Structure and Activity in Chemical Carcinogenesis. Comparison of the Reactions of 7-Bromomethylbenz[*a*]anthracene and 7-Bromomethyl-12-methylbenz[*a*]anthracene with Mouse Skin Deoxyribonucleic Acid *in Vivo*†

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ABSTRACT: The extents and products of reaction of 7-bromomethylbenz[*a*]anthracene and 7-bromomethyl-12-methylbenz[*a*]anthracene with mouse skin DNA *in vivo* were compared. The time required to achieve a maximum extent of reaction was far greater for the former than for the latter compound. Carcinogenic potency for these bromo compounds was not positively correlated with either the total extent of reaction with DNA or the extent of attack on any specific nucleoside residue in DNA.

Studies of the 7-bromomethylbenz[*a*]anthracenes have been undertaken because of their possible relevance to the mechanism of action of the methyl-substituted polycyclic hydrocarbon carcinogens (Dipple *et al.*, 1968; Flesher and Sydnor, 1971), and because they provide a means by which the nature of the initiating event in chemical carcinogenesis might be defined (Rayman and Dipple, 1973). 7-Bromomethylbenz[*a*]anthracene is a much less effective carcinogen for mouse skin than is 7-bromomethyl-12-methylbenz[*a*]anthracene (Dipple and Slade, 1971), and more than 90% of the products of reaction of both of these compounds with DNA has been identified (Dipple *et al.*, 1971; Rayman and Dipple, 1973). It has been possible, therefore, to extend previous studies by comparing the reactions of these compounds with DNA in mouse skin in order that the relevance of the interactions between chemical carcinogens and cellular DNA to tumour initiation might be investigated.

Experimental Section

[^{3}H]7-Bromomethylbenz[a]anthracene and [^{3}H]7-Bromomethyl-12-methylbenz[a]anthracene. These compounds were prepared by the methods of Dipple and Slade (1970) as previously described (Rayman and Dipple, 1973). [^{3}H]7-Bromomethylbenz[*a*]anthracene and [^{3}H]7-bromomethyl-12-methylbenz[*a*]anthracene at respective specific activities of 165 and 687 Ci per mol were used to establish the time course of binding to mouse skin DNA, and preparations at respective specific activities of 2.65 and 2.29 Ci per mmol were used for experiments involving the analysis of the products of binding to mouse skin DNA.

Animal Experiments. The hair was shaved from the backs of Swiss S female mice (age 8–10 weeks) and 2 days later radioactive bromo compound (1 μmol), dissolved in benzene (0.05 ml), was applied to the back of each animal. (These conditions were the same as those used previously for assessing papilloma initiation by these compounds in mouse skin (Dipple and Slade, 1971).) At various times groups of treated mice were killed and the treated area of skin was excised and frozen in liquid nitrogen.

Time Course of Binding of Bromo Compounds to Mouse Skin DNA. Fat and muscle were removed from the excised skins (usually from a group of 25 mice but in some experiments only 12 mice) by the method of Wiest and Heidelberger (1953). The frozen skins (liquid nitrogen) were ground to a fine powder with a pestle and mortar, and lysed in sodium triisopropylnaphthalenesulfonate, butan-2-ol, sodium 4-aminosalicylate, and sodium chloride solution (Kirby and Cook, 1967). The lysate was extracted with an equal volume of phenol–*m*-cresol–8-hydroxyquinoline reagent (Kirby, 1965) and reextracted with a half-volume of this same reagent. DNA was then purified from the aqueous solution by precipitation with 2-ethoxyethanol, treatment with ribonuclease, and partitioning between 1.25 M potassium phosphate solution (pH 7.2) and 2-methoxyethanol (Diamond *et al.*, 1967). For determinations of specific radioactivity, DNA was dissolved by heating in 5% trichloroacetic acid, after which the optical density at 260 nm and the radioactivity of this solution were recorded. The value for ϵ_p for DNA was taken as 8750.

