Comparison of the Products of the Reaction of 7-Methylbenz[a]anthracene 5,6-Oxide and RNA, with Those Formed in 7-Methylbenz[a]anthracene-treated Cells

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SUMMARY

RNA was isolated by a phenol extraction method from mouse embryo cells treated in culture with either [G-3H]-7-methylbenz[a]anthracene or [G-3H]-7-methylbenz[a]anthracene 5,6-oxide (the K-region epoxide). The RNA was degraded to ribonucleosides, mixed with ultraviolet-absorbing quantities of the epoxide ribonucleoside products isolated from RNA that had reacted with 7-methylbenz[a]anthracene 5,6-oxide in aqueous ethanol solution, and chromatographed on a column of Sephadex LH-20 eluted with a methanol:water gradient. The 7-methylbenz[a]anthracene 5,6-oxide ribonucleoside products formed in cells were identical to those formed in aqueous solution, although the relative amounts of the products varied. The majority of these epoxide-ribonucleoside products were not identical to the products formed in cells treated with the parent hydrocarbon. These results suggest that the major reactive form of 7-methylbenz[a]anthracene that binds to RNA in mouse embryo cells is not the K-region epoxide of this hydrocarbon.

INTRODUCTION

Elucidation of the mechanism of activation that results in the binding of carcinogenic aromatic hydrocarbons to cellular macromolecules may be of value in determining the mechanism whereby polycyclic hydrocarbons induce tumor formation. Several lines of evidence suggest that metabolic activation of the hydrocarbons within the cells is necessary for this binding (reviewed in Ref. 13), and one possible type of reactive hydrocarbon metabolite that has been proposed is an epoxide (5). Although the formation of epoxides may be one of the metabolic routes responsible for the activation of hydrocarbons in cells (reviewed in Refs. 13 and 18), comparisons of the DNA-bound products formed by hydrocarbons in cells with the products formed by the reaction of their K-region epoxides with DNA failed to support the concept that K-region epoxides are the reactive intermediates responsible for this binding (2, 3).

It is conceivable, however, that different ultimate reactive species of hydrocarbons are responsible for their binding to different cellular macromolecules and to macromolecules in different parts of the cell. Therefore, we have compared the RNA-bound products from 7-MeBA-treated mouse embryo cells with the RNA-bound products formed when 7-MeBA K-region epoxide reacts with RNA in aqueous solution or in mouse embryo cells in culture.

MATERIALS AND METHODS

Chemicals and Reactions with RNA. 7-MeBA-epoxide (6) was prepared from 7-MeBA by the method of Newman and Blum (15). After cyclization of the dialdehyde, the crystalline epoxide was filtered off, washed, and recrystallized from cyclohexane as colorless needles (17). The 7-MeBA-epoxide, which moved as 1 spot on thin-layer chromatograms and had the required spectral properties, was reacted with RNA from Torula yeast (Sigma Chemical Co., St. Louis, Mo.), as described by Swaisland et al. (20).

Radiochemicals. 7-MeBA (specific radioactivity, 29 Ci/mmol) was tritiated by exchange at the Radiochemical Centre, Amersham, England, and purified by TLC prior to use (9). [H]-7-MeBA-epoxide (specific radioactivity, 3.6 Ci/mmol) was prepared from [H]-7-MeBA as described above (10, 15). The recrystallized compound was added to cell cultures within 3 hr after preparation.

Isolation of RNA from Cells in Culture. Primary cultures of C57BL mouse embryo cells were treated with radiochemicals by the procedure described previously (1). RNA was isolated from the cell pellet by the technique of Diamond et al. (7) using a modified aqueous phase (8). In later experiments, RNA was isolated by a procedure using disodium naphthalene-1,5-disulfonate (14, 16) that yielded approxi-
mately 14 mg/10^9 cells. All RNA samples had A_{260}/A_{280} ratios of 1.9 or greater.

**Degradation of RNA.** RNA samples were degraded to ribonucleosides by alkaline hydrolysis and treatment with alkaline phosphatase (20). To determine whether any of the RNA-bound products were destroyed by alkaline hydrolysis, samples of [^3H]-7-MeBA-RNA and [^3H]-7-MeBA-epoxide RNA were degraded with RNase A (Worthington Biochemical Corp., Freehold, N. J.), phosphodiesterase (bovine spleen type 1; Sigma), and alkaline phosphatase (Escherichia coli type III; Sigma). Examination on Sephadex LH-20 of these digests showed no significant differences from the products obtained in the alkaline hydrolysis procedure (Charts 1 and 2). This enzyme procedure may not result in a complete degradation of the RNA, however, since there was a 15% increase in the amount of radioactivity that eluted coincident with undegraded RNA (Charts 1 and 2; elution volume, 75 to 95 ml) and a corresponding 15% decrease in the amount of radioactivity in the product region (Charts 1 and 2; Peaks I, II, and III). Therefore, the alkaline hydrolysis procedure was used for all samples.

