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Isolation and Degradation of DNA from Cells Treated with Tritium-labeled 7,12-Dimethylbenz(a)anthracene: Studies on the Nature of the Binding of This Carcinogen to DNA[1]

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SUMMARY

In an attempt to obtain a source of material for chemical studies, the binding of 7,12-dimethylbenz(a)anthracene (DMBA) was studied in *E. coli*, *B. subtilis*, and ascites tumor cells. For the bacteria a dose-dependent binding was found, but the level of binding was low. In the case of the ascites tumor cells in the mouse, binding of DMBA to DNA, RNA, and protein showed a dependence on time after injection of the hydrocarbon, but again the level of binding was much less than that found earlier for mouse skin. Mouse L-cells and chicken fibroblasts in tissue culture again gave a low level of DMBA binding, but rodent embryo cells in culture, as reported earlier, were a good source of DNA having bound DMBA. Enzymic degradation of this material and subsequent fractionation suggested that the DMBA was bound to the bases of DNA, resulting in partial inhibition of the enzymic degradation of the DNA. A similar partial inhibition was observed for DNA alkylated with half-sulphur mustard but not for methylated DNA. The low level of *in vivo*, binding obtained suggests that alternative methods will be needed to establish both the nature of the bound hydrocarbon moiety and the base at which it is attached.

INTRODUCTION

The first demonstration of carcinogenicity by a pure chemical was that of Kennaway (13) who showed that dibenz(a,h)anthracene induced tumors at the site of application to mouse skin. Subsequently many other polycyclic aromatic hydrocarbons were found to possess this property which, it was generally assumed, resulted from interaction of the hydrocarbon with some cellular constituent.

The finding of hydrocarbon bound to soluble proteins of mouse skin, and the possible significance of these results, has been summarized by Heidelberger (11), and preliminary evidence for the binding of dibenz(a,h)anthracene to mouse skin DNA was reported by Heidelberger and Davenport (12). More definite evidence for and the possible significance of binding to the DNA and RNA of mouse skin was reported by Brookes and Lawley (4, 5). In a recent review, Brookes (3) discussed briefly his reason for favoring DNA rather than protein as the critical site for reaction of most chemical carcinogens including the hydrocarbons.

A problem that arises in any attempt to investigate the chemistry of the *in vivo* binding of hydrocarbons to cellular constituents is a source of material. In the earlier studies (1, 4), mouse skin was used, but extraction of the required cellular constituents from this tissue was somewhat tedious. Furthermore, it was necessary to apply a considerable quantity of hydrocarbon to each mouse to get a detectable extent of intracellular binding, and the yield of nucleic acids was poor.

In an attempt to find an alternative source of starting material, ascites tumors in mice and bacterial cells were used, but the most satisfactory system was found to be rodent embryo cells in tissue culture (7). DNA from this source was used in all degradation and fractionation studies reported here.

The studies on the binding of hydrocarbons to DNA in mouse skin (4, 8) have shown DMBA[4] to be most readily bound; this hydrocarbon was also the most toxic towards cells in culture (7). It was therefore used in the present studies.

The choice of DNA rather than RNA or protein for degradative study was made because of its easier isolation in a pure state and of its likely significance in the biologic properties of the hydrocarbons.

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[2]Recipient during part of this work of an Eleanor Roosevelt International Cancer Fellowship of the American Cancer Society awarded by the International Union Against Cancer.

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[4]The following abbreviations are used: DMBA, 7,12-dimethylbenz(a)anthracene; DMSO, dimethylsulfoxide; EDTA, ethylenediaminetetraacetate.

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MATERIALS AND METHODS

Labeled Materials

Generally labeled DMBA-^{3}H (sp. act. 9–21 c/mmole), 2-chloroethyl-2-hydroxyethyl sulfide-^{35}S (sp. act. 0.6 c/mmole), and methanesulphonate-methyl-^{14}C (sp. act. 28.1 mc/mmole) were supplied by The Radiochemical Centre, Amersham, England.

DMBA-^{3}H Binding to Bacterial Cells

E. coli B/r and *B. subtilis* uvr$^+$ ind$^-$ were grown overnight in M9 medium supplemented with 0.5% casamino acids (in addition, 10 µg/ml tryptophan was added to the *B. subtilis* medium) to approx. 2 × 10^9 cells/ml. The cells were diluted 1:100 into fresh medium and aerated. Under these conditions the division time for both organisms was about 35–40 min, and the exponential phase of growth continued for at least 2 hr.

The log phase cells were treated by adding the required amount of DMBA-^{3}H in a volume of DMSO such as to give a final DMSO concentration of 1%. After a further 2-hr growth, the cells were harvested, washed by resuspension in M9 medium, and again pelleted.

For EDTA treatment of *E. coli*, cells in early log phase were harvested, washed with 0.12 M Tris-chloride, pH 8.0, resuspended in this buffer at approximately 5 × 10^9 cells/ml, and EDTA was added to a concentration of 2 × 10^{-4} or 10^{-3} M. After incubation at 37°C for 3 min, the cells were diluted 100-fold into growth medium and treated as for control cells.

Cell growth was followed by measurement of the absorbance at 590 mµ and by colony assay after appropriate dilution.

Spheroplasts were prepared from *E. coli* essentially as described by Guthrie and Sinsheimer (9). They were treated in suspension with DMBA-^{3}H and incubated for 2 hr.