Analysis of Products of Binding of Bromo Compounds to Mouse Skin DNA. Two groups of forty mice were separately treated with the two bromo compounds as described above. At selected times, ten mice from one group were killed and the skins were divided into two groups. DNA was then isolated from each of these groups, as described above, up to and including the ribonuclease treatment. After extracting with phenol to remove the ribonuclease, DNA was then precipitated with two volumes of ethanol, washed, and dried. Usually, 2–3 mg of DNA was obtained from five skins, and its purity was checked by centrifugation in CsCl solution (Flamm *et al.*, 1969). Thus, each DNA sample was dissolved in 0.01 M Tris buffer, pH 7.3 (8 ml), and to each of these solutions CsCl (10.5 g) was added. After thorough mixing, the solutions were centrifuged at 42,000 rpm for 41 hr (fixed-angle Ti 50 rotor, Beckman Model L centrifuge). Fractions of 25 drops were collected and an aliquot (0.05 ml) from each fraction was diluted with water (0.5 ml) for determination of radioactivity and ultraviolet (uv) absorption. The fractions

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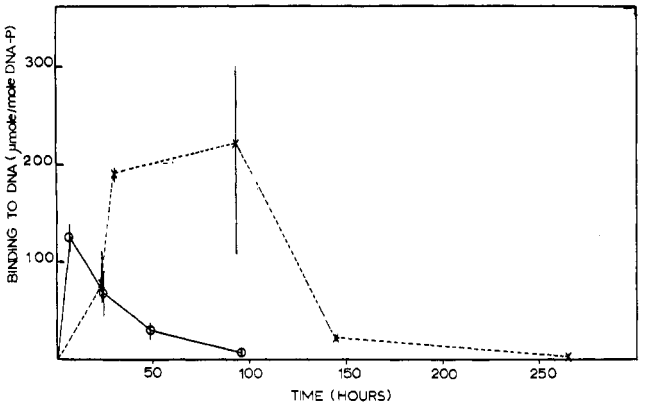

FIGURE 1: Time course for binding of [^{3}H]7-bromomethylbenz[*a*]-anthracenes to mouse skin DNA *in vivo*. Mice were treated with 1 μmol of tritiated bromo compound and DNA isolated and assayed as described under Experimental Section. Symbols are averages of duplicate or triplicate determinations and the extremities of the vertical bars represent the highest and lowest values obtained. At long time points the range of the experimental determinations was very narrow. This is indicated in the figure although the vertical bar is shorter than the symbol height. (○) 7-Bromomethyl-12-methylbenz[*a*]anthracene; (×) 7-bromomethylbenz[*a*]anthracene.

containing DNA (usually four or five fractions) were pooled and dialyzed against distilled water at 4°. Aliquots (0.05 ml) from the resultant viscous solutions (usually 1.5–2.0 ml) were diluted with water (0.5 ml), hydrolyzed by the addition of deoxyribonuclease solution (1 mg/ml) plus 0.01 M Tris buffer (pH 7) containing 0.01 M magnesium chloride (0.02 ml of each), and used for the determination of specific activity and extent of reaction. To the remaining portions of the solutions from dialysis were added 0.01 M Tris buffer (pH 7), 0.01 M in magnesium chloride (0.15 ml), and deoxyribonuclease solution (0.15 ml of a 1-mg/ml solution in 0.001 M Tris buffer, pH 6). The solutions were incubated at 37° for 1 hr. To each solution 1 M Tris buffer (pH 9), 0.02 M in sodium chloride and 0.02 M in magnesium chloride (0.15 ml), was added, together with 0.16 unit of snake venom phosphodiesterase. These solutions were kept at 37° for 44–48 hr. *Escherichia coli* alkaline phosphatase (0.15 ml of a 1-mg/ml solution) was then added and incubation at 37° was continued for a further 17 hr. During all these procedures exposure of the DNA solutions to light was avoided.

