The Binding of Polycyclic Aromatic Hydrocarbons to the DNA, RNA, and Proteins of Transformable Cells in Culture

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SUMMARY

The binding of several labeled carcinogenic hydrocarbons to macromolecular constituents of various cells involved in systems for chemical carcinogenesis in vitro was studied. The hydrocarbons were firmly bound to the DNA, RNA, and proteins of cultured embryonic cells from C3H mice and hamsters and of various cells derived from C3H mouse prostates. The extent of binding in the embryonic cells was very similar to that previously found with mouse skin in vivo. Chemically and spontaneously transformed cells bound 5 to 10 times less hydrocarbon than did the parental nontransformed prostate cells, although less malignant variants obtained from highly malignant clones bound the hydrocarbons to two-thirds the extent of the nontransformed cells. In a series of 5 hydrocarbons, benzo[a]pyrene was bound to proteins to the highest extent, and 7,12-dimethylbenz[a]anthracene was bound most to nucleic acids. The weakly carcinogenic dibenz[a,c]anthracene was bound to all constituents in all cells to a greater extent than its more carcinogenic isomer, dibenz[a,h]anthracene. There was no significant difference in the binding of hydrocarbons to exponentially growing and nongrowing cells, and the binding was stable and persisted up to 5 weeks when there was no cell growth. However, in growing cells part of the bound material was partitioned by cell division, but some was also lost.

INTRODUCTION

In recent years, 2 systems of chemical carcinogenesis in vitro have been established. These include the transformation of primary and secondary cultures of hamster embryonic cells with carcinogenic hydrocarbons (3, 14, 40), 4-nitroquinoline 1-oxide (27, 30, 36) and dimethylnitrosamine (25). The transformation of cells derived from C3H mouse prostate with carcinogenic hydrocarbons has also been accomplished (10–12). In these systems, the carcinogens have been shown to produce malignant as well as morphological transformation, which are tightly correlated (12). Furthermore, a good correlation has been found between carcinogenic potency in vivo and transforming activity in vitro (3, 12, 30). Thus, these in vitro systems are now considered by us and many others to be reliable models of chemical carcinogenesis and provide a new approach to investigate its cellular and molecular mechanisms.

This work investigates the interaction between carcinogenic hydrocarbons and cellular constituents and has confirmed and extended the observations that these compounds are firmly bound in vivo to nucleic acids and proteins of mouse skin (1, 6, 8, 21, 43).

MATERIALS AND METHODS

Cells and Cell Culture. Most of the experiments were carried out with nontransformed or chemically or spontaneously transformed fibroblastic cell lines derived from C3H mouse prostates (10–12). Secondary cultures of embryonic cells from C3H mice (Charles River Breeding Laboratories, Boston, Mass.) and Syrian hamsters (Con Olson Co., Verona, Wis.) were also used. The cells used and their characteristics are summarized in Table 1.

Primary cultures of embryonic cells were prepared by enzymatic dispersion (0.1% trypsin) of minced whole embryos (3, 30) and were cultured in Eagle's basal medium and 10% fetal calf serum; hamster embryonic cells were cultured in Dulbecco's medium without phenol red and containing 10% fetal calf serum. For subculturing, cells were dispersed with 0.1% trypsin in Dulbecco's phosphate buffer solution. All media and sera were obtained from Grand Island Biological Corp., Grand Island, N. Y. The cells were grown in plastic Petri dishes (100 x 20 or 150 x 25 mm, Falcon Plastics, Los Angeles, Calif.). In some experiments, roller bottle cultures operating at a speed of 0.5 rpm were also used.