DMBA-^{3}H Binding to Ascites Cells in Mice

Twelve Swiss mice were injected intraperitoneally with approximately 10^6 Ehrlich ascites tumor cells. After 6 days the mice were injected i.p. with 0.2 ml DMSO containing 215 µc (i.e., 2.6 µg) of DMBA-^{3}H. Two mice were killed immediately after the injection, and after 2, 5.5, 19, 23, and 48 hr, the ascitic fluid was drained and the tumor cells isolated. DNA, RNA, and protein were isolated as described below.

In other experiments mice bearing the ascites tumor received various doses of DMBA-^{3}H, and cellular constituents were isolated from the tumor cells after 12 hr.

DMBA-^{3}H Binding to Cells in Tissue Culture

Mouse embryo cells were initiated by trypsinization of 15- to 17-day-old embryos. Secondary cultures growing in 14-cm plastic tissue culture dishes (Falcon Plastics) were treated by replacing the medium (Eagle's basal medium + 10% calf serum) with fresh medium containing the required concentration of DMBA-^{3}H and 0.5% DMSO.

Mouse L-cells and chicken fibroblasts (kindly provided by Drs. R. R. Reuckert and H. M. Temin, McArdle Lab.) were treated similarly.

After 24 hr the cells were harvested with trypsin, washed with fresh medium, and the cellular constituents isolated as described below.

Isolation of Cellular Constituents

DNA, RNA, and total proteins were isolated from *E. coli* as described previously (16). *B. subtilis* was suspended in 5% sodium *p*-aminosalicylate (pH 7.0) and lysed by the addition of 0.5 µg/ml lysozyme before proceeding as for *E. coli.*

Ascites tumor cells and mouse embryo cells were treated as described previously (7). Detailed analyses of nucleic acid isolated by these procedures have been reported (4).

Radioactive Assays

^{14}C- and ^{3}H-labeled DNA, RNA, and proteins were assayed in aqueous solution by liquid scintillation methods as outlined previously (7).

Alkylation of DNA

To 4 ml of a 2-mg/ml solution of salmon sperm DNA (Mann Research Labs) in 0.1 M sodium acetate at 37°C, was added 0.01 ml of an ether solution of 2-chloroethyl-2-hydroxyethyl sulfide-^{35}S (5 mg/ml; 57 µc/mg). After 15 min the DNA was precipitated with 2 volumes of ethanol, washed with ethanol and ether, and dried. The DNA had a sp. act. of 125 µc/gm, corresponding to an extent of reaction of 5.2 mmoles/mole P. In a similar experiment, DNA (8 mg) was alkylated with methanesulphonate-methyl-^{14}C (2 mg; 255 µc/mg) in 0.2 M acetate. The isolated DNA had a sp. act. of 1227 µc/gm, corresponding to an extent of reaction of 14.8 mmoles/mole P.

Degradation and Column Chromatography of Labeled DNA

The DNA (either DMBA-DNA or alkylated DNA) was treated in 0.01 M magnesium acetate (pH 7.0) at a concentration of 1 mg/ml with deoxyribonuclease (Worthington), 15 µg/ml, at 37°C for 1 hr, before raising the pH to approximately 8.5 and adding 25 µg/ml of snake venom diesterase (Worthington). Incubation was continued for 2.5–48 hr.

The DNA digest was applied to a column (5 x 1.5 cm) of DE-23 cellulose (Whatman), precycled as recommended, and finally made up in 7 M urea:0.02 M Tris-chloride, pH 7.5. This column was developed with a linear gradient of 0–0.3 M sodium chloride in 7 M urea; 0.02 M Tris buffer (500 ml total volume); 5- to 6-ml fractions were collected automatically. The UV absorption at 260 mµ of the fractions was determined with a Unicam SP500 spectrophotometer; 0.1-ml samples were taken for liquid scintillation assay of radioactivity, using a Packard Tri Carb counter model 3375, with automatic external standardization.

In a typical experiment, the fractions of the radioactive peak immediately following the UV-absorbing material were combined, diluted with water, and reabsorbed on a DE-23 cellulose column; after washing with water, the product was eluted with 1 M triethylammonium bicarbonate, pH 8.0. After lyophilization the material remaining was dissolved in 1 ml of water. The

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solution had a radioactivity of 86 mμc/ml.

For monoesterase treatment of this solution, *E. coli* alkaline phosphatase (Worthington) was used (25 μg/ml).

DMBA-DNA (0.1 mg; 75 mμc) was treated with 0.02 ml of 70% perchloric acid at 100°C for 1 hr, 1 ml of water was added, and the total digest applied to a column (5.0 x 0.5 cm) of Dowex-50 ($^{+}$H). The column was developed with a linear gradient of 0.5–2.5 N hydrochloric acid (200 ml total volume). Fractions of approximately 5 ml were collected automatically and assayed for UV absorption and radioactivity.

DNA [0.75 mg; 200 mμc of DMBA-DNA (from mouse skin) + 5.25 mg of salmon sperm DNA] was degraded for 1 hr with deoxyribonuclease as described above, before raising the pH to 8.5 and adding 1 mg/ml of lyophilized *Crotalus adamanteus* venom (Calbiochem). During the next 3 hr, periodic additions of 0.1 N sodium hydroxide were made to keep the pH in the range 8–8.7.