**Chromatography.** Column chromatography of RNA hydrolysates was carried out on 90- x 1.5-cm columns packed to a height of 80 cm with Sephadex LH-20 (Pharmacia Fine Chemicals, Uppsala, Sweden) and was eluted with water:methanol gradients as described previously (1). Recovery of radioactivity from all columns was 90 to 100%.

TLC was carried out on Polygram Sil N-HR/UV254 sheets (Camlab, Cambridge, Eng.) developed in acetone. The chromatograms were cut into 1- x 2-cm strips, and the products on each strip were extracted with 2 ml methanol for 18 to 24 hr. One ml of each sample was used for scintillation counting, 0.2 ml was diluted with 2.0 ml methanol, and the absorbance (A_{260}) was measured with a Unicam SP 500 spectrophotometer (Pye Unicam Ltd., Cambridge, England).

**RESULTS**

To determine whether the hydrocarbon-RNA products formed in 7-MeBA-treated mouse embryo cells could also be obtained by causing RNA to react with the K-region epoxide of 7-MeBA in aqueous ethanol or by exposing cells in culture to tritiated 7-MeBA-epoxide, the following experiments were carried out. In the 1st experiment, 7-MeBA-epoxide was reacted with RNA in aqueous ethanol and, after removal of unreacted epoxide by extraction with ether, the RNA was hydrolyzed and chromatographed on a Sephadex LH-20 column. The column elution profile was similar to the A_{260} profile described in Chart 1, although a very large quantity of UV-absorbing, unreacted ribonucleosides was eluted in the 1st 300 ml. The fractions containing the UV-absorbing epoxide-ribonucleoside products (elution volumes greater than 400 ml) were then pooled, evaporated to a small volume, mixed with a digest of [^3H]-7-MeBA-RNA from cells, and chromatographed on a Sephadex LH-20 column (Chart 1). The ribonucleoside digest contained an early peak (elution volume, 75 to 90 ml), some radioactivity that eluted in the same region as the unreacted ribonucleosides (elution volume, 90 to 180 ml), 3 radioactive hy-

![Chart 1. Sephadex LH-20 column elution profile of a mixture of UV markers of the 7-MeBA-epoxide-ribonucleoside peaks (E1, E2, and E3) obtained from reaction in aqueous solution, and a digest of RNA from mouse embryo cells treated in culture with [^3H]-7-MeBA. The sample applied was a digest of 5 mg of RNA (bound radioactivity, 0.36 μCi/mg) from mouse embryo cells treated for 24 hr with [^3H]-7-MeBA (specific radioactivity, 29 Ci/m mole) at a concentration of 0.1 μg/ml medium plus the 7-MeBA-epoxide-ribonucleoside markers isolated on a similar chromatographic column from 85 mg RNA that had reacted with 5 mg 7-MeBA-epoxide in water:ethanol (2:1). The gradient used was a 30% methanol:water to 100% methanol gradient as described previously (1). A, UV absorption of the unreacted ribonucleosides monitored continuously at 254 nm; O, UV absorption of the markers measured for each fraction at 260 nm; C, cpm/1.0-ml sample of each fraction.](Image)
Chart 2. Sephadex LH-20 column elution profile of a mixture of UV markers of the 7-MeBA-epoxide-ribonucleoside peaks (E1, E2, and E3) obtained from reaction in aqueous solution, and a digest of RNA from mouse embryo cells treated in culture with [3H]-7-MeBA-epoxide. The sample applied was a digest of 2.6 mg of RNA (bound radioactivity, 0.1 μCi/mg) from mouse embryo cells treated for 4 hr with [3H]-7-MeBA-epoxide (specific radioactivity, 3.6 Ci/m mole) at a concentration of 1.0 μg/ml medium plus the 7-MeBA-epoxide-ribonucleoside markers isolated on a similar column from 200 mg of RNA that had reacted with 10 mg of 7-MeBA-epoxide for 4 hr in water:ethanol (2:1). The column was eluted as described for Chart 1. ▲, UV absorption of the untreated ribonucleosides monitored continuously at 254 nm; ○, UV absorption of the markers measured for each fraction at 260 nm; ○, cpm/1.0 ml sample of each fraction.