Carcinogens and Carcinogen Treatment. Generally labeled, tritiated hydrocarbons BP, DBA, DB[a,c]A, DMBA, and MCA were obtained from Amershamsearle, Arlington Heights, Ill. The specific activities of the 4 carcinogenic compounds (BP, DBA, DMBA, and MCA) were 500 mCi/mmol, whereas that of the weakly carcinogenic DB[a,c]A was 203 mCi/mmol. The purity of the compounds was examined by radioactive scanning of thin-layer chromatograms (Eastman Chromagram Sheet No. 6060, Eastman Kodak Co., Rochester, N. Y.) in benzene (100%) and ethanol:benzene (1:9). With all labeled compounds, cells were dropped on glass slides, fixed in methanol, and exposed to photographic emulsions for 12 to 56 days.

The abbreviations used are: BP, benzo[a]pyrene; DBA, dibenz[a,h]anthracene; DB[a,c]A, dibenz[a,c]anthracene; DMBA, 7,12-dimethylbenz[a]anthracene; MCA, 3-methylcholanthrene; SSC, sodium citrate solution; TCA, trichloroacetic acid; TdR, thymidine.
carcinogens, the radiochemical purity was greater than 95%. A
small amount of radioactivity often observed at the origin was
an artifact due to air oxidation during development of the
thin-layer plate, because rechromatography of a sample eluted
from the hydrocarbon region (Rf = 0.95) again gave some
radioactivity at the origin. No other radioactive spots other
than the hydrocarbon and at the origin were found in the
scans. Thus, the labeled hydrocarbons were deemed pure
enough to use in these experiments.

The labeled carcinogens were dissolved in dimethyl
sulfoxide after the benzene was evaporated with nitrogen and
were added to the medium to give a final concentration of
0.5% dimethyl sulfoxide. The concentration of the compounds
in the medium was, unless otherwise stated, 4 × 10⁻⁶ M
(about 1 μg/ml) or 2 μCi/ml (0.8 μCi/ml of DB[ac] A). At this
concentration, the cells can be transformed.

After the incubation with tritiated hydrocarbons, the
monolayer of cells was washed once with 0.9% NaCl solution
and harvested with 0.02% EDTA in 0.9% NaCl solution. Each
experiment required about 50 million cells for isolation of the
cellular constituents. The cell pellet was frozen at —80° until
use.

<table>
<thead>
<tr>
<th>Cells</th>
<th>History and characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prostate cells</td>
<td>A clone obtained from B1, line of prostate cells (10). Contact-inhibited, low saturation density, and failure to produce tumors in X-irradiated syngeneic C3H mice. More readily transformable with epoxides of hydrocarbons (23). PR</td>
</tr>
<tr>
<td>PR</td>
<td>A line of prostate cells, less susceptible to transformation, passages 20 to 40 were used.</td>
</tr>
<tr>
<td>Secondary culture of C3H mouse embryonic cells</td>
<td></td>
</tr>
<tr>
<td>Transformed cells</td>
<td></td>
</tr>
<tr>
<td>4C</td>
<td>Transformed cells obtained after the treatment of B1, line with MCA, high saturation density, criss-crossed arrangement, and production of tumors in nonconditioned C3H mice (33).</td>
</tr>
<tr>
<td>7C</td>
<td>Another clone obtained in the same way as 4C cells.</td>
</tr>
<tr>
<td>T-10</td>
<td>Tumor cells obtained from C3H mice after the in vitro treatment of prostate cells with MCA, passed serially through mice, and then cultured.</td>
</tr>
<tr>
<td>T-24</td>
<td>Obtained in the same way as T-10 cells.</td>
</tr>
<tr>
<td>SP-48</td>
<td>Spontaneously transformed cells obtained at the 48th passage of PR cells, malignant and high saturation density.</td>
</tr>
<tr>
<td>SP-24</td>
<td>Spontaneously transformed cells obtained at the 28th passage of PR cells.</td>
</tr>
<tr>
<td>Revertant cells</td>
<td></td>
</tr>
<tr>
<td>RR9B</td>
<td>Less malignant variant of 4C cells, obtained by plating on glutaraldehyde-fixed cells (S. Mondal et al., manuscript in preparation).</td>
</tr>
<tr>
<td>4C, F1, B1</td>
<td>Less malignant variant of 4C cells, obtained after treatment with 5-fluoro-2-deoxyuridine (S. Mondal et al., manuscript in preparation).</td>
</tr>
</tbody>
</table>

