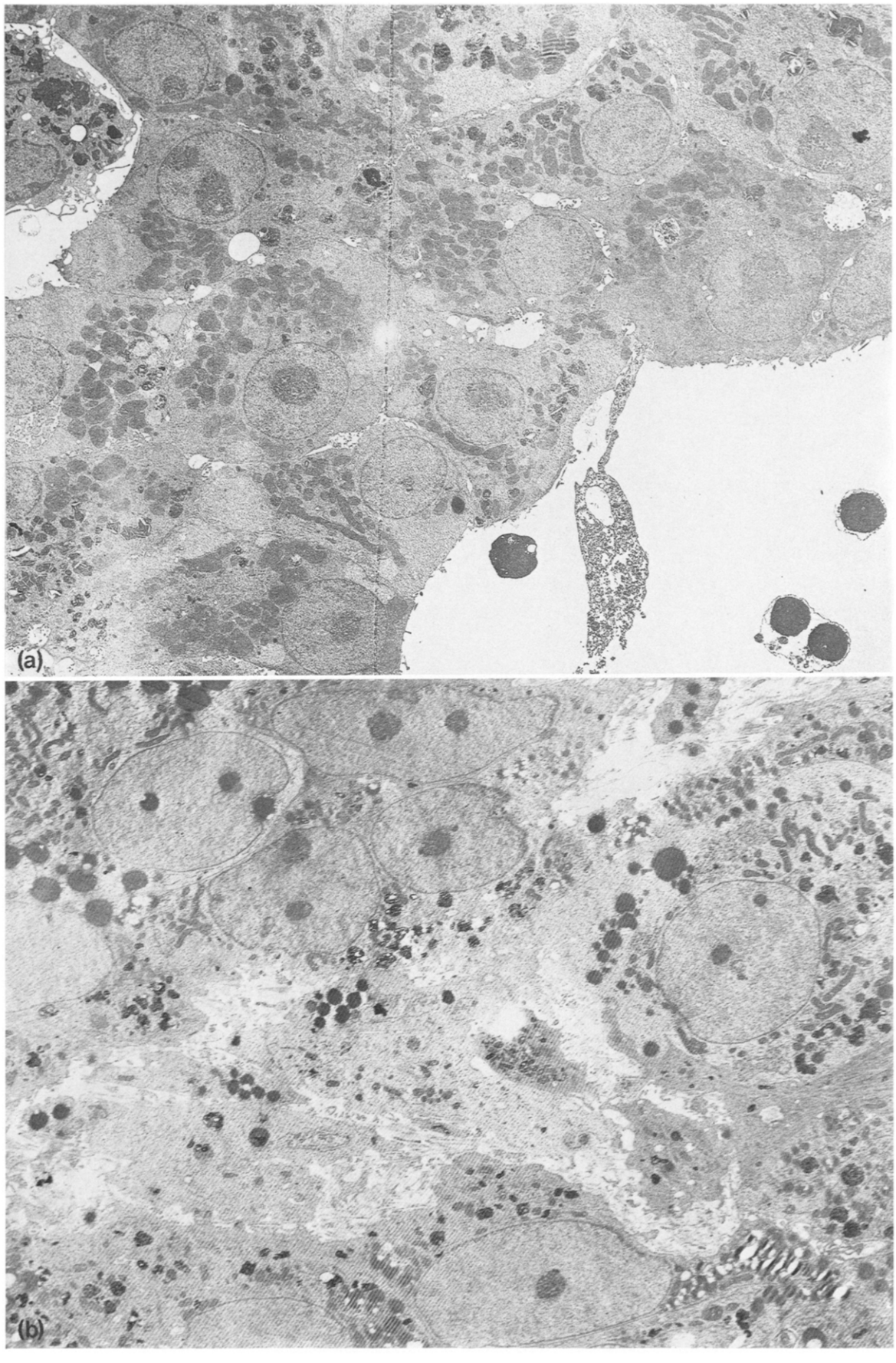

Fig. 3. Electron micrographs of control and MDAB-treated hepatocytes in culture. Control cultures (a) received 50 µl of propylene glycol and MDAB cultures (b) 25 µg of MDAB. The cultures were prepared for electron microscopic examination after three days of culture. Magnification × 3000.