drocarbon-ribonucleoside product peaks (I, II, and III), and the 3 UV-absorbing 7-MeBA-epoxide ribonucleoside marker peaks (E1, E2, and E3) described above. The 2 larger epoxide-ribonucleoside product peaks (E2, E3) were clearly separated from the hydrocarbon-ribonucleoside peaks (I, II, and III), but the small epoxide-ribonucleoside peak (E1) eluted in the same position of the gradient as 1 of the 7-MeBA-ribonucleoside peaks (Peak II). Thus, although the major products of the reaction of 7-MeBA-epoxide with RNA in aqueous solution (Peaks E2 and E3) were different from those found in RNA from cells treated with the parent hydrocarbon, the results shown in Chart 1 suggested that a minor epoxide-RNA product peak (E1) might contain products identical to those found in [3H]-7-MeBA-RNA from cells.

To investigate this further, UV-absorbing epoxide-RNA products were prepared as described above, mixed with a digest of [3H]-7-MeBA-epoxide-RNA from mouse embryo cells, and chromatographed on Sephadex LH-20 (Chart 2). The elution profile of this mixture also contained a radioactive early peak, 3 radioactive product peaks (I, II, and III), and the 3 UV-absorbing marker peaks (E1, E2, and E3). Although the tritium (I, II, and III) and UV-absorbing peaks (E1, E2, and E3) occurred in the same fractions, the tritium profile of the 2 major peaks was not coincident with the UV absorption profile. In the case of Peaks I and E1 (Chart 2), however, the UV absorption and tritium profiles did coincide.

To determine whether the cellular hydrocarbon-ribonucleoside products in Peak II of Chart 1 or the cellular epoxide-ribonucleoside products in Peak I of Chart 2 were identical to those in Peak E1 of the digest of RNA that had reacted with the epoxide in aqueous solution, the materials eluted in these peaks were chromatographed on Sephadex LH-20 columns eluted with 45 to 65% methanol gradients (Chart 3). Although the maxima differed by only 2 fractions, it is apparent that, in the mixture of the [3H]-7-MeBA-RNA and the UV-epoxide-RNA (Chart 1, Peaks II and E1), the tritium peak was not coincident with the peak of UV absorption (Chart 3A). In the mixture of the [3H]-7-MeBA-epoxide-RNA and the UV-epoxide-RNA (Chart 2, Peaks I and E1), the tritium peak was coincident with the peak of UV absorption (Chart 3B).

To confirm that the bulk of the material in Peak II from cells treated with the parent hydrocarbon was not identical to the epoxide-RNA products in Peak E1, although the material in this peak from cells treated with the epoxide and from RNA reacted with the epoxide in aqueous solution may be the same, the same material described in Chart 3 was also subjected to TLC in acetone (see Table 1). The radioactivity in Peak II (Chart 1) from cells treated in culture with the
parent hydrocarbon remained at the origin, while the majority of the UV-absorbing epoxide-RNA material (Peak E1) formed a peak of $R_v$ 0.2 and only 20% remained at the origin. On the chromatogram of the mixture of $[\text{H}]$-7-MeBA-epoxide-RNA products from cells (Chart 2, Peak I) and the UV-absorbing epoxide markers (Peak E1), the tritium was associated with both the UV-absorbing peaks (Table 1).

The slight differences between the column elution profiles of the major peaks of the tritiated 7-MeBA-epoxide-RNA products from cells (Peaks II and III, Chart 2) and the UV-absorbing 7-MeBA-epoxide-RNA products (Peaks E2 and E3, Chart 2) were also investigated by TLC in acetone. The data for the 2 mixed peaks (II-E2 and III-E3) in Table 1 show that each column peak was resolved into 2 peaks by TLC. Although the $R_v$'s of the 2 tritium and 2 UV-absorbing products were the same, the relative amounts of material in the 2 spots were different in $[\text{H}]$-epoxide-RNA from cells and in epoxide-RNA from the aqueous solution. This result could explain the slight differences in the Sephadex LH-20 elution profiles of the major tritiated and UV-absorbing peaks.

**DISCUSSION**

The mechanism of activation of 7-MeBA by which it becomes bound covalently to RNA in mouse embryo cells in culture does not appear simply to involve the formation of the K-region epoxide and the reaction of this species with RNA. The present results show that the products formed by the reaction of the K-region epoxide of 7-MeBA with RNA in aqueous solution or in cells in culture have similar chromatographic properties (although they are not formed in the same relative amounts), but the majority of these products differ from those formed in cells treated with the parent hydrocarbon.