Fractionation of Cells into Alcohol-soluble and -insoluble Fractions. The cells were fractionated into alcohol-soluble and -insoluble fractions as follows. The cells after removal of the medium and addition of 1 mg of bovine serum albumin as carrier were suspended in 4 ml of 80% ethanol for 10 min at room temperature and centrifuged. The resulting supernatant alcohol-soluble fraction was counted in Scintisol (Isolab, Akron, Ohio), whereas the alcohol-insoluble precipitate was washed twice with ethanol, once with ether, then dried, dissolved with Soluene (Packard Instrument Company, Inc., Downers Grove, Ill.), and counted in toluene PPO. This procedure made it possible to extract loosely associated hydrocarbon into the alcohol-soluble fraction; such material is removed only with difficulty by the usual acid precipitation procedures.

Isolation of the Cellular Constituents. DNA, RNA, and protein were isolated by modification of the procedure of Diamond et al. (13). The cells were lysed for 10 min in 6 to 10 ml of cold Kirby's 2-stage extraction medium, which consisted of 1% sodium triisopropylphosphaplate sulfonate, 6% sodium 4-aminosalicylate, 1% NaCl and 6% sec-butyl alcohol in water (28). The resulting lysate was extracted with an equal volume of phenol:m-cresol:8-hydroxyquinoline:water (500:70:0.5:55, by weight) for 30 min with stirring at room temperature.

After centrifugation at 12,000 × g for 30 min, the upper aqueous phase was removed and centrifuged to remove the contaminating interphase. The DNA was precipitated from the aqueous phase by addition of an equal volume of 2-ethoxyethanol, fished out on a glass rod, and dissolved in 0.01 M phosphate buffer. The solution of DNA was treated with 50 μg/ml of RNase (preheated at 80° for 10 min and carefully chosen to be free of DNase) at 37° for 30 min. Then the DNA was deproteinized by 4 washings with chloroform:isoamyl alcohol (24:1), precipitated with 70% ethanol containing 2% sodium acetate, and washed with ethanol, then with ether, and dried. Finally, the DNA was dissolved in 0.15 M NaCl and 0.015 M SSC.

The RNA was obtained from the upper aqueous phase following removal of the DNA by addition of 2 volumes of ethanol. After standing in a freezer overnight, the RNA precipitate was centrifuged at 2500 rpm for 20 min and dissolved immediately in SSC containing 5 μg/ml of polyvinyl sulfate. Then the RNA was reprecipitated by the addition of 2 volumes of ethanol at —20° overnight. Finally, the RNA was dissolved in SSC containing 5 μg/ml of polyvinyl sulfate.

The intermediate and phenol layers were extracted again with the detergent mixture, and the protein was precipitated by addition of 3 volumes of methanol. The protein was washed 5 times with methanol and once with ether and was dried and dissolved in 0.5 ml of 10% triethylammonium hydroxide to which Scintisol was added for counting.

Quantitation and Radioactivity Assays. The DNA and RNA were determined quantitatively by absorbance at 260 μM and by the diphenylamine reaction for DNA (9) and the orcinol reaction for RNA (38). The ratio of absorbance at 260 μM to that at 280 μM was usually 1.9:2.1 for DNA and 2.0:2.3 for RNA. No protein could be detected in the samples of DNA and RNA. Protein was determined by the procedure of Lowry et al. (31) with a standard of bovine serum albumin. The
radioactivity of the solutions of RNA and protein was counted in Scintisol in a Packard Tri-Carb liquid scintillation counter. DNA was counted after digestion with 50 µg/ml of DNase for 30 min at 37°C in the counting vial. All samples were counted for a sufficiently long time so that those with the lowest radioactivity were accurate to ±7.5%.