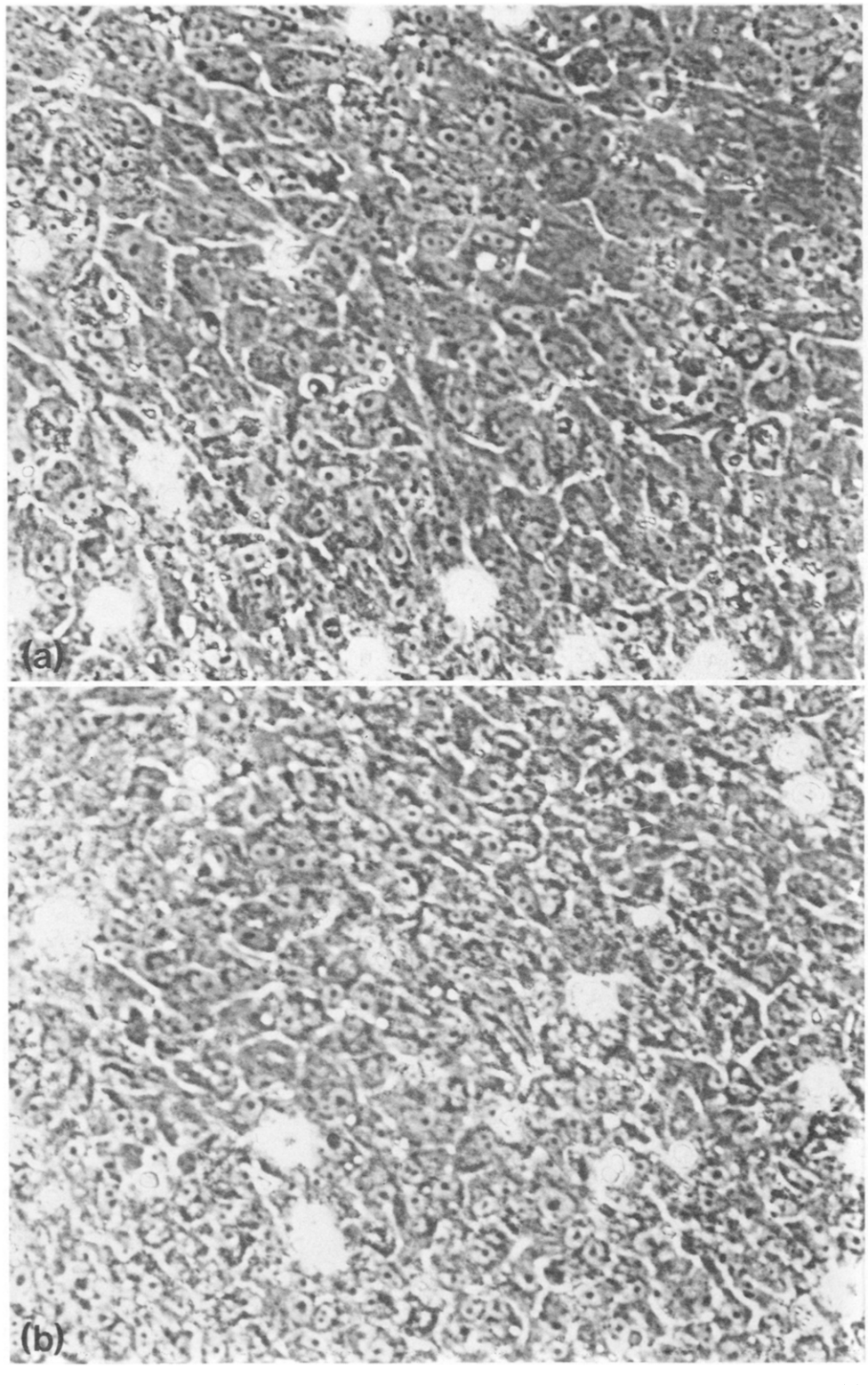

Fig. 5. *Phase-contrast photomicrographs of control and MDAB-treated hepatocytes in culture. Control cultures* (*a*) *received* 50 µl *of propylene glycol and MDAB cultures* (*b*) 25 µg *of carcinogen. The cultures were photographed on day* 3. *Magnification* × 125.