Chromatography on Sephadex LH-20. Elution of nucleoside mixtures from Sephadex LH-20 with methanol was as previously described (Dipple *et al.*, 1971; Rayman and Dipple, 1973). The fraction size collected was 200 drops and in most of these experiments the whole fraction was transferred to a counting vial, Triton-X 100–toluene–phosphor (10 ml) was added, and radioactivity was determined by liquid scintillation counting.

Results

In a previous study (Dipple and Slade, 1971) it was found that the topical application of 1 μmol of 7-bromomethyl-12-methylbenz[*a*]anthracene to the backs of Swiss S mice evoked a far higher papilloma incidence (88%) than did a similar treatment with 1 μmol of 7-bromomethylbenz[*a*]-anthracene (16%). When the time course of binding of these two compounds to the DNA of whole mouse skin (after treatment of the mice under the conditions used for the above biological experiments) was examined, the results presented

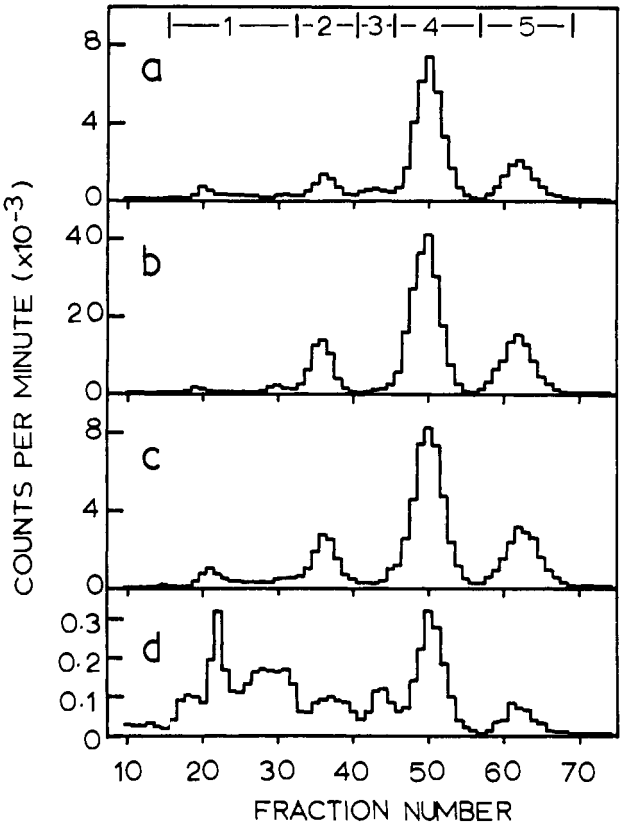

FIGURE 2: Column chromatograms on Sephadex LH-20 eluted with methanol of nucleoside products from mouse skin DNA treated for (a) 31 hr, (b) 96 hr, (c) 144 hr, and (d) 264 hr with [^{3}H]7-bromomethylbenz[*a*]anthracene. Isolation and degradation procedures are given under Experimental Section.

in Figure 1 were obtained. In accord with previous studies on the reaction of these two compounds with either 4-(*p*-nitrobenzyl)pyridine (Dipple and Slade, 1970) or with DNA *in vitro* (Rayman and Dipple, 1973), it was found that the less carcinogenic bromo compound was more extensively bound to DNA than was the more carcinogenic bromo compound. It was also noted that the time required for maximum binding to DNA was far greater for the less carcinogenic 7-bromomethylbenz[*a*]anthracene than for 7-bromomethyl-12-methylbenz[*a*]anthracene.

The inverse correlation between carcinogenic potency and extent of reaction with mouse skin DNA indicated that the overall capacity of these compounds for reaction with DNA could not be the major determinant of their respective carcinogenic potencies. However, it was possible that the more carcinogenic agent might be capable of introducing more aralkylations of a specific type into DNA than the less carcinogenic 7-bromomethylbenz[*a*]anthracene and this possibility was also examined.