Preparative CsCl Density Equilibrium Centrifugation. The initial CsCl (Pierce Chemical Co., Rockford, Ill.) solution, containing 0.15 M NaCl solution, 0.015 M sodium citrate, and an appropriate amount of DNA, was adjusted to a density of 1.700 as determined from its refractive index. The density equilibrium was attained by centrifugation at 38,000 rpm (140,000 × g) in an SB-405 rotor of an International Model B-6 (International Equipment Co., Needham Heights, Mass.) ultracentrifuge for 48 hr at 20°C. After centrifugation, 8-drop fractions were collected from the bottom of the tube, and the absorbance at 260 nm was read. The radioactivity was counted after precipitation of DNA with cold 5% TCA to avoid abnormal quenching by CsCl (21).

Sucrose-Gradient Centrifugation of RNA. The sedimentation constants of RNA species were determined by centrifugation in 15 to 30% (w/w) sucrose (RNase-free, Schwarz/Mann, Orangeburg, N. Y.) gradients in 5 mM Tris-HCl, pH 7.3, and 0.1 M NaCl for 18 hr at 28,000 rpm in a SW-25.1 rotor of a Spinco Model L ultracentrifuge (35). All gradients were collected from the bottom of the tube in 40-drop fractions, and the absorbance at 260 nm was measured. After addition of carrier RNA and protein, the material precipitable with 5% TCA was centrifuged, dissolved in 0.5 ml of formic acid, and counted in Scintisol. The sedimentation constants were determined by comparison with the cytoplasmic RNA species of HeLa cells (35).

RESULTS

When prostate cells were treated with tritiated MCA for 24 hr, approximately 3% of the compound that was added in the medium was incorporated into the cells (Table 2), of which 99.7% was extracted with alcohol; only 0.3% of the incorporated radioactivity (0.01% of that added) was found in the alcohol-insoluble fraction of the cells. Since the hydrocarbon is readily soluble in alcohol, the radioactivity in the alcohol-insoluble fraction presumably represents covalent, rather than physical, binding of the compound or a metabolite to the cellular constituents since physical binding is completely disrupted by extraction with organic solvents (4, 34, 44). After isolation and purification of the proteins and nucleic acids, most of the bound carcinogen was associated with proteins. As shown in Table 3, when MCA was added to G23 cells for 24 hr, 96% of the radioactivity was found in the protein fraction, while 3 and 1% were found in the RNA and DNA fractions, respectively. However, the specific activities of binding of MCA (µmole/mg) to the macromolecules were almost the same in the DNA, RNA, and protein fractions. Table 4 shows the recovery of radioactivity in the acid-insoluble fraction after enzymatic digestion and denaturation of the DNA samples. The radioactivity was not released by incubation with RNase and Pronase, whereas treatment with DNase resulted in complete loss of radioactivity. Heat and alkaline denaturation of DNA did not cause any significant release of radioactivity into acid-insoluble form. Thus, the conversion of labeled DNA from a double-stranded to a single-stranded form did not release radioactive material; this eliminates intercalation as the explanation for this binding.

The DNA sample that was labeled with MCA-3H was analyzed by CsCl equilibrium density gradient centrifugation. As shown in Chart 1, the radioactivity was completely associated with the absorbance profile of DNA at 260 nm. The specific activity at the peak was 9.5 µmole/mg, which is very close to that obtained before centrifugation (9.9 µmole/mg). All these results clearly demonstrate that the carcinogen was...
Hydrocarbon Binding to DNA, RNA, and Protein

Chart 1. Cesium chloride density gradient centrifugation of DNA from PR prostate cells treated with $4 \times 10^{-6}$ M MCA-$^3$H for 24 hr. The DNA was isolated and purified as described in "Materials and Methods" and centrifuged in a solution of CsCl (1.700 g/ml), 0.15 M NaCl, and 0.015 M sodium citrate for 48 hr at 38,000 rpm (140,000 $\times g$) in an SB-405 rotor of an International R-60 ultracentrifuge. Fractions (8 drops) were collected from the bottom, and the absorbance at $A_{260}$ was read. Radioactivity was counted after precipitation of DNA with 5% TCA.