The mixture of nucleosides was fractionated on a column of Celite 545 (11.5 x 1.2 cm) using the procedures of Hall (10) with the solvent system ethyl acetate:2-ethoxyethanol:2% formic acid (4:1:2, v/v/v). The fractions containing the nucleosides, identified by their UV absorption, were combined and concentrated, and their radioactivity was measured.

Paper Chromatography and Electrophoresis

Isobutyric acid:ammonia:water (66:2:32, v/v/v) was used with Whatman No. 1 paper, descending.

Electrophoresis employed a simple apparatus (Made by Dr. S. Lappla, Biochemistry Department, University of Wisconsin) in which the paper, supported on a plastic frame, was cooled by immersion in an organic solvent, and had its ends dipping into the buffer compartments. The buffer used was 7 M urea: 0.025 M Tris phosphate, pH 7.0.

RESULTS

Hydrocarbon Binding in Bacterial Cells

Preliminary studies had failed to show any toxic effect of DMBA towards *E. coli*. The report by Lieve (17) that brief pretreatment with 2×10^{-4}M EDTA resulted in sensitivity of *E. coli* to actinomycin D suggested the use of this procedure with DMBA. However, when the binding of DMBA-^{3}H to DNA, RNA, and protein of cells pretreated with EDTA was compared with that to untreated cells, no significant difference was observed (Table 1), even when the EDTA concentration was increased to 10^{-3} M. Furthermore, in other experiments (not reported here in detail) no toxicity of hydrocarbon towards EDTA-treated cells was observed. The variation of extent of reaction with the dose of DMBA, for both *E. coli* B/r and *B. subtilis*, is shown in Table 1, as also is the binding data obtained with protoplasts of *E. coli*.

Hydrocarbon Binding by Ascites Cells

The extent of reaction of DMBA with DNA, RNA, and protein of ascites cells in mice at various times after injection, expressed as the specific activity of the isolated materials, is shown in Chart 1. Each mouse yielded approximately 10 mg of ascites cell DNA. The extent of binding at various dose levels of DMBA is shown in Table 2.

Hydrocarbon Binding by Cells in Tissue Culture

The binding of DMBA-^{3}H to normal and virus-transformed rodent and mammalian cells in culture has been reported previously (7). The DNA with bound DMBA (DMBA-DNA) used in this study was obtained by treatment of secondary mouse embryo cells. The binding data for these cells in comparison with mouse L-cells and chicken fibroblasts are shown in Table 3.

Fractionation of Hydrolyzed DNA

Chart 2 shows the column chromatography of a perchloric acid digest of DMBA-DNA. Essentially all the applied radioactivity passed directly through the column and none was present in the four bases.

When DMBA-DNA was enzymically degraded to nucleosides and fractionated on Celite as described by Hall (10), about

Table 1

Bacterial species	Dose of DMBA-^{3}H (μg/ml)	Extent of reaction with DNA (μmoles/mole P)	RNA (μmoles/mole P)	Protein (μmoles/100 gm)
E. coli B/r after pretreatment with 10^{-3} M EDTA	0.012	0.030	0.020	0.013
E. coli B/r	0.012	0.028	0.031	0.013
E. coli B/r	1.0	0.64	0.85	0.16
E. coli B/r	10.0	9.8	9.8	3.6
B. subtilis	0.024	0.055	0.033	
B. subtilis	1.0	1.07	0.98	0.56
B. subtilis	10.0	15.0	9.5	7.1
E. coli Protoplasts	0.024	0.006	0.003	0.025

Extent of reaction of DMBA-^{3}H with cellular constituents of *E. coli* B/r and *B. subtilis*. EDTA, ethylenediaminetetraacetate; DMBA, 7,12-dimethylbenz(a)anthracene.

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Table 2

Dose of DMBA-^{3}H (μg/mouse)	Extent of reaction with DNA (μmoles/mole P)	RNA (μmoles/mole P)
2.4	0.13	0.011
24	0.58	0.12
200	1.78	0.55

Extent of reaction of DMBA-^{3}H with the DNA and protein isolated from Ehrlich ascites cells 12 hr after i.p. injection of tumor-bearing mice with various doses of the labeled hydrocarbon. DMBA, 7,12-dimethylbenz(a)anthracene.