[^{3}H]Bromo compounds of fairly high specific radioactivity (2–3 Ci/mmol) were prepared and applied to the backs of mice as before. At each time point selected duplicate samples of DNA were isolated from mouse skin and finally purified by isopyknic centrifugation in cesium chloride solutions. After dialysis to remove cesium chloride, the extents of reaction of the bromo compounds with DNA were measured (Tables I and II). Each DNA sample was then enzymically converted to nucleosides and examined by chromatography on Sephadex LH-20 eluted with methanol. One of the two elution profiles obtained for each time point studied is illustrated in

TABLE I: Products of Reaction of 7-Bromomethylbenz[*a*]anthracene with Mouse Skin DNA *in Vivo*.[a]

Time (hr)	Binding to DNA in μmol/mol of DNA-P	Products as % Total Radioactivity					Products in μmol/mol of DNA-P				
		Peak 1	Peak 2	Peak 3	Peak 4	Peak 5	Peak 1	Peak 2	Peak 3	Peak 4	Peak 5
31	165	7.8	9.1	4.9	54.9	19.0	12.9	15.1	8.1	90.5	31.2
	158	4.2	6.7	1.9	68.4	16.9	6.7	10.7	3.0	108.4	26.8
96	212	5.4	17.3	1.8	51.7	22.8	11.4	36.6	3.9	109.6	48.4
	353	4.2	15.8	1.0	55.0	23.0	15.0	55.8	3.6	194.1	81.2
144	41.2	8.2	15.1	1.2	49.8	22.0	3.4	6.2	0.5	20.5	9.1
264	2.2	35.3	8.5	6.7	28.5	8.8	0.78	0.19	0.15	0.63	0.19
	1.3	41.3	10.3	7.5	23.4	8.5	0.54	0.13	0.10	0.31	0.11

[a] Mice were treated and DNA was isolated and analyzed as described under Experimental Section. Column headings are defined in Figure 2a.

Figure 2 for the 7-bromomethylbenz[*a*]anthracene experiments.

These profiles, particularly at the early times, are qualitatively very similar to those obtained after reaction of this bromo compound with DNA *in vitro* (Rayman and Dipple, 1973), where it has been shown that the product represented by peak 4 of Figure 2a is N^2-(benz[*a*]anthracenyl-7-methyl)-deoxyguanosine, by peak 5 is N^6-(benz[*a*]anthracenyl-7-methyl)deoxyadenosine, and by peak 2 is probably N^4-(benz[*a*]anthracenyl-7-methyl)deoxycytidine (Dipple *et al.*, 1971). In order to confirm that these same products were obtained from mouse skin DNA, the fractions comprising the deoxyguanosine product (peak 4) from a chromatogram of mouse skin DNA were pooled, evaporated to dryness, and subjected to mild acid hydrolysis to remove the sugar residue from the radioactive product. The hydrolysate was then mixed with authentic N^2-(benz[*a*]anthracenyl-7-methyl)-guanine and the uv absorption of this marker compound and radioactivity from the mouse skin DNA product were shown to be inseparable by chromatography on Sephadex LH-20 eluted with methanol (Figure 3a) or on thin-layer sheets (Polygram Sheets SIL N-HR/UV, Camlab) developed in the following solvents: acetone, ethyl acetate, methanol, and benzene–butan-1-ol (1:1). A similar experiment with an acid hydrolysate of peak 5 of Figure 2a and authentic N^6-(benz-[*a*]anthracenyl-7-methyl)adenine showed identity between the mouse skin DNA product and the marker compound (Figure 3b).

