Eur J Cancer Clin Oncol. Vol. 18, No. 11, pp. 1163–1170, 1982.
Printed in Great Britain.

0277-5379/82/111163-08$03.00/0

The Effect of Carcinogens on the Accumulation of Tyrosine Aminotransferase by Foetal Rat Hepatocytes in Culture

GEORGE YEOH,* MACHELL GODFREY,* ROBERT TOIA† and MAXWELL CAKE‡

*Department of Physiology, University of Western Australia, Nedlands 6009, Western Australia, †School of Chemistry, The University of New South Wales, Kensington 2033, New South Wales, and ‡School of Environmental and Life Sciences, Murdoch University, Murdoch 6150, Western Australia

Abstract—*The hepatocarcinogen 3'-methyl-4-dimethyl-aminoazobenzene (MDAB) suppresses the accumulation of tyrosine aminotransferase in cultured foetal hepatocytes. Experiments involving liver derived from foetuses of various ages reveals that a response is only obtained with rats older than 16-day gestation. It has been proposed that the lack of an effect in less mature hepatocytes is due to their inability to activate the carcinogen. Chemically synthesized analogues of MDAB which are considered likely to be activated forms of the procarcinogen are shown to be effective in the less mature cells. This supports the proposal that these cells may be unreponsive because they are unable to activate MDAB. Tests with other carcinogens reveal that the hepatocarcinogen dimethylbenzanthracene is also effective in 19-day gestation hepatocytes. However, the non-hepatocarcinogens azaserine and benz(a)pyrene are ineffective. Treatment with MDAB is shown not to alter the level of steroid receptor and reduce its translocation into the nucleus, suggesting that this is not the mechanism by which TAT is suppressed. The effect of the tumour promoter phorbol-myristate acetate (PMA) administered together with MDAB was shown not to modify the response to the carcinogen alone.*

INTRODUCTION

IT HAS previously been shown that foetal rat hepatocytes differentiate in culture and in the presence of dexamethasone accumulate tyrosine aminotransferase during culture [1]. The presence of the hepatocarcinogen, 3'-methyl-4-dimethyl-aminoazobenzene (MDAB), suppresses the increase in tyrosine aminotransferase [2]. This finding is consistent with the notion that blocked ontogeny is the underlying basis of oncogeny [3–5]. There are several ways to interpret the blockade of tyrosine aminotransferase accumulation. One which we favour is that hepatocytes which are about to acquire the capacity to synthesize the enzyme fail to do so in the presence of the carcinogen. This diversion from their normal differentiation pathway, we propose, may be associated with the early event of initiation of the tumorigenic process.

Many aspects of the suppression of tyrosine aminotransferase by MDAB remain to be clarified. Firstly, this effect was only observed with hepatocytes obtained from more mature foetuses (19-day gestation). The carcinogen was totally ineffective when added to cultures of hepatocytes derived from 15-day gestation foetuses [2]. In order to ascertain the stage of development when the hepatocytes become sensitive to the carcinogen, the effect of MDAB on cells from different aged foetuses was studied. Hepatocytes from foetuses aged 16-days gestation or older were responsive.

A possible explanation for the apparent resistance of the less mature hepatocytes may be their inability to activate the procarcinogen.

Accepted 10 June 1982.

Abbreviations: Tyrosine aminotransferase (TAT); 3'-methyl-4-dimethylaminoazobenzene (MDAB); 2'-methyl-4-dimethylaminoazobenzene (2'-MDAB); 6-aminochrysene (6-AC); dimethylbenzanthracene (DMBA); phorbol-myristate-acetate (PMA).

In order to test this proposal, possible metabolic activation products of MDAB were prepared by chemical means and added to cultures of 15-day gestation hepatocytes. In addition, several related compounds were also tested, including two which from considerations of their chemical properties may be anticipated as a means of generating a potent alkylating agent, viz. a benzene diazonium ion, within the cell. The compound considered most likely to be a proximate form of MDAB, i.e. 3'-methyl-4-dimethyl-aminoazobenzene-4-*N*-oxide, was shown to be effective.