Table 5
Recovery of acid-insoluble radioactivity after enzymatic digestion of RNA

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>None$^a$</td>
<td>100</td>
</tr>
<tr>
<td>DNase, 50 $\mu$g/ml</td>
<td>91</td>
</tr>
<tr>
<td>RNase, 50 $\mu$g/ml</td>
<td>8</td>
</tr>
<tr>
<td>Pronase, 50 $\mu$g/ml</td>
<td>97</td>
</tr>
</tbody>
</table>

$^a$ RNA solution (0.60 $A_{260}$) was incubated with the enzymes. Experimental conditions were identical to those described in Table 4.

bound to DNA and not to RNA or protein, which might have been present as contaminants. Similarly, a typical RNA sample was proved by enzymatic digestion to be free of contamination by DNA and protein (Table 5). The labeled RNA was fractionated by sucrose gradient centrifugation (Chart 2). This yielded 3 fractions of RNA's with sedimentation coefficients of 28, 18, and 4 S, corresponding to tRNA's and rRNA. The radioactivity was approximately proportional to the UV absorption throughout the fractions.

Extent and Time Course of Binding of Hydrocarbons to the Macromolecules. Charts 3 and 4 show the time course of binding of the different hydrocarbons to DNA, RNA, and proteins of the prostate G23 cells and hamster embryonic cells, respectively. The tritiated hydrocarbons were added to exponentially growing cells on an equimolar basis of $4 \times 10^{-6}$ M (approximately 1 $\mu$g/ml) and were left in the medium throughout the experiment. The extent of binding always increased with time of cultivation, although the hydrocarbons were bound more extensively to hamster embryonic cells than to G23 cells during the 1st 6 hr.

The extent of binding of MCA to 3 types of transformable cells in culture was compared with the previous findings obtained after application to mouse skins (21). In all cases, MCA was added for 24 hr at a concentration at which transformation or tumors can be induced. As summarized in Table 6, there was no significant difference in the binding to 2 embryonic cell types and mouse skin, although there was 30 to 50% less binding to the macromolecules of G23 cells than to mouse embryonic cells. The specific activity of 3.3 $\mu$moles/mg

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Chart 4. Time course of binding of a series of 5 hydrocarbons to DNA, RNA, and protein from hamster embryonic cells. The exponentially growing secondary cultures were treated with \(4 \times 10^{-6}\) M labeled compound for 3, 6, or 24 hr, and the DNA, RNA, and protein were isolated.

Table 6

<table>
<thead>
<tr>
<th>Specific activity ((\mu)moles/mg)</th>
<th>Mouse skin</th>
<th>Mouse embryo</th>
<th>Hamster embryo</th>
<th>G23</th>
<th>DNA</th>
<th>RNA</th>
<th>Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
<td>28</td>
<td>28</td>
<td>29</td>
<td>15</td>
<td>1.7</td>
<td>1.8</td>
<td>4.6</td>
</tr>
<tr>
<td>RNA</td>
<td>20</td>
<td>17</td>
<td>24</td>
<td>17</td>
<td>1.5</td>
<td>0.9</td>
<td>2.2</td>
</tr>
<tr>
<td>Protein</td>
<td>36</td>
<td>25</td>
<td>60</td>
<td>19</td>
<td>1.2</td>
<td>1.7</td>
<td>2.1</td>
</tr>
</tbody>
</table>

\(a\) The values obtained by Goshman and Heidelberger (21) 24 hr after 100 \(\mu\)g of MCA-\(^3\)H were painted on mouse skin.