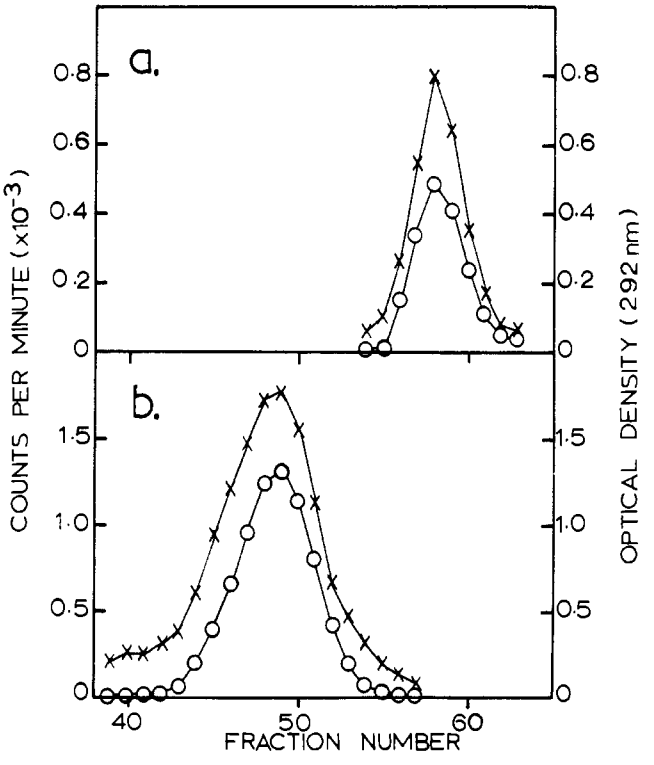

FIGURE 3: Elution profiles from Sephadex LH-20 eluted with methanol of: (a) peak 4 of Figure 2a was pooled, evaporated to dryness, and then hydrolyzed by heating in 0.1 N HCl at 100° for 15 min. Authentic nonradioactive N^2-(benz[*a*]anthracenyl-7-methyl)-guanine (Dipple *et al.*, 1971) was then added and the solution was neutralized and chromatographed on Sephadex LH-20 as described under Experimental Section. (b) Peak 5 of Figure 2a was treated as above and chromatographed with authentic N^6-(benz[*a*]anthracenyl-7-methyl)adenine (Dipple *et al.*, 1971). (×) Tritium counts per minute; (○) optical density at 292 nm.

The elution profiles obtained from mouse skin DNA at various times after treatment with 7-bromomethyl-12-methylbenz[*a*]anthracene are illustrated in Figure 4. Again, the profiles at early times are essentially the same as those obtained after reaction with DNA *in vitro* (Rayman and Dipple, 1973), where it was shown that the products represented in Figure 4a by peaks 2, 4, and 5 are probably N^4-(12-methylbenz[*a*]anthracenyl-7-methyl)deoxycytidine, N^2-(12-methylbenz[*a*]anthracenyl-7-methyl)deoxyguanosine, and N^6-(12-methylbenz[*a*]anthracenyl-7-methyl)deoxyadenosine, respectively.

The radioactive counts under each peak specified in Figures 2a and 4a were summed and are expressed as percentages of the total radioactive products obtained in Tables I and II. The amounts (in micromoles per mole of DNA phosphorus) of each product present in mouse skin DNA at various times are also given. It can be seen that the maximum amount of any radioactive product formed is never significantly larger for the more carcinogenic 12-methyl derivative than for 7-bromomethylbenz[*a*]anthracene.

Discussion

Inspection of the extents of reaction of the two bromo compounds with mouse skin DNA (Figure 1 and Tables I and II)

TABLE II: Products of Reaction of 7-Bromomethyl-12-methylbenz[*a*]anthracene with Mouse Skin DNA *in Vivo*.[a]