It has been reported that MDAB will modify the function of the glucocorticoid receptor [6] and reduce its uptake into the nucleus of hepatocytes. Since the level of tyrosine aminotransferase in cultured hepatocytes is very much dependent upon the level of glucocorticoid added to the culture medium [7], it is possible that this is the underlying mechanism for the suppression of tyrosine aminotransferase by MDAB. For this reason, the level of glucocorticoid receptor and its translocation into the nucleus of foetal hepatocytes in culture in the presence of MDAB is also included in this study.

An investigation of the effect of a limited range of carcinogens was undertaken to determine whether the response is specific for hepatocarcinogens and not obtained when non-hepatic carcinogens are used. The results suggests that only hepatocarcinogens are effective. Finally, the effect of the tumour promoter phorbol-myristate acetate (PMA) on the MDAB suppression of tyrosine aminotransferase is examined.

MATERIALS AND METHODS

Chemicals

L-[3,5-^{3}H]-Tyrosine and [1,2-^{3}H]-dexamethasone were obtained from the Radiochemical Centre, Amersham, Bucks. Biochemicals dimethylbenzanthracene and benz(a)pyrene were purchased from Sigma Chemical Co., St. Louis, MD. Collagenase was a product of Boehringer Mannheim, Federal Republic of Germany. Tokyo Kasei Kogyo, Japan supplied 3'-methyl-4-dimethyl-aminoazobenzene. 6-Aminochrysene (6-AC) and 2-methyl-4-dimethyl-aminoazobenzene (2-MDAB) were purchased from Aldrich Chemical Co., Milwaukee, WI. 3'-Methyl-4-dimethyl-aminoazobenzene-4-*N*-oxide (C) was prepared by *m*-chloroperbenzoic acid oxidation of MDAB, after the method of Craig and Purushothaman [8] as described by Kadlubar *et al.* [9]. The product was recrystallized thrice from hexane–acetone as orange plates, m.p. 69–71°C (Kofler hot stage microscope, uncorrected). 1-(3-Methylphenyl)-3-methyl-3-phenyltriazine (A) and 1-(3-methylphenyl)-3-phenyltriazine (E) were prepared by coupling the diazonium salt obtained from diazotization of 3-methylaniline with either *N*-methylaniline or aniline respectively in the presence of excess sodium acetate [10]. The products were recrystallized from light petroleum as dark yellow prisms, m.p. 66–67°C and orange plates, m.p. 87–89°C respectively. 3'-Methyl-4-methyl-aminoazobenzene (B) and 3'-methyl-4-aminoazobenzene (F) were prepared by rearrangement of the triazines (A and E respectively) in the presence of 3 molar equivalents of the appropriate amine and 0.7 molar equivalents of that amine hydrochloride [10]. Recrystallization from light petroleum gave B as bronze needles, m.p. 113–114°C and F as light brown plates, m.p. 119–121°C. 4-Aminoazobenzene (D) was prepared as described by Vogel [11] and obtained as light orange needles from carbon tetrachloride, m.p. 126°C. The structural formulae of compounds A–F which were synthesized are depicted in Fig. 1.

Eagle's Minimal Essential Medium (MEM) was obtained in powder form from Flow Laboratories, Annandale, New South Wales, Australia and Newborn Calf Serum from Commonwealth Serum Laboratories, Parkville, Victoria, Australia. Fungizone was obtained from Grand Island Biological Co., Grand Island, NY, U.S.A. Penicillin and streptomycin were supplied by Calbiochem-Behring (Aust), Pty. Ltd., Carlingford, New South Wales, Australia.

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.

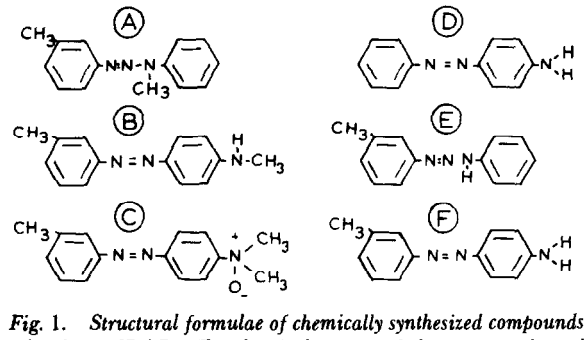

Fig. 1. Structural formulae of chemically synthesized compounds related to MDAB. The chemical names of the compounds and their respective codes are outlined in Materials and Methods.

