factors, including interactions between mRNA–codon and tRNA–anticodon, tRNA and ribosome, and trinucleotide and ribosome.

Some codons such as AAA, bound to ribosomes to a greater extent than others, such as UUC. Even in the case of the synonym codons, UUU and UUC, differences in the extent of codon–ribosome–aa-tRNA complex formation were observed.

Ribosomal-bound UUC and Phe-tRNA were found to exchange with unbound UUC and Phe-tRNA. The rate of exchange of bound with unbound UUC was more rapid than the exchange of bound with unbound Phe-tRNA. Ribosomal-bound [^{3}H]UUC exchanged more rapidly with an unbound *synonym* codon, UUU, than with codons for leucyl-, seryl-, and aspartyl-tRNA, respectively.

These results strongly suggest that the anticodon of ribosomal-bound aa-tRNA can act as a template for a *set* of synonym trinucleotides during the exchange process much as a trinucleotide acts as a template for aa-tRNA. In this way, the specificity of a species of aa-tRNA for a set of codons can be elucidated with unfractionated tRNA by determining the rate of release of a radioactive trinucleotide from the trinucleotide–ribosome–aa-tRNA complex in the presence of unlabeled synonym triplets.

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Reaction of 7-Bromomethylbenz[*a*]anthracene with Nucleic Acids, Polynucleotides, and Nucleosides*

A. Dipple,† P. Brookes, D. S. Mackintosh, and M. P. Rayman

ABSTRACT: In order to investigate the mode of action of the carcinogen 7-bromomethylbenz[*a*]anthracene, the chemistry of its reaction with nucleic acids and their constituents has been studied. It appears that in dimethylacetamide this reagent reacts with nucleosides mainly at the same position as do methylating agents *i.e.*, N-7 of guanine derivatives, N-1 of adenine derivatives, and N-3 of cytosine derivatives. However, in aqueous solution, reaction with nucleosides, nucleic acids, or polynucleotides leads mainly to reaction on the amino groups of guanine, adenine, and possibly cytosine.

In earlier studies 7-bromomethylbenz[*a*]anthracene did not induce sarcoma in the rat, and was thereby shown to be a less active carcinogen than its parent hydrocarbon, 7-methylbenz[*a*]anthracene (Dipple and Slade, 1970). However, at a higher dose, tumors are produced in the rat (A. Dipple and T. A. Slade, unpublished data), and this bromo compound is also known to be active in the initiation of papilloma in mouse skin (J. D. Scribner, personal communication; Dipple and Slade, 1971). An understanding of the chemistry of the reaction of this agent with cellular constituents could obviously clarify the mechanism by which it evokes the carcinogenic response and could conceivably, though not necessarily, cast some light on the mechanism of action of the aromatic hydrocarbons themselves.

Earlier studies (Brookes and Lawley, 1964) have suggested that DNA may be the critical receptor for the aromatic hydrocarbon carcinogens, and for this reason we have studied in the first instance the reactions of this new chemical carcinogen with nucleic acids.

Brookes and Dipple (1969) presented preliminary data

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† To whom correspondence should be addressed.

showing that 7-bromomethylbenz[*a*]anthracene reacted readily with nucleic acids, polynucleotides, nucleotides, and nucleosides and that this reagent exhibited a preference for reaction with purines, and in particular with guanine. The present study extends these findings and describes the exact nature of the products formed. It is found that in aqueous solution nucleic acids are alkylated by 7-bromomethylbenz[*a*]anthracene mainly on the amino groups of the purines, and the chemistry of this reagent is, therefore, quite dissimilar to that of the simpler and more extensively studied alkylating agents (Lawley, 1966).

Experimental Section

Melting points are corrected and were determined on a microscope hot-stage apparatus. Absorption spectra were recorded with a Unicam SP 800 spectrophotometer and optical densities at selected wavelengths with a Unicam SP 500 spectrophotometer. The homogeneity of all products was routinely confirmed by thin-layer chromatography on silica gel using either Eastman Chromatogram Sheet 606D, or Polygram S1L N-HR/UV (Camlab, Cambridge). Analyses were by Strauss, Microanalytical Laboratories, Oxford.

7-Bromomethylbenz[a]anthracene. Unlabeled reagent was routinely prepared by bromomethylation of benz[*a*]anthracene, and tritium-labeled reagent was prepared by the bromination of radioactive 7-methylbenz[*a*]anthracene using the procedures described previously (Dipple and Slade