\(b\) At 24 hr after treatment of exponentially growing cells with \(1 \times 10^{-6}\) M MCA-\(^3\)H (approximately 1.0 \(\mu\)g/ml).

Table 7

<table>
<thead>
<tr>
<th>Specific activity ((\mu)moles/mg)</th>
<th>DNA</th>
<th>RNA</th>
<th>Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMBA</td>
<td>112</td>
<td>42</td>
<td>39</td>
</tr>
<tr>
<td>MCA</td>
<td>28</td>
<td>17</td>
<td>25</td>
</tr>
<tr>
<td>BP</td>
<td>19</td>
<td>28</td>
<td>123</td>
</tr>
<tr>
<td>DB[ac]A</td>
<td>7.7</td>
<td>7.7</td>
<td>61</td>
</tr>
<tr>
<td>DBA</td>
<td>2.3</td>
<td>4.4</td>
<td>11</td>
</tr>
</tbody>
</table>

DNA obtained with mouse skin and embryonic cells corresponds to 12 \(\mu\)moles/mole phosphorus of DNA (calculated on the basis that DNA molecules contain 8\% of phosphorus) or to 1 mole of carcinogen per \(10^8\) nucleotides [calculation made on the assumption that 20,000 nucleotides give a molecular weight of DNA of about \(6.4 \times 10^6\) (6)].

The distribution of bound carcinogen among the 3 cellular constituents differed markedly for the individual hydrocarbons. (Charts 3 and 4 and Table 7). For example, BP was bound preferentially to proteins, while DMBA was bound most to nucleic acids. The 2nd highest binding to protein was obtained with the weakly carcinogenic DB[ac]A (Table 7). BP and MCA were bound to the RNA with specific activities about one-half of that of DMBA; to the DNA, BP and MCA were bound to about one-fourth the extent of DMBA. In all cells, the extent of binding of the weakly carcinogenic DB[ac]A to all macromolecules was greater than that of the isomeric and more carcinogenic DBA. The relevance of this observation to the mechanism of carcinogenesis is not clear.

**Binding of MCA to Transformed and Revertant Cells.** Table 8 shows the binding of MCA to the DNA, RNA, and proteins of chemically and spontaneously transformed mouse prostate cells. The transformed cells bound 5 to 10 times less MCA than did their parental nontransformed cells (Table 8). There was no significant difference in binding between spontaneously and chemically transformed cells. This is in good agreement with the observations of Diamond et al. (13). In the less malignant variant 4C1, F1, C and RR9B cells, which were obtained from highly malignant 4C cells by treatment with 5-fluoro-2'-deoxyuridine and by plating on glutaraldehyde-fixed cells, respectively, the extent of binding was approximately two-thirds that of nontransformed cells (Table 9). These cells are about 500-fold less malignant than...
the clone from which they were derived (S. Mondal, M. J. Embleton, H. Marquardt, and C. Heidelberger, Intern. J. Cancer, in press).

Comparison of Binding of MCA to Macromolecules of Exponentially Growing and Nongrowing Cells. It was reported from this laboratory (12) and by Berwald and Sachs (3) that no transformation was obtained when hydrocarbons were applied to a confluent sheet of nongrowing cells. To investigate the reason for this, we measured the binding of MCA to macromolecules in exponentially growing and in nongrowing cells, which were obtained 3 or 4 days after a confluent sheet was formed and in which no mitotic figures were detected. Conditioned medium containing MCA-3H was used in order to avoid the induction of DNA synthesis by addition of fresh medium. As shown in Table 10, there was no significant difference between exponentially growing and nongrowing cells in the binding of MCA to nucleic acids and proteins.