Culture medium

The basic culture medium was modified MEM supplemented with 10% foetal calf serum, glutamine (2.4 mM final concentration), Fungizone (2.8 μg/ml) and penicillin/streptomycin (57 units/ml and 5700 μg/ml respectively). The culture medium was supplemented with 10 nM dexamethasone. This level of the steroid analogue has previously been shown to maximize the yield of enzyme.

Hepatocyte isolation

Livers from 17 to 19-day foetal rats were chopped and incubated with collagenase in balanced salts solution as described by Yeoh *et al.* [1]. Cells were harvested and washed twice in balanced salts solution by centrifugation at 50 ***g*** for 2 min, then suspended in culture medium. Livers from 15 and 16-day gestation rats were removed aseptically then dispersed by aspiration using a wide bore Pasteur pipette.

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. Control cultures received 50 μl of propylene glycol. Carcinogens were added in 50 μl of propylene glycol to a final concentration of 2.5 μg/ml, except where indicated in the text.

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, suspended in balanced salts solution and collected by centrifugation at 1600 ***g*** for 2 min.

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 contrifugation in a Beckman Airfuge at about 210 kPa (165,000 ***g*** max) for 12 min.

Enzyme assays

Tyrosine aminotransferase was assayed by a radiochemical method described previously [1]. The enzyme was assayed in fractions eluted from a CM-Sephadex column to which extracts from cultured hepatocytes were applied.

Protein and DNA assay

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

Steroid receptor assay

To determine the specific nuclear binding of glucocorticoid in the intact cell, cell cultures were incubated with 50 nM [^{3}H]-dexamethasone in the presence and absence of 5 μM non-radioactive steroid. After a 1-hr incubation at 37°C the cultures were subjected to the following fractionation procedures, which were performed at 0–4°C. The cells were washed with ice-cold buffered salts solution, harvested using a Teflon policeman and then homogenized in 4 volumes of 0.05 M Tris–HCl, pH 7.6, containing 3 mM $MgCl_2$ and 10 nM 2-mercaptoethanol in a Potter–Elvehjem homogenizer. The homogenate was adjusted to 0.25 M sucrose, an aliquot taken for DNA estimation and the remainder centrifuged at 1500 ***g*** for 10 min. The supernatant was used to obtain a cytosol fraction by further centrifugation in a Beckman Airfuge. Specifically bound steroid was determined using the dextran-coated charcoal technique [15].

Nuclei were purified from the pellet of the low-speed centrifugation of the cell homogenate. The pellet was suspended in 2 volumes of 0.05 M Tris–HCl, pH 7.6, containing 0.25 M sucrose and 3 mM $MgCl_2$, and then diluted with 2.5 volumes of 0.05 M Tris–HCl, pH 7.6, containing 2.2 M sucrose and 3 mM $CaCl_2$. The resulting suspension was centrifuged through a cushion of 2.15 M sucrose–calcium buffer at 73,000 ***g*** for 30 min. After washing the pellet with 0.05 M Tris–HCl, pH 4.6, containing 0.25 M sucrose and 3 mM $MgCl_2$, the nuclear fraction was resuspended in this buffer and duplicate samples assayed for DNA and radioactivity. Specific binding represents the amount of radioactivity bound in the absence of unlabelled competitor less the amount bound in the presence of competitor [16].

Results for both cytosol and nuclear binding are expressed as pmol steroid bound per mg DNA.

RESULTS

Cultures of foetal hepatocytes derived from 15 and 19-day gestation rats accumulate the enzyme tyrosine aminotransferase if dexamethasone (10 nM) is present in the medium

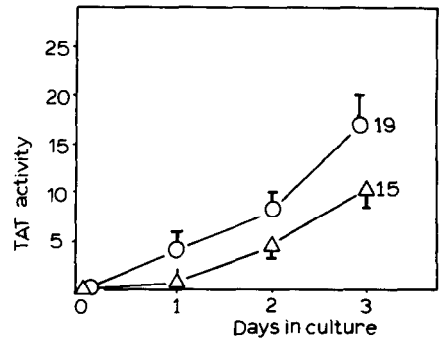

Fig. 2. Accumulation of tyrosine aminotransferase in 15 and 19-day foetal hepatocytes. Cultures were prepared and maintained as described in Materials and Methods. The medium was supplemented with 10 nM dexamethasone. Cells were harvested immediately after preparation (Day 0) and on each day after. Tyrosine aminotransferase activity is expressed as μmol/hr/mg protein. Error bars indicate ± S.E.