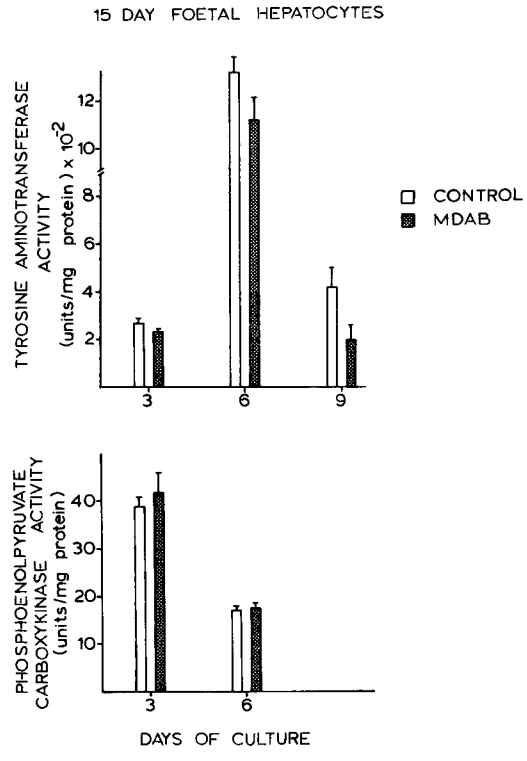

Fig. 4. Tyrosine aminotransferase and phosphoenolpyruvate carboxykinase activity in 15-day gestation hepatocytes exposed to MDAB in culture. Control cultures received 50 µl of propylene glycol/10 ml of culture medium (open bars) and MDAB-treated cultures (hatched bars) received 25 µg MDAB in 50 µl of propylene glycol in 10 ml of culture medium. The cultures were harvested on the days indicated and prepared for enzyme assay as described in Materials and Methods. Nine cultures were assayed for tyrosine aminotransferase and six cultures for phosphoenolpyruvate carboxykinase. Error bars indicate S.E.M.

19-day gestation hepatocytes are synthesizing tyrosine aminotransferase and that the carcinogen interferes with the continued production of enzyme. Alternatively, it may be argued that 19-day gestation liver contains a mixture of hepatocytes; some of which have acquired the enzyme and others in which the tyrosine aminotransferase gene is not yet active. The effect of the carcinogen would then be to block the differentiation of the latter group of cells and prevent them from acquiring the capacity to synthesize tyrosine aminotransferase. At the same time genes which were already expressible prior to carcinogen exposure, e.g., the phosphoenolpyruvate carboxykinase or the glucose-6-phosphatase genes, would be unaffected. This mechanism would be akin to the mechanism of viral blockade of myogenesis [6] or chondrogenesis [7] or the effect of the tumour promoter phorbol myristate acetate in preventing myogenesis [3], chondrogenesis [4] or erythrogenesis. [18]. In these instances, the genes to be expressed during differentiation are affected by the transforming agent, while genes already active appear to be unaltered. Although this study provides no conclusive evidence that the short term exposure to MDAB in fact produces transformed cells, the selective supression of tyrosine aminotransferase suggests that the exposed hepatocytes have been diverted from a normal differentiation program and that this process may be associated with the ultimate production of tumour cells.

On the basis of the hypothesis that carcinogenesis results from transformation of differentiating precursor cells, it would have been expected that a greater effect would have been found in 15-day foetal hepatocytes than in 19-day ones. Since 15-day hepatocytes have not yet begun synthesizing tyrosine aminotransferase at the time the culture is initiated, the hypothesis would predict a complete blockade of enzyme appearance as a result of carcinogen treatment. The lack of an effect on these cells may be due to their inability to activate the carcinogen. This possibility is currently under investigation.

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