Time (hr)	Binding to DNA in μmol/mol of DNA-P	Products as % Total Radioactivity					Products in μmol/mol of DNA-P				
		Peak 1	Peak 2	Peak 3	Peak 4	Peak 5	Peak 1	Peak 2	Peak 3	Peak 4	Peak 5
7	179	8.1	8.2	1.0	50.6	27.5	14.5	14.8	1.7	91.0	49.3
	129	11.3	11.9	1.4	44.6	28.8	14.5	15.3	1.8	57.3	37.0
24	108	12.4	9.3	1.2	49.0	26.7	13.3	10.1	1.7	53.0	28.8
	90	12.8	11.0	2.5	45.5	27.1	11.5	9.9	2.3	41.0	24.4
49	52	14.1	11.3	5.9	41.4	23.9	7.3	5.9	3.0	21.5	12.4
	52	11.5	11.5	6.5	42.2	24.0	6.0	6.0	3.4	22.0	12.5
96	7.3	46.1	6.4	16.5	19.1	7.5	3.4	0.5	1.2	1.3	0.5
	7.1	28.5	9.0	13.6	29.6	14.9	2.0	0.6	1.0	2.1	1.1

[a] Mice were treated and DNA was isolated and analyzed as described under Experimental Section. Column headings are defined in Figure 4a.

shows that these measurements are subject to some variation particularly in the region of maximum binding for 7-bromomethylbenz[*a*]anthracene. However, the difference between the levels of binding of the two compounds is greater than the variation in the measured binding for either compound, and it is clear that the less carcinogenic compound is bound more extensively to mouse skin DNA than is 7-bromomethyl-12-methylbenz[*a*]anthracene. The less carcinogenic 7-bromomethylbenz[*a*]anthracene also requires a longer time to attain a maximum binding, and therefore its products are present in mouse skin DNA for a longer time than those from the more carcinogenic 7-bromomethyl-12-methylbenz[*a*]anthracene. Thus, there is no positive correlation between the overall extents of reaction of these bromo compounds with mouse skin DNA or the persistence of their reaction products in mouse skin and their carcinogenic potencies for this tissue.

Loveless (1969) has suggested, however, that the biological effects of certain alkylating agents may be related to the extent of reaction at specific sites on the DNA bases rather than to the overall reaction with DNA. The applicability of this proposal to these bromo compounds was investigated by comparing the amounts of the various products present in mouse skin DNA after treatment with the two bromo compounds (Tables I and II, and Figures 2 and 4). The extents of reaction on the amino groups of the DNA bases (peaks 2, 4, and 5 of Figures 2 and 4) reached higher levels for the less carcinogenic 7-bromomethylbenz[*a*]anthracene than for 7-bromomethyl-12-methylbenz[*a*]anthracene. A similar situation obtained for the product represented by peak 3 in Figures 2 and 4 which was observed in *in vitro* studies after reaction with heat-denatured DNA but not after reaction with native DNA (Rayman and Dipple, 1973). The group of unidentified products represented by peak 1 in Figures 2 and 4 were present in similar amounts in mouse skin DNA after treatment with either bromo compound. (These products are interesting because they constitute an increasing percentage of the total products as overall binding to DNA decreases with time. Since peak 1 is the region of the chromatograms where photoproducts were eluted in the *in vitro* studies (Rayman and Dipple, 1973) it is possible that reactions analogous to the light-mediated *in vitro* reactions occur in the mouse skin. Alternatively, since the products of reaction of 7-bromomethylbenz[*a*]anthracene with DNA are chemically stable under the conditions of temperature and pH prevailing *in vivo*, the change in the proportions of various products with time could result from specific enzymatic excision processes which have been demonstrated in *in vitro* systems for 7-bromomethylbenz[*a*]anthracene (Lieberman and Dipple, 1972; Venitt and Tarmy, 1972).)

Thus, no evidence was obtained to suggest that the more effective carcinogen, 7-bromomethyl-12-methylbenz[*a*]-