(Fig. 2). The level of enzyme activity is lower in the less mature hepatocytes. On day 1 of culture the level is barely detectable in 15-day gestation liver cultures. Hepatocytes prepared from foetuses of varying gestational age were exposed to MDAB for three days in culture and then assayed for tyrosine aminotransferase. The results (Table 1) show that livers derived from 15 and 16-day gestation foetuses did not respond, whereas hepatocytes from older foetuses were affected and displayed significantly reduced levels of the enzyme when compared with controls.

Fifteen-day gestation hepatocytes were exposed to extracts prepared from (i) 19-day gestation liver cultures which were exposed to MDAB *in vitro*, (ii) the liver of adult rats maintained on a diet of 0.06% MDAB for two weeks and (iii) the S9 fraction of microsomes derived from rats given phenobarbitol and incubated with MDAB. In all cases no diminution of tyrosine aminotransferase was observed.

Several compounds (B, C, D and F) closely related to MDAB and two diazoamino compounds (A and E) were synthesized as described in Materials and Methods (for structural formulae see Fig. 1). By analogy with the proposed pathway for metabolic activation of *N*-methyl-4-aminoazobenzene [9], it is possible that some of these may be metabolic intermediates in the pathway of MDAB activation to its ultimate carcinogenic form. The results of experiments using these compounds are summarized in Table 2. In 19-day hepatocytes, B and C proved to be as effective as the parent compound MDAB, while in contrast, F was only marginally effective and D totally ineffective. The diazoamino compound E was also ineffective, but interestingly, the *N*-methyl analogue A proved toxic and cell death occurred after 3 days exposure.

Compounds B and C were subsequently tested on the less mature hepatocytes. On these cells, B marginally diminished tyrosine aminotransferase activity (30%) but C produced a level of inhibition of 72% (Table 3), which is about the level seen with the parent compound in tests with 19-day gestation hepatocytes.

The total glucocorticoid receptor activity and its distribution between the cytoplasm and the nucleus in hepatocytes exposed to MDAB was determined. The results (Table 4) indicate that there is no marked difference between the values in controls and MDAB-treated cultures.

Table 1. Effect of MDAB on hepatocytes derived from foetuses of various ages

Gestational age (days)	Tyrosine aminotransferase activity Control	MDAB
15	666 ± 120	736 ± 67 (6)
16	990 ± 59	823 ± 107 (3)
17	1044 ± 136	276 ± 11 (3)
18	2125 ± 187	704 ± 81 (3)
19	1986 ± 212	714 ± 34 (3)

Cultures were exposed to 2.5 μg MDAB/ml administered in 50 μl of propylene glycol at the time of plating. Controls received 50 μl of the solvent. Both cultures were maintained in the presence of 10 nM dexamethasone. Cells were harvested and tyrosine aminotransferase assayed on the third day of culture. Enzyme activity is expressed as μmol/hr/mg protein. Results are presented as the means ± S.E. Number of cultures are indicated in parentheses.

Table 2. Effect of compounds structurally related to MDAB on tyrosine aminotransferase activity in 19-day gestation hepatocytes

Compound code	Enzyme activity	Percentage inhibition
Control	1265 ± 215 (4)	0
MDAB	408 ± 27 (3)	68
A	*	
B	478 ± 215 (4)	62
C	374 ± 97 (4)	70
D	1185 ± 256 (3)	6
E	1286 ± 273 (2)	0
F	842 ± 266 (3)	33

Cultures were exposed to 2.5 μg/ml of each compound administered in 50 μl of propylene glycol at the time of plating. Controls received 50 μl of the solvent. All cultures were maintained in the presence of 10 nM dexamethasone. Cells were harvested and tyrosine aminotransferase assayed on the third day of culture. Enzyme activity is expressed as μmol/hr/mg protein. Results are presented as the means ±S.E.
*Toxic.