The chemical structure of the 7-MeBA-epoxide-ribonucleoside products has not yet been established. The products obtained from digests of 7-MeBA-epoxide-RNA were similar to the previously described products obtained from digests of RNA reacted with benz[a]anthracene 5,6-oxide or 7,12-dimethylbenz[a]anthracene 5,6-oxide (20). The reactivity of hydrocarbon epoxides with purine polynucleotides (4, 11, 20) suggests that the epoxide-RNA products are probably some form of epoxide-purine nucleoside adduct.

The 2 methods of releasing the 7-MeBA-bound products from RNA and their chromatographic properties are con-

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**Table 1**

<table>
<thead>
<tr>
<th>TLC of peaks eluted from Sephadex LH-20 columns</th>
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<tbody>
<tr>
<td>TLC on silica gel sheets developed in acetone of the materials obtained from the column chromatograms described in Charts 1 and 2. 7-MeBA, cis- and trans-5,6-dihydro-5,6-dihydroxy-7-MeBA, and 5-hydroxy-7-MeBA all had $R_v$ 0.7 in this system.</td>
</tr>
<tr>
<td>Peak on Sephadex LH-20 column</td>
</tr>
<tr>
<td>Hydrolysate of RNA from cells treated with $[\text{H}]$-7-MeBA and UV markers</td>
</tr>
<tr>
<td>Chart 1, II-E1</td>
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<tr>
<td>Hydrolysate of RNA from cells treated with $[\text{H}]$-7-MeBA-epoxide and UV markers</td>
</tr>
<tr>
<td>Chart 2, I-E1</td>
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<td>Chart 2, I-E1</td>
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<td>Chart 2, II-E2</td>
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<td>Chart 2, II-E2</td>
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<td>Chart 2, III-E3</td>
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<td>Chart 2, III-E3</td>
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sistent with those expected for hydrocarbon-ribonucleoside products, but chemical characterization of these products is impossible with the small amount of material obtained. Brookes et al. (6) demonstrated that when cells containing DNA prelabeled with a tritiated purine precursor were treated with unlabelled 7-MeBA, the Sephade LH-20 profile of the DNA digest contained labeled peaks in positions identical to those found in DNA from cells treated with [3H]-7-MeBA. Although similar studies have not yet been done with RNA, the identical Sephade LH-20 elution positions of the products from [3H]-7-MeBA-RNA (Chart 1) and [3H]-7-MeBA-DNA (1) support the conclusion that these are hydrocarbon-ribonucleoside products.

The nature of the radioactivity eluted in the 1st half of the gradients (Charts 1 and 2) has not been completely resolved. Previous studies with hydrolysates of DNA from [3H]-7-MeBA-treated cells showed that the radioactivity in the region of the gradient in which unreacted deoxyribonucleosides are eluted for DNA (1) and the experiment described in Chart 1, the cells had been treated with [3H]-7-MeBA at a radioactivity concentration of 18 µCi/ml medium. When enzyme digests of DNA from mouse embryo cell cultures that had been treated with 3 to 5 µCi of [3H]benzo(a)pyrene per ml medium (3) or from hamster embryo cell cultures that had been treated with 1.5 µCi of [3H]benzo(a)pyrene or [3H]-7,12-dimethylbenz[a]anthracene per ml medium (W. M. Baird and L. Diamond, unpublished results) were chromatographed on similar columns, no significant amount of radioactivity cochromatographed with the unreacted deoxyribonucleoside peaks.

The early peak (elution volume, 75 to 90 ml) seen in both Charts 1 and 2 is more difficult to characterize. The radioactivity present in this peak accounted for only 12% of the radioactivity probably represents RNA that was not completely digested by the procedure used but may represent unreacted ribonucleosides, including guanosine, and residual proteins with bound hydrocarbon derivatives.

Comparison of the 7-MeBA-RNA Sephade LH-20 column elution profile (Chart 1) with that of 7-MeBA-DNA (1) reveals a striking similarity. A 3rd product peak (Chart 1, l) is apparent in the hydrocarbon-ribonucleoside products, and a very small peak is seen in a similar location in the column profile of the hydrocarbon-deoxyribonucleoside products. This suggests that the same reactive form of 7-MeBA may be responsible for its binding to both nucleic acids in mouse embryo cells.

Although the possibility still exists that the hydrocarbon-bound products do result from formation of a K-region epoxide preceded or followed by other metabolic steps (an externally applied K-region epoxide might be excluded from such metabolism), it is more probable that 7-MeBA becomes bound to nucleic acids in mouse embryo cells by some other mechanism of activation. Recent studies suggest that benzo[a]anthracene and benzo(a)pyrene become bound to DNA in hamster embryo cells through a non-K-region dihydrodiol-epoxide (19, 21). A similar mechanism could also be involved in the metabolic activation of 7-MeBA in mouse embryo cells.

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