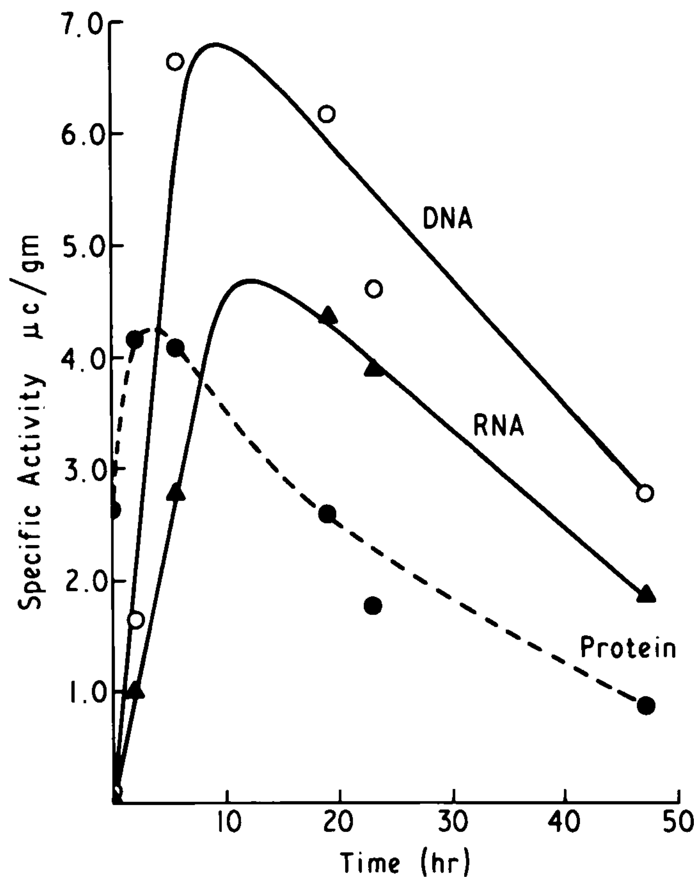

Chart 1. The extent of reaction of 7,12-dimethylbenz(a)anthracene, as measured by the specific radioactivity of the isolated cellular macromolecules of Ehrlich ascites tumor cells in mice, at various times after intraperitoneal injection of the labeled hydrocarbon.

30% of the applied radioactivity came off the column in the first few fractions and the rest was not recovered. The isolated purine and pyrimidine nucleosides were free of activity.

Degradation of DMBA-DNA with deoxyribonuclease plus purified snake venom diesterase for various periods of time, and subsequent fractionation on DEAE-cellulose with a 7 M urea:salt gradient, gave the results shown in Charts 3 and 4. Even the shortest enzyme treatment reduced the UV absorption to a single peak while the radioactivity was spread through many fractions.

Similar enzymic degradation and fractionation on DEAE-cellulose of DNA alkylated with the monofunctional alkylating agents 2-chloroethyl-2-hydroxyethyl sulfide-^{35}S, or methanesulphonate-methyl-^{14}C, gave the results shown in Charts 5 and 6.

Chromatography and Electrophoresis

The radioactive product eluted from the DE-23 column immediately after the UV-absorbing mononucleotide band was isolated. Its paper chromatographic and electrophoretic behavior and the changes produced by treatment with monoesterase and acid are shown in Chart 7.

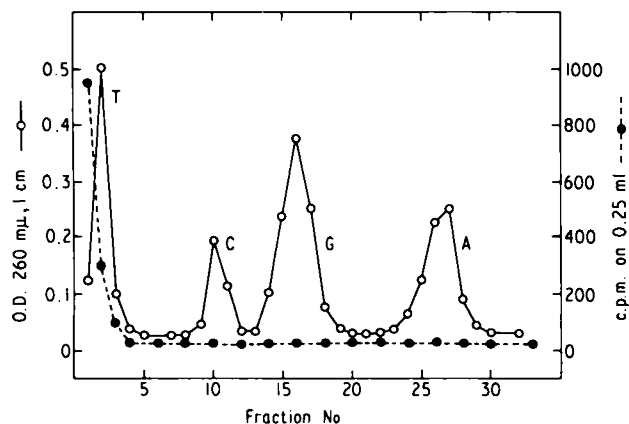

Chart 2. DNA labeled in mouse embryo cultures with tritiated 7,12-dimethylbenz(a)anthracene was degraded with 70% perchloric acid and fractionated on Dowex-50 ($^{+}$H) using a linear gradient of 0.5–2.5 N hydrochloric acid. The UV-absorbing peaks were identified by their spectra: T, thymine; C, cytosine; G, guanine; A, adenine. Radioactivity was determined on 0.25-ml samples of each fraction by liquid scintillation methods.

Table 3

Type of cell	Dose (μg/ml)	Extent of reaction with DNA (μmoles/mole P)	RNA (μmoles/mole P)	Protein (μmole/100 gm)
Mouse L-cell	0.01	0.12	0.11	0.041
Chicken fibroblast	0.01	0.01		
Mouse embryo fibroblast	0.01	4.05		0.18

Extent of reaction of DMBA-^{3}H with cellular constituents of various cells in culture. DMBA, 7,12-dimethylbenz(a)anthracene.

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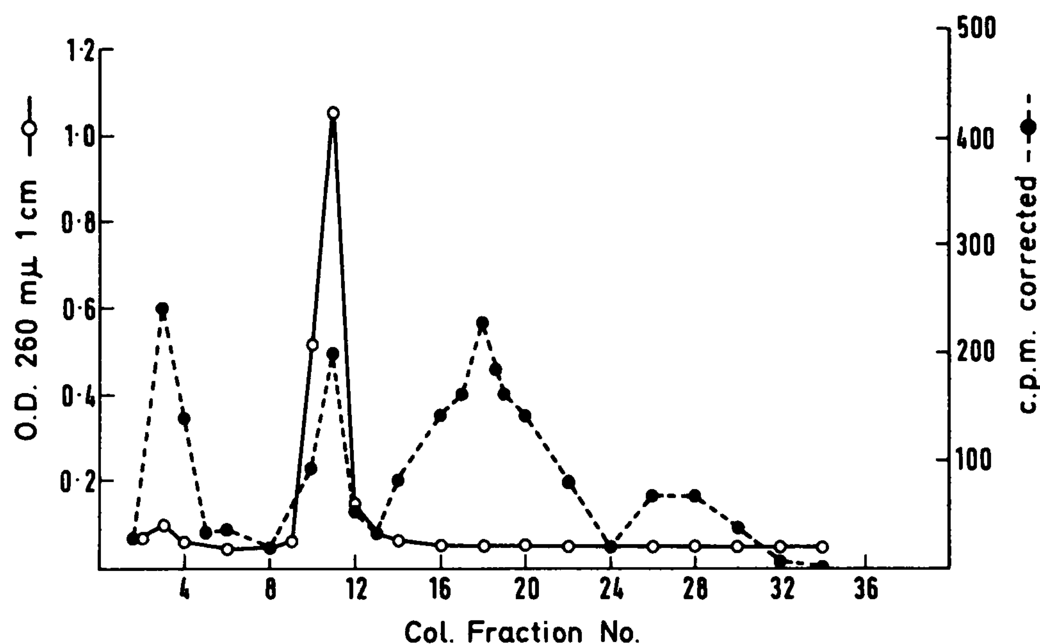