Table 3. Effect of compounds structurally related to MDAB on tyrosine aminotransferase activity in 15-day gestation hepatocytes

Compound code	Enzyme activity	Percentage inhibition
Control	456 ± 131 (5)	0
B	323 ± 142 (5)	29
C	129 ± 28 (5)	72

Cultures were exposed to 2.5 μg/ml of each compound administered in 50 μl of propylene glycol at the time of plating. Controls received 50 μl of the solvent. All cultures were maintained in the presence of 10 nM dexamethasone. Cells were harvested and tyrosine aminotransferase assayed on the third day of culture. Enzyme activity is expressed as μmol/hr/mg protein. Results are presented as the means ±S.E.

A limited range of carcinogens, some of which are hepatocarcinogens and others not generally believed to have the liver as a prime target, were tested on 19-day gestation hepatocytes. The data (Table 5) indicates that a response is obtained with the known hepatocarcinogens, i.e. MDAB, the 2′ analogue of MDAB and dimethylbenzanthracene (DMBA). The other compounds, 6-aminochrysene, azaserine and benz(a)pyrene, do not suppress the level of tyrosine aminotransferase.

To determine whether PMA is capable of modifying the response of 19-day gestation hepatocytes to MDAB, cultures were exposed simultaneously to both agents for three days. The results of this experiment shown in Table 6 suggests that the tumour promoter has no effect on this system.

Table 4. Steroid-receptor levels in 19-day gestation hepatocytes exposed to MDAB

Fraction	Receptor concentration Control	MDAB
Cytoplasmic	1.54 ± 0.22	2.35 ± 0.82
Nuclear	0.99 ± 0.30	1.01 ± 0.23
Total	2.53 ± 0.51	3.35 ± 1.05

Hepatocytes from 19-day gestation foetal rats were cultured for 72 hr in the absence or presence of 2.5 μg MDAB/ml. The cell cultures were then incubated for a further hour at 37°C with 50 nM [^{3}H]-dexamethasone in the presence or absence of a 100-fold excess of unlabelled steroid. The cells were harvested, cytosol and nuclear fractions prepared and specific binding of dexamethasone estimated as described in Materials and Methods. The data is expressed as pmol steroid bound/mg DNA and the results are the means ± S.E. of 4 experiments.

Table 5. Effect of carcinogens on tyrosine aminotransferase accumulation in 19-day gestation hepatocytes

Carcinogen	Hepatocarcinogen	Percentage inhibition
MDAB	Yes	72
DMBA	Yes	78
2-MDAB	Yes	75
6-AC	?	2
Azaserine	No	−3
Benz(a)pyrene	No	5

Cultures were exposed to 2.5 μg/ml of each carcinogen administered in 50 μl of propylene glycol at the time of plating. Controls received 50 μl of the solvent. Cells were harvested and tyrosine aminotransferase assayed on the third day of culture. Enzyme activity is expressed as μmol/hr/mg protein. Results are presented as the means ±S.E.

DISCUSSION

Under normal conditions, the enzyme tyrosine aminotransferase is not detectable in the liver of 15 and 19-day gestation foetal rats. However, when hepatocytes from these rats are cultured in the presence of dexamethasone, the enzyme accumulates. Under the conditions of assay, on day 1 the level of activity is either barely detectable or not detectable in 15-day gestation liver. This may be due to the precise age of the animals used to prepare the cultures. In contrast, there is significant activity in 19-day gestation hepatocytes maintained in culture under identical conditions (Fig. 1). Enzyme continues to accumulate in both sets of cultures for 6 days [2].

The accumulation of enzyme in 19-day but

Table 6. Effect of PMA on MDAB suppression of tyrosine aminotransferase

	Tyrosine aminotransferase activity
Control	1161 ± 43 (3)
PMA	1173 ± 34 (3)
MDAB	596 ± 68 (3)
PMA + MDAB	729 ± 17 (3)

Cultures were exposed to 1.25 μg MDAB/ml administered in 50 μl of propylene glycol, and where appropriate PMA (0.25 μg/ml final concentration) was added twice each day from the time of plating. Controls received 50 μl of the solvent. Cells were harvested and tyrosine aminotransferase assayed on the third day of culture. Enzyme activity is expressed as μmol/hr/mg protein. Results are presented as the means ± S.E. Number of determinations in each group is indicated in brackets.

not 15-day gestation liver was shown to be suppressed by the presence of the hepatocarcinogen MDAB in the medium [2]. The reason for this differential response is not clear. Since MDAB, like methylaminoazobenzene [9], is a procarcinogen and requires activation before it can exert its effect, it is possible that 15-day gestation hepatocytes were unresponsive because of an inability to carry out the step(s) necessary for activation.