Persistent Binding of the Hydrocarbon to Macromolecules. The stability of binding of the hydrocarbons to the macromolecules was investigated. The prostate cells (PR cells) in exponentially growing phase were treated for 24 hr with 4 X 10^-6 M MCA-3H, then washed twice with fresh medium, and kept for a period up to 5 weeks with twice-weekly changes of medium. The extent of binding was measured at 2 and 3 days and 1, 3, and 5 weeks after the treatment. As shown in Chart 5, the specific activities dropped during the 1st 2 days and then, after the cells reached saturation density, they remained rather constant for 5 weeks. Such persistent binding has also been observed during hepatocarcinogenesis, where relatively little cellular proliferation takes place (17, 42, 45).

The following experiment was undertaken to investigate the relationship between cell growth and stability of binding. Exponentially growing prostate cells were double-labeled with MCA-3H and TdR-14C, and were transferred. Thereafter, the growth of the cells (○) and radioactivity from TdR-14C (○) and from MCA-3H (●) were measured in the alcohol-insoluble fraction.

Table 10
Comparison of binding of MCA-3H to macromolecules of exponentially growing and nongrowing PR cells after treatment with 4 X 10^-6 M MCA-3H for 24 hr

<table>
<thead>
<tr>
<th>Growth phase</th>
<th>DNA (μmoles/mg)</th>
<th>RNA (μmoles/mg)</th>
<th>Protein (μmoles/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Growing</td>
<td>7.4</td>
<td>18</td>
<td>10</td>
</tr>
<tr>
<td>Nongrowing</td>
<td>12</td>
<td>30</td>
<td>14</td>
</tr>
<tr>
<td>Experiment 2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Growing</td>
<td>12</td>
<td>20</td>
<td>18</td>
</tr>
<tr>
<td>Nongrowing</td>
<td>9.9</td>
<td>17</td>
<td>20</td>
</tr>
</tbody>
</table>

Chart 6. Correlation between cell growth and stability of binding. The prostate cells in exponentially growing phase were double-labeled with MCA-3H and TdR-14C, and were transferred. Thereafter, the growth of the cells (○) and radioactivity from TdR-14C (○) and from MCA-3H (●) were measured in the alcohol-insoluble fraction.
DISCUSSION

The binding of carcinogenic hydrocarbons to nucleic acids and proteins has been demonstrated first in animals (1, 6–8, 21, 24, 32, 43, 46) and then in tissue culture systems (2, 7, 13, 15, 16, 47, 48). The present work has been carried out mostly with fibroblast cells derived from mouse prostate, which can be transformed to cancer under the conditions of these experiments (11, 12).

At least 2 mechanisms are involved in the interaction of the hydrocarbons with the cellular constituents. The 1st is the weak physical interaction with DNA molecules as demonstrated by Ts'o and Lu (44). Boyland and Green (4), and Nagata et al. (34). Frank (18) reported a similar weak hydrophobic interaction of polycyclic hydrocarbons with protein. The 2nd mechanism is a firm, covalent binding of the compound to the macromolecules. The type of binding is generally considered to be more important for the biological phenomena of carcinogenesis and mutagenesis. The interactions described here appear to be a covalent, rather than physical, binding of the compounds to macromolecules, since each fraction was extracted repeatedly with organic solvents. The fact that the binding of hydrocarbons to DNA is resistant to heat or alkaline denaturation eliminates intercalation and suggests that the binding may be directly to nucleotides. The extent of this firm binding is almost the same among different target cells in vivo and in vitro.

Considerable discussion has been devoted to the question of whether or not there is any correlation between carcinogenic potency and the binding of carcinogens to cellular constituents. Heidelberger and Moldenhauer (24) reported in 1956 such a quantitative correlation between the extent of binding of hydrocarbons to the soluble proteins of mouse skin in vivo and carcinogenic activity, except that DB[a]A was bound extensively. Brookes and Lawley (8) have found a good correlation between carcinogenicity, which they expressed as the lbill index, and the extent of covalent binding to the DNA in a series of 6 hydrocarbons. Goshman and Heidelberger (21) pointed out 2 instances in which binding of a hydrocarbon to DNA was not correlated with a carcinogenic event, i.e., the finding that the weakly carcinogenic DB[a]A was bound to a greater extent than was its more carcinogenic isomer DBA and that the binding of hydrocarbons to epidermal DNA was almost identical in susceptible Swiss mice and in hairless mice that are not susceptible to carcinogenesis (20). The former finding is confirmed here in tissue culture.