Chart 3. DNA labeled in mouse embryo cultures with tritiated 7,12-dimethylbenz(a)anthracene was degraded with DNase (3 hr) and snake venom diesterase (18 hr) and fractionated on DEAE-cellulose using a linear gradient of 0–0.3 M sodium chloride in 7 M urea: 0.02 M Tris-chloride, pH 7.5. Fractions (5–6 ml) were collected, the UV absorption at 260 mμ measured, and 0.1-ml samples taken for determination of radioactivity.

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DISCUSSION

The experiments reported earlier (4) on the binding of labeled carcinogenic hydrocarbons to mouse skin nucleic acids, which were subsequently confirmed (8), strongly suggested that a covalent interaction of a hydrocarbon derivative with DNA and RNA occurred within the cells exposed to the hydrocarbon.

There remains the problem of the nature of the reactive moiety. The hydrocarbons themselves are highly water-insoluble compounds devoid of any chemical reactivity towards cellular macromolecules, and it must be assumed that the binding to both protein and nucleic acids which has been established must involve a reactive metabolite. The possible importance of the phenanthrene double bond or "K" region in any such reactions has been proposed by the Pullmans (18), and evidence for this view in the case of dibenz(a,h)anthracene and protein was reported by Bhargava *et al.* (1). For the nucleic acids, the only criteria of hydrocarbon binding was the presence of radioactivity in the DNA and RNA which could not be removed by physical means. It might therefore be argued that this activity was due to degradation of the hydrocarbon and subsequent utilization of the tritium for normal cellular synthesis. This explanation seemed very unlikely, as discussed earlier (4), but had not been fully eliminated. The experiments now reported showed that the bases and nucleosides derived from DMBA-DNA were free of radioactivity and that, following perchloric acid degradation, the radioactivity passed straight through a Dowex-50 (^+H) column, possibly as a result of the degradation of the hydrocarbon moiety to water containing the tritium originally in the hydrocarbon. Enzymic degradation to nucleosides and subsequent column fractionation gave the four normal nucleosides free of radioactivity, and, in this case, the major part of the activity applied to the column was not eluted. This failure to recover all the radioactivity was not surprising, since early attempts at fractionation of the DMBA-containing product, both on columns and on paper, had indicated a tendency to absorb irreversibly.

The finding that DMBA was bound to DNA in mouse skin did not of itself prove that this binding had any biologic significance. The availability of mutants of *E. coli* known to respond differently to agents which react with their DNA suggested the use of this system with the hydrocarbons.

Since DMSO had been used effectively to keep hydrocarbons in solution for treatment of cells in tissue culture (6), it was used similarly here. Exponentially growing cultures of *E. coli* or *B. subtilis* were treated with up to 10 μg/ml of DMBA, but no toxic effect was observed. Higher concentrations of hydrocarbon could not be kept in solution even when 5% DMSO was used. Attempts to sensitize *E. coli* by brief pretreatment with 2×10^{-4} M EDTA, a procedure which induces sensitivity to actinomycin D (17), were not successful in this case.

When the binding to DNA of DMBA-^3H was measured, it was found that EDTA pretreatment did not increase binding (Table 1); therefore, it was omitted in subsequent experiments. The binding data obtained with *E. coli* B/r and *B. subtilis* indicated a dose dependence, but the extent of reaction, very similar for both organisms, was very low (Table 1). In view of the failure to observe toxicity of the hydrocarbon towards the bacteria used, it was of interest to note that, at the maximum dose of DMBA of 10 μg/ml, the level of DNA