In order to establish at which point during development hepatocytes became responsive to MDAB, livers from foetuses of various ages were cultured and exposed to the hepatocarcinogen *in vitro*. The most immature cells to show a response in terms of a diminution of tyrosine aminotransferase accumulation were 17-day gestation hepatocytes (Table 1). It is concluded that at this age the hepatocytes have acquired sufficient enzyme(s) involved in the metabolic activation of MDAB to become susceptible. This result is consistent with the generally low or undetectable levels of hepatic microsomal mono-oxygenases which have been reported in foetal rat liver by Guenther and Mannering [17].

If 15-day gestation hepatocytes are unresponsive to MDAB because of an inability to activate the hepatocarcinogen, then it should be possible to elicit an effect by exposing the cells to activated forms of MDAB. Two approaches were used to test this. Firstly, extracts prepared from cells exposed to MDAB were added to 15-day gestation hepatocytes in culture. The extracts were prepared from (i) cultures of 19-day gestation hepatocytes exposed to MDAB *in vitro*, (ii) the liver of adult rats maintained on a diet of 0.06% MDAB for two weeks and (iii) the S9 microsomal fraction of the liver taken from rats treated with phenobarbital then incubated *in vitro* with MDAB. In no instance could any effect be demonstrated. Several explanations can be forwarded to account for the failure of these experiments. However, the most likely one is that the activated form of MDAB, assuming it was formed using the above systems, was either present at insufficient levels or unavailable (possibly due to binding to other cellular macromolecules) to exert its effect on the cells in culture.

The second approach was to test synthetically prepared compounds which are likely to be metabolic activation products of MDAB. In light of the two proposed pathways, P-450-dependent and P-450-independent, for the activation of *N*-methyl-4-aminoazobenzene [9] the compounds B, C and F were synthesized and tested. Closely related compounds A, D and E were also tested, for although there is no evidence that they are intermediates in the metabolism of MDAB, it was considered that they would serve as controls for non-specific toxic effects. In the 19-day gestation hepatocytes, B and C were as active as MDAB whilst F showed only mild activity. Interestingly, the closely related compound D, which varies from F only in the substitution in the remote aromatic ring, was essentially inactive. This is also in agreement with previous results from hepatocarcinogenicity testing of this compound [10]. Considering the relative inactivity of F in the test system, it is unlikely that di-*N*-demethylation of MDAB is involved in the activation process.

In subsequent experiments compounds B and C were added to the 15-day hepatocyte cultures. A marginal decrease in tyrosine aminotransferase activity was obtained with B and a significant depression was observed with C. The effect observed with C, i.e. 72% suppression (Table 3), compares favourably with the effect of the parent compound in 19-day gestation hepatocytes and suggests that any subsequent transformations required to form the ultimate carcinogen can now be readily effected by the cells. At the present time it is a moot point whether compound C undergoes simple derivatization or requires *N*-demethylation to achieve this end, and work to resolve this question is now proceeding. It should also be noted that on some occasions when C was used the yield of cells was decreased. Whether this reflects a decreased plating efficiency or is due to a loss of cells which rounded up and were lost during medium replacement is unclear. The presence of haemopoietic cells in the initial inoculum makes it impossible to define the cause of cell loss. Nevertheless, of the cells which were harvested the specific activity of aspartate aminotransferase and phosphoenolpyruvate carboxykinase were shown to be the same as in controls. The rate of albumin production, expressed on a per cell basis, also remained unchanged. This evidence suggests that the effect of C is specific and it is concluded that the observed insensitivity of 15-day gestation hepatocytes is due to their inability to activate the carcinogen.

