Sulfhydryl reagents have been reported to be necessary in maintaining activity of adenylate cyclase prepared from avian and amphibian erythrocytes.[10,11] The apparent importance of sulfhydryl groups for activity of this enzyme in mammalian brain has not been previously noted.

The results of this study indicate that free sulfhydryl groups are necessary for full expression of adenylate cyclase activity in brain homogenates. Presumably these sulfhydryl groups are located in the enzyme itself. However, an alternate possibility is that ethacrynic acid and DTNB may interact with sulfhydryl groups of structural proteins in membranes. This, in turn, could alter the configuration of the membrane bound adenylate cyclase and inhibit its enzymatic activity.

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SHORT COMMUNICATION

Effects of methylcholanthrene on 2-acetamidofluorene association with liver cell components

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2-ACETAMIDOFLUORENE (AAF) can be converted to a highly reactive, ultimate carcinogen which is capable of forming conjugates with cell components under physiological conditions.[1] The reactions involved are *N*-hydroxylation followed by esterification to a molecule which can ionize and react with cell nucleophiles.[2] Resistance to the hepatocarcinogenic effects of AAF is observed in male rats treated simultaneously with AAF and small quantities of methylcholanthrene (MC).[3,4] One metabolic effect of MC in AAF-dosed rats is to reduce the proportion of AAF excreted as the glucuronide of *N*-hydroxy-AAF.[5] This result is due to a reduction in *N*-hydroxylating activity, an increase in *N*-hydroxy-AAF reduction or to an increase in the reactions producing phenolic metabolites. Because *N*-hydroxy-AAF is more carcinogenic than the parent compound,[6] it seemed that, through diminishing the concentration of *N*-hydroxy-AAF for further reaction, MC blocked an essential step in the activation of AAF. Consequently, interaction of the carcinogen with key components in target cells should be reduced below a threshold carcinogenic level in MC-treated animals and provide a basis for the observed inhibition of AAF induced hepatocarcinogenesis.[3,4] These key components have not been identified.

By comparing AAF associations in stock diet and MC diet fed rats, it might be possible to distinguish carcinogen–cell interactions important to carcinogenesis from interactions which are not. This was the purpose behind the experiments to be described.

Male Ash/Wistar rats (Scientific Products Farm, Ltd., Margate, England) weighing 180–220 g were given i.p. injections of 2-acetamido-9-[^{14}C]fluorene (New England Nuclear Corp., Frankfurt, Germany) with a specific activity of 3·00 mCi/mmole and dissolved in aqueous dimethylformamide (1:2). The dose level was 1 μl and 0·1 μCi/g rat.

Rats were fed stock diet alone or containing 0·003% methylcholanthrene. Injections were made at 09·00–09·30 and the animals fasted until they were killed 24 hr later.

4·5 g of liver was homogenized in 40 ml of cold 0·32 M sucrose, 10 mM $MgCl_2$, 25 mM KCl, 1 mM ethyleneglycol-bis-(β-aminoethyl ether) N_1N^1-tetraacetic acid, 50 mM Tris–HCl pH 7·6 at 0°. The homogenate was fractioned by differential centrifugation to give nuclei (N),[7] mitochondria (M),[8] light mitochondria (LM),[9] smooth endoplasmic reticulum (SER),[9] rough endoplasmic reticulum (RER),[9] and cell sap (CS).[9]

DNA and rRNA were extracted and purified from the remainder of each liver by the Kirby methods.[11] Nucleotides from rRNA were separated on Dowex-1 columns with formic acid.[12]

Cell sap was fractionated by gel filtration on a column of Sephadex G-25 (fine) equilibrated with 1·0 M NaCl, 50 mM Tris–HCl pH 7·8 at 2°. The void volume eluate was further fractioned on a column of Sephadex G-200 equilibrated with the same solution as the G-25 column.

Protein content was estimated according to the Folin–Ciocalteu reagent method.[13] Measurements of RNA[14] and DNA[15] content were made after their physical separation.[16]

Liquid scintillation counting of particulate samples was performed after their dissolution in Hyamine hydroxide. Aqueous solutions were incorporated directly with Insta-gel (Packard Instruments, Downers Grove, U.S.A.) Counting efficiencies were determined using the external standard channels ratio method on a Tracerlab Corumatic Series 200 spectrometer.