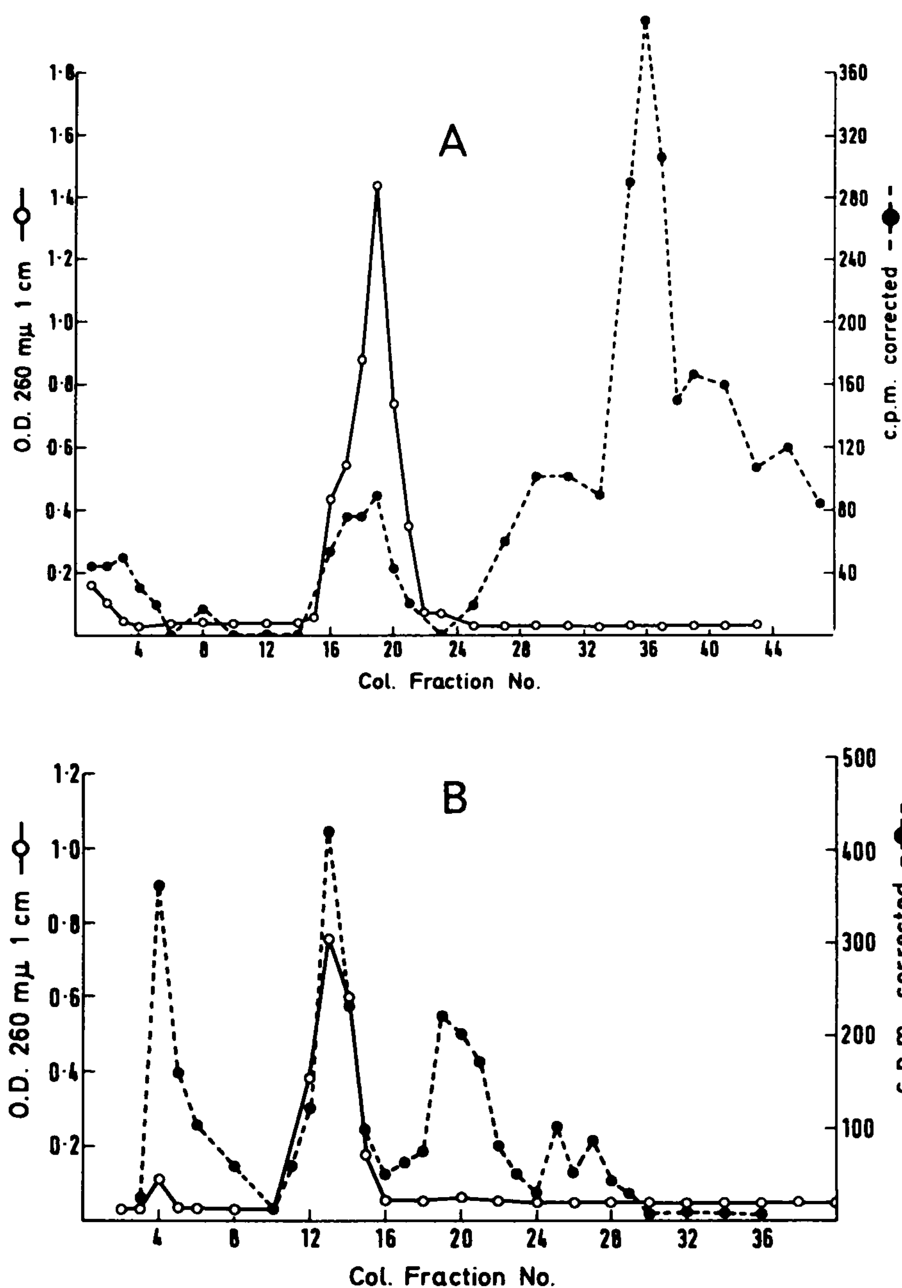

Chart 4. The same data as for Chart 3, except that degradation was: *A*, DNase (1 hr) and snake venom diesterase (2.5 hr), and *B*, DNase (1 hr) and snake venom diesterase (42 hr).

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binding was about 9.5 μmoles/mole P, whereas at the D37 dose of the monofunctional alkylating agent 2-chloroethyl-2-hydroxyethyl sulfide, Lawley (14) found the level of reaction to be of the order of 1000 μmoles/mole P. Although it is possible that other bacterial systems might give higher levels of hydrocarbon binding, other sources of material were thought to offer better possibilities.

The binding of DMBA to ascites cell DNA (Chart 1) was of interest in that a time dependence was observed, as with the mouse skin system. The ascites cells were a convenient source of DNA in quantity, but the efficiency of binding was low and, in the case of DNA, was not proportional to dose (Table 2).

Attention was directed to rodent embryo cells in tissue culture by the work of Diamond (6) in which the cytotoxicity of hydrocarbons was measured quantitatively. A study of DMBA-^{3}H binding by these cells suggested that there was a correlation between binding and cytotoxicity and showed that secondary mouse or hamster cells were an excellent source of DMBA-DNA (7). Since these cells can be grown only on a glass or plastic surface, the problem arose of obtaining a number sufficient to give enough DNA for chemical studies. As mouse