Compound A was found to be toxic. The reason for this is unclear; it may be due to its potential alkylating activity, which could render most cellular macromolecules non-functional.

To define the rate-limiting step it is necessary to assay for the individual enzymes as well as to use a range of activated intermediates includ-

ing the proposed sulphated ultimate carcinogenic form of MDAB [18].

It was previously shown that the activity of tyrosine aminotransferase in cultured foetal hepatocytes is extremely sensitive to the level of glucocorticoid in the medium [7]. The current model of steroid action in rat liver involves entry of the steroid into the cell, its binding to a specific receptor, an activation step and the transfer of the steroid–receptor complex into the nucleus (for review see Cake and Litwack [19]). The steroid–receptor complex then increases the level of tyrosine aminotransferase transcription [20, 21]. Kensler *et al.* [6] reported that hepatocarcinogen administration to rats *in vivo* results in a diminished binding efficiency of the steroid–receptor complex to rat liver nuclei. The suppression of tyrosine amiontransferase activity, observed previously [2] and in this study, may be indirectly due to an effect of the carcinogen on the binding efficiency of the steroid–receptor complex to the nucleus. It was therefore necessary to measure the level of receptor and its translocation into the nucleus in cells exposed to MDAB *in vitro*. The result of this experiment (Table 5) shows that neither the total amount of receptor nor its distribution between the cytosol and the nucleus is significantly altered by exposure of the cells to MDAB. Therefore these results eliminate the possibility of an indirect effect of the carcinogen on the expression of the tyrosine aminotransferase gene.

Phorbol-myristate acetate (PMA) has been shown to influence gene expression in a variety of cultured cells [22–24]. It does not appear to inhibit the accumulation of tyrosine aminotransferase in cultured hepatocytes [25]. Nevertheless it was considered worthwhile to see if it could modify the response of hepatocytes to MDAB. The results of this study (Table 6) show that there is no enhancement of the suppression of tyrosine aminotransferase by MDAB when cells are exposed to both MDAB and PMA simultaneously. It appears that PMA will affect only certain cell types and that liver cells are not among these.

It was of interest to learn whether the effect of MDAB on tyrosine aminotransferase accumulation was specific for hepatocarcinogens or whether all carcinogens were capable of eliciting this response. Preliminary experiments using carcinogens which were readily available commercially revealed that the 2-analogue of MDAB was effective, as was dimethylbenzanthracene. Both these compounds are known to be hepatocarcinogenic. In contrast, azaserine and benz(a)pyrene, which are not generally considered to be hepatocarcinogens, were ineffective. The carcinogen 6-aminochrysene, for which there is both evidence for and against being a hepatocarcinogen [26], is without effect. So thus far it appears that liver carcinogens are effective; however, it would require a broader survey of carcinogens before this system could be said to be specific for hepatocarcinogens. For the present, its potential as a screening agent for suspect hepatocarcinogens remains. In addition, the differential response between mature and less mature hepatocytes could be used to ascertain whether certain carcinogens require activation before they become effective.

Acknowledgements—These studies were supported by grants from the National Health & Medical Research Council, the Australian Research Grants Commission and the Cancer Council of Western Australia.

REFERENCES

1. YEOH GCT, BENNETT FA, OLIVER IT. Hepatocyte differentiation in culture: appearance of tyrosine aminotransferase. *Biochem J* 1979, **180**, 153–160.
2. YEOH GCT. The effect of 3'-methyl-4-dimethyl-aminoazobenzene on foetal rat hepatocytes in culture. *Eur J Cancer* 1981, **17**, 743–752.
3. BARRY PIERCE G. Neoplasms, differentiation and mutations. *Am J Pathol* 1974, **77**, 103–113.
4. POTTER VR. Phenotypic diversity in experimental hepatomas: the concept of partially blocked ontogeny. *Br J Cancer* 1978, **38**, 1–23.
5. MARKERT CL. Cancer: the survival of the fittest. In: SAUNDERS GF, ed. *Cell Differentiation and Neoplasia*. New York, Raven Press, 1978.
6. KENSLER TW, BUSBY WF, JR, DAVIDSON NE, WOGAN GN. Effect of hepatocarcinogens on the binding of glucocorticoid–receptor complex in rat liver nuclei. *Cancer Res* 1976, **36**, 4647–4651.
7. YEOH GCT, ARBUCKLE T, OLIVER IT. Tyrosine aminotransferase induction in hepatocytes cultured from rat foetuses treated with dexamethasone *in utero*. *Biochem J* 1979, **180**, 545–549.
8. CRAIG JC, PURUSHOTHAMAN KK. An improved preparation of tertiary amine oxides. *J Org Chem* 1970, **35**, 1721–1726.