Recently, Duncan et al. (16) reported that the “binding index” of a hydrocarbon (the amount bound divided by the amount metabolized) is more closely related to its carcinogenic activity than is the binding itself. However, we could not find a significant correlation between the binding index (as defined above) and carcinogenic activity in a series of 5 hydrocarbons (the present work plus Ref. 26). This is probably because in this laboratory the production of water-soluble metabolites is determined directly after solubilization of the cells by detergent (26), whereas Duncan et al. (16) measured metabolism indirectly.

Grover and Sims (22) and Gelboin (19) have independently demonstrated that carcinogenic hydrocarbons that are not themselves chemically reactive are metabolically activated by microsomal enzyme(s) to some form(s) capable of binding firmly to DNA. In this laboratory, Huberman et al. (26) have examined the metabolic activity of the same cells under conditions identical to those of this work. We have found a good correlation between metabolic activity and the extent of binding of polycyclic hydrocarbons in the following respects. The binding is greater to macromolecules obtained from cells with a high rate of metabolism (for example, embryonic cells) than to those of cells with a slow metabolic rate, such as transformed cells; and the order of metabolic activity among different hydrocarbons is identical with that of their binding to the protein fraction, to which most of the hydrocarbon is bound. These results also suggest that the metabolic conversion of hydrocarbons must be a prerequisite to their covalent binding to cellular constituents. A recent investigation in this laboratory (39) has confirmed experimentally the original suggestion of Boyland and Sims (5) that an epoxide is formed as an intermediate in the metabolism of DBA, and we have also demonstrated that K-region epoxides are more active than hydrocarbons, dihydrodiols, and phenols in the production of malignant transformation both in hamster embryonic and in mouse prostate cells (23). The binding to macromolecules of the epoxides and other metabolites of carcinogenic hydrocarbons is currently under investigation.

Although hydrocarbons were bound much less to the DNA, RNA, and proteins of transformed than of nontransformed cells, the binding to less malignant variant cells derived from highly malignant clones is almost as great as to nontransformed cells. A plausible explanation is the restoration of metabolic activity, but the possibility that some change in the target constituents takes place after loss of malignancy should also be considered.

There has been no agreement concerning the preferred state of cells in which the carcinogens are incorporated and bound. Süss et al. (41) found a significant decrease in the binding of BP and DMBA to DNA in mouse skin during the period of inhibition of DNA synthesis produced by hydroxyurea. Alfred and DiPaolo (2) reported that the weakly carcinogenic benz[a]anthracene reached a maximum binding to hamster embryonic cell DNA during the 1st 24 hr after resumption of DNA synthesis that had been blocked by excess thymidine. Similarly, Marquadt et al. (32) found a 4-fold increase in the binding of hydrocarbons to DNA of regenerating as compared with normal liver 24 hr after partial hepatectomy. On the contrary, Yuspa et al. (47, 48) demonstrated clearly in tissue culture that the binding of DMBA and benz[a]anthracene was reproducibly greater to the DNA of nonreplicating cells than that of replicating cells. It was reported by Kuroki et al. (29), using specific inhibitors of macromolecular synthesis and synchronous cultures of hamster embryonic cells, that the binding of 4-nitroquinoline-1-oxide-14C was not affected by the progress either of macromolecular synthesis or of the cell cycle. These latter findings are in accord with the present investigation. Thus, the lack of transformation in confluent sheets of cells (3, 12) cannot be explained on the basis of lack of binding of the carcinogens to macromolecules.
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The Binding of Polycyclic Aromatic Hydrocarbons to the DNA, RNA, and Proteins of Transformable Cells in Culture

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