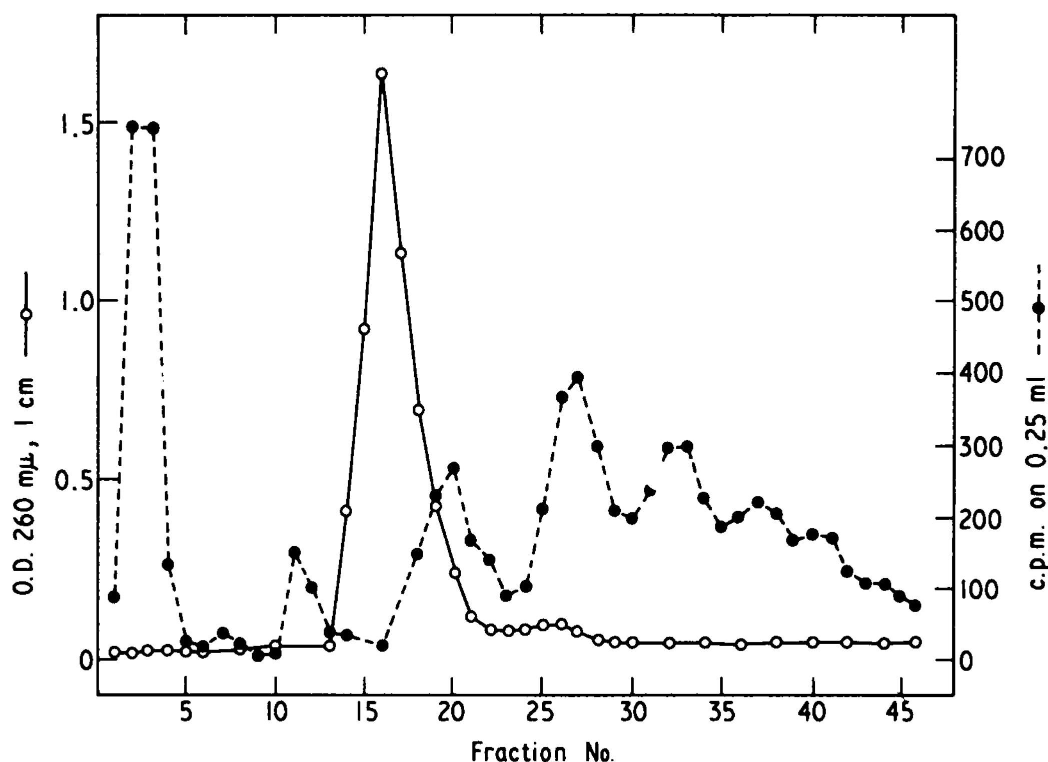

Chart 5. DNA alkylated *in vitro* with 2-chloroethyl-2-hydroxyethyl sulfide-^{35}S was degraded with DNase (1 hr) and snake venom diesterase (3.5 hr) and chromatographed on DE-23 cellulose in 7 M urea as described for Chart 3.

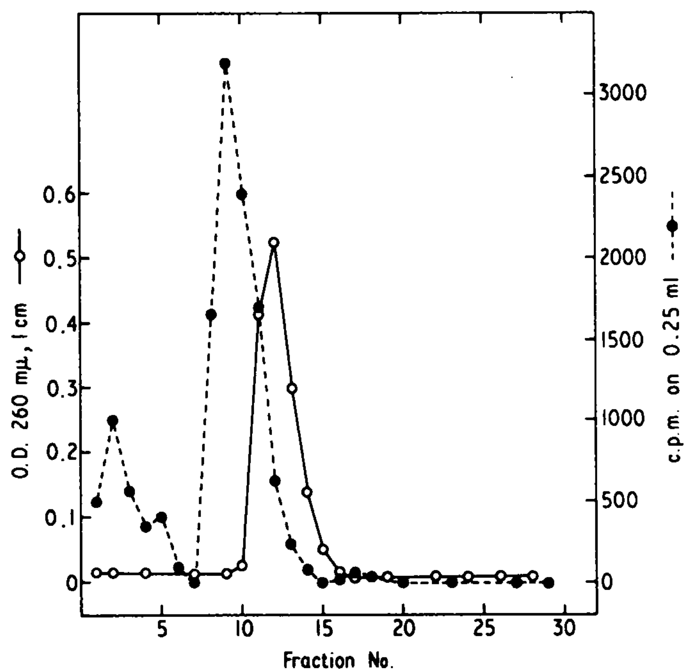

Chart 6. DNA methylated *in vitro* with methanesulphonate-methyl-^{14}C was degraded with DNase (1 hr) followed by snake venom diesterase (3.5 hr) and chromatographed on DE-23 cellulose in 7 M urea as described for Chart 3.

L-cells and chicken fibroblasts were more easily available in quantity, their ability to bind DMBA was studied, but as can be seen from Table 3, they were clearly inferior to mouse embryo cells in this respect. DNA from this latter source was therefore used in the subsequent chemical studies.

DMBA-DNA was enzymically degraded using conditions known to convert control DNA to mononucleotides. Since preliminary experiments had indicated that the radioactivity in such a digest was absorbed irreversibly to DEAE-cellulose, fractionation was attempted using a 7 M urea:salt gradient as described by Tomlinson and Tener (19). The urea supresses secondary binding forces that normally allow the separation of purine and pyrimidine nucleotides, and fractionation occurs only on the basis of charge. The urea proved effective in reducing the powerful absorption of the hydrocarbon-containing moieties in the enzyme digest, and a fractionation as shown in Chart 3 was obtained after 18 hr of enzyme treatment. The pattern of elution of the labeled material suggested that the enzymic digestion had not completely degraded the hydrocarbon-containing regions of the DNA to mononucleotides although the mass of the DNA had been so degraded as evidenced by the single peak of UV-absorbing products. Degradation for a shorter (Chart 4*A*) or longer (Chart 4*B*) time supported this concept, since the relative amount of material eluted in the mononucleotide region varied, as would be predicted. The radioactive product eluted in the first few fractions, possibly nucleosides, also increased on enzymic digestion, suggesting a contamination of the diesterase with a trace of monoesterase activity.