9. KADLUBAR FF, MILLER JA, MILLER EC. Microsomal *N*-oxidation of the hepatocarcinogen *N*-methyl-4-aminoazobenzene and the reactivity of *N*-hydroxy-*N*-methyl-4-aminoazobenzene. *Cancer Res* 1976, **36**, 1196–1206.
10. MILLER JA, MILLER EC. The carcinogenicity of certain derivatives of *p*-dimethylaminoazobenzene in the rat. *J Exp Med* 1948, **87**, 139–156.
11. VOGEL AI. *Practical Organic Chemistry*. London, Longmans, 1956.
12. BRADFORD MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976, **72**, 248–254.
13. BEARDEN JC. Quantitation of submicrogram quantities of protein by an improved protein-dye binding assay. *Biochem Biophys Acta* 1978, **533**, 525–529.
14. HINEGARDNER RT. An improved fluorometric assay for DNA. *Anal Biochem* 1976, **39**, 197–201.
15. BEATO M, FEIGELSON P. Glucocorticoid-binding proteins of rat liver cytosol: I. Separation and identification of the binding protein. *J Biol Chem* 1972, **247**, 7890–7896.
16. ROUSSEAU GG, BAXTER JD, HIGGINS SJ, TOMKINS GM. Steroid-induced nuclear binding of glucocorticoid receptors in intact hepatoma cells. *J Mol Biol* 1973, **539**, 539–554.
17. GUENTHER TM, MANNERING GJ. Induction of hepatic mono-oxygenase systems in fetal and neonatal rats with phenbarbital, polycyclic hydrocarbons and other xenobiotics. *Biochem Pharmacol* 1977, **26**, 567–575.
18. MILLER JA, MILLER EC. Perspectives on the metabolism of chemical carcinogens. In: EMMELOT P, KRIEK E, eds. *Environmental Carcinogenesis*. Amsterdam, North-Holland Biomedical Press, 1979.
19. CAKE MH, LITWACK G. The glucocorticoid receptor. In: LITWACK B, ed. *Biochemical Action of Hormones*. New York, Academic Press, 1975.
20. NICKOL JM, LEE KL, KENNEY FT. Changes in hepatic levels of tyrosine aminotransferase messenger RNA during induction by hydrocortisone. *J Biol Chem* 1978, **253**, 4009–4015.
21. HOFER E, SEKERIS CE. Cycloheximide causes increased accumulation of translatable mRNA for tyrosine aminotransferase and tryptophan oxygenase in livers of cortisol-treated rats. *Eur J Biochem* 1978, **86**, 547–554.
22. LOWE M, PACIFICI M, HOLTZER H. Effects of phorbol-12-myristate-13-acetate on the phenotypic program of cultured chondroblasts and fibroblasts. *Cancer Res* 1978, **38**, 2350–2356.
23. DIAMOND L, O'BRIEN TG, ROVERA G. Inhibition of adipose conversion of 3T3 fibrolasts by tumor promoters. *Nature (Lond)* 1977, **269**, 247–249.
24. ROVERA G, O'BRIEN TG, DIAMOND L. Tumor promoters inhibit spontaneous differentiation of Friend erythroleukemia cells in culture. *Proc Natl Acad Sci USA* 1977, **74**, 2894–2898.
25. YEOH GCT, BRIGHTON VJ. Phorbol myristate acetate (PMA) fails to prevent the appearance of markers associated with muscle and liver differentiation in culture. *Cell Biol Int Report* 1981, **5**, 607–616.
26. ROE FJC, CARTER RL, ADAMWAITE S. Induction of liver and lung tumours in mice by 6-aminochrysene administered during the first 3 days of life. *Nature (Lond)* 1969, **221**, 1063–1065.