TABLE 1. THE RADIOACTIVITY IN VARIOUS CELL FRACTIONS FROM THE LIVERS OF RATS INJECTED 24 hr BEFORE DEATH WITH 0·1 μCi 2-ACETAMIDO-9-[^{14}C]FLUORENE/g RAT*

Diet	CS	LM	RER	SER	M	N
Methylcholanthrene	1138 ± 107	1110 ± 106	810 ± 104	773 ± 250	765 ± 84	381 ± 63
Stock	1087 ± 196	962 ± 151	742 ± 139	735 ± 115	729 ± 122	583 ± 145

* Mean values and S.D. are expressed as dis./min/mg protein and refer to four animals for each diet. Methylcholanthrene was incorporated as 0·003 per cent of the stock diet and fed to the rats for the last 3 weeks of life.

Association of AAF metabolites was greatest with cell sap and light mitochondrial components (Table 1) while association was least evident with the nuclear fractions. The ranking of AAF association with the cell fractions was not altered by treatment with MC and in none of the fractions was there a reduction in association attributable to MC.

TABLE 2. THE RADIOACTIVITY IN LIVER DNA AND rRNA FROM RATS INJECTED 24 hr BEFORE DEATH WITH 0·1 μCi 2-ACETAMIDO-9-[^{14}C]FLUORENE/g RAT*

Diet	DNA	rRNA
Methylcholanthrene	66 ± 11	145 ± 25
Stock	78 ± 16	124 ± 37

* Mean values and S.D. are expressed as dis/min/mg nucleic acid and refer to four animals for each diet. Methylcholanthrene was incorporated as 0·003 per cent of the stock diet and fed to the rats for the last 3 weeks of life.

AAF binding to DNA was lower than to rRNA in both groups of rats (Table 2). Again, MC did not have a demonstrable effect on the association of AAF with cell components. Analysis of the RNA nucleotides following alkaline hydrolysis did not reveal differences between the two groups since all the radioactivity remained bound to the Dowex 1 column until eluted as a single peak with 20 N formic acid. Virtually all the 260 nm wavelength absorbing material was eluted with 4 N formic acid.

Cell sap macromolecules bound most of the labelled AAF; only 20 per cent of the radioactivity was associated with material retarded on the Sephadex G-25 column. The macromolecular components could be fractionated on the Sephadex G-200 column to give four regions of relatively high radioactivity. These regions occurred at $V_e/V_0 = 1{\cdot}1$, $1{\cdot}5$, $2{\cdot}1$ and $2{\cdot}5$. No variation in relative binding specificity could be seen if the results were plotted as dis/min/mg protein against the relative elution volumes.

These results suggest that, despite reports that the balance of urinary products from AAF metabolism is changed by MC treatment, this is not reflected quantitatively in an altered association of AAF metabolites with liver cell components. The nature of the associations could conceivably be altered by MC, a short coming in the analyses which applies especially to the particulate fractions. Association is presumably firm, however, between carcinogen metabolites and the chromatographed cell sap macromolecules and it is unlikely that associations other than by covalency would be retained throughout the isolation and purification of the nucleic acids. Kriek[18] has demonstrated two reaction products in liver DNA of rats given a single injection of *N*-hydroxy-AAF. Whereas one of these products remains associated with DNA for at least 8 weeks, the main (80 per cent) product has a half life of 7 days. MC might cause this pattern to change in a way which would not have been detected in the present experiments. If, however, the nature and extent of the carcinogen cell component associations are similar in the two groups then it suggests that such associations are not important to carcinogenesis. Alternatively, the property of MC to inhibit a neoplastic response is due to some mechanism of cell regulation other than foreign compound metabolism. There may exist in the MC treated cells the environment for more error-free repair of cell damage caused by AAF. The form this might take is unknown at present, but MC does have an effect on the template activity of rat liver chromatin[19] and the study of induction of enzymes other than those involved in microsomal oxidations, e.g. DNA repair enzymes, could be interesting. If this hypothesis is correct, then MC might inhibit carcinogenesis by chemicals other than aromatic amines and related compounds.

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