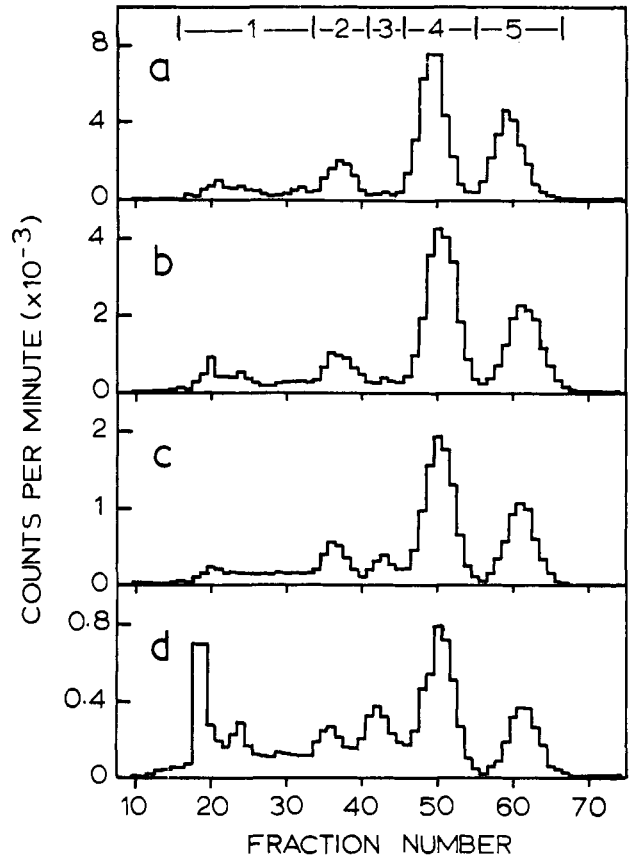

FIGURE 4: Column chromatograms on Sephadex LH-20 eluted with methanol of nucleoside products from mouse skin DNA treated for (a) 7 hr, (b) 24 hr, (c) 49 hr, and (d) 96 hr with [^{3}H]7-bromomethyl-12-methylbenz[*a*]anthracene. Isolation and degradation procedures are given under Experimental Section.

anthracene, attacked any site on the DNA bases more extensively than did 7-bromomethylbenz[*a*]anthracene and this work does not, therefore, support the view that DNA is the critical cellular receptor for chemical carcinogens. However, these findings do not exclude this view for the following reasons. (1) These studies involve the assumption that reactions with the DNA of whole mouse skin are representative of those occurring in the cells which eventually give rise to papilloma. (2) Since the time course of binding for the two bromo compounds was quite different, a positive correlation between carcinogenic potency and reaction with DNA does exist in the first few hours after initiation (Figure 1). (3) A positive correlation between carcinogenic potency and the amounts of some minor unidentified product eluted among other unidentified products under peak 1 of Figures 2 and 4 cannot be excluded. (4) The different chemical properties of 7-bromomethylbenz[*a*]anthracene and 7-bromomethyl-12-methylbenz[*a*]anthracene dictate that the latter compound should react less extensively with DNA than the former compound, and that the 12-methyl derivative should exhibit a greater preference for attack on adenine residues in DNA than does 7-bromomethylbenz[*a*]anthracene (Rayman and Dipple, 1973, and Tables I and II). It is conceivable, therefore, that these chemical differences might confer a greater selectivity for attack at the specific chromosomal site involved in tumor initiation on the more carcinogenic 7-bromethyl-12-methylbenz[*a*]anthracene than on 7-bromomethylbenz[*a*]-anthracene.

Consistent with this latter possibility is the work of Fahmy and Fahmy (1972) which demonstrates that chemical carcinogens display a remarkable specificity for mutation at the rRNA genes (resulting in *bobbed* mutations) in *D. melanogaster*. These authors have also shown that 7-bromomethyl-12-methylbenz[*a*]anthracene exhibits a greater specificity for the production of *bobbed* mutations in *Drosophila* than does 7-bromomethylbenz[*a*]anthracene (Fahmy and Fahmy, 1970, and personal communication). Chemical studies of the interaction of 7,12-dimethylbenz[*a*]anthracene with mouse skin DNA did not reveal any specific attack with respect to either mouse eipdermal satellite or main-band DNA (Zeiger *et al.*, 1972). It remains possible, however, that differences correlated with carcinogenic potency might be observed when a wider range of carcinogens is studied, or when the sensitivity of the chemical methods can be increased to parallel that of genetic analysis.

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