These experiments implied that the presence of the hydro-

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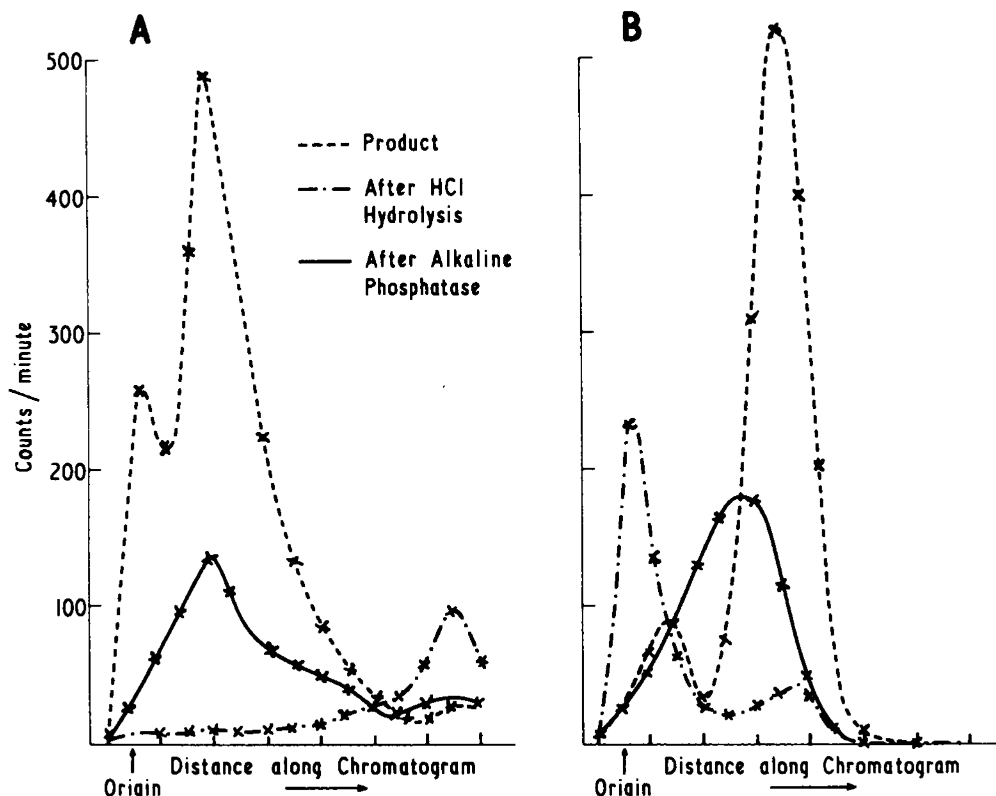

Chart 7. Following degradation and fractionation of DMBA-DNA, as described in Chart 3, the material from the radioactive peak immediately following the UV-absorbing material was reisolated free of urea and salts. A part of this material (the Product) was treated with *E. coli* alkaline phosphatase and another part with 0.1 N hydrochloric acid at 100°C for 15 min. The initial product and the materials resulting from acid and monoesterase treatment were applied to Whatman No. 1 paper strip (3 cm wide) and: *A*, chromatographed using isobutyric acid:ammonia:water (66:2:32 v/v/v); or *B*, subjected to paper electrophoresis in 7 M urea:0.025 M Tris-phosphate, pH 7.0, at 750 volts for 3 hr. The strips were cut into 1-cm lengths, and the radioactivity was determined by liquid scintillation methods.

carbon moiety bound to the DNA partially inhibited the hydrolytic enzymes so that, after a limited digestion, hydrocarbon was recovered in an oligonucleotide fragment.

Since nothing was known of the mode of attachment of the hydrocarbon to the DNA, it seemed of interest to examine enzymic degradation of alkylated DNA where the chemistry of the reaction is clearly understood (13, 14). Charts 5 and 6 show that, whereas DNA methylated with methanesulphonate-methyl-^{14}C gave only a single labeled product, namely the expected methyldeoxyguanylic acid-7-^{14}C, DNA alkylated with 2-chloroethyl-2-hydroxyethyl sulfide-^{35}S gave a result very similar to that obtained with DMBA-DNA. This suggests that enzymic degradation of DNA, alkylated at N-7 of guanine is inhibited if the alkyl group is larger than a certain size. The similar behavior of alkylated DNA and DMBA-DNA supports the view that the hydrocarbon is bound covalently rather than by some form of physical interaction such as implied in the intercalation model (2). It seems totally unlikely that physically bound hydrocarbon would not be removed by the procedures used for isolation of the DNA, and even less likely that it would remain bound after degradation of the DNA to give a fractionation pattern as shown in Charts 3 and 4.

Paper chromatography and electrophoresis of the material reisolated from the peak eluted from DEAE-cellulose immediately after the mononucleotides was consistent with its being an oligonucleotide or mixture of oligonucleotides (Chart 7).

Digestion with monoesterase changed the chromatographic behavior as did treatment with acid. After this latter treatment, the material chromatographed in a manner reminiscent of a base moiety, remaining at the origin in electrophoresis and running near the solvent front in the powerfully solvating isobutyric acid:ammonia system. This conversion in acid could be effected by 0.1 N HCl at 37°C, which suggested the hydrolysis of a substituted purine rather than pyrimidine nucleoside.

Further identification on the base involved, its position of substitution, and the nature of the hydrocarbon moiety will require more detailed fractionation of the products, preferably as mononucleotides. Fractionation after degradation to the level of free bases is made difficult by the insolubility of the product, and any progress is limited by the very small amount of material available. With the mouse embryo DNA used in these experiments, 10 mg of DMBA-DNA, which is the yield from approximately 10^9 cells, would contain less than 0.5 μg of actual hydrocarbon base product. Positive identification is clearly difficult on this scale, and alternative approaches are being investigated. These involve the synthesis of model compounds derived from hydrocarbons and possessing the ability to react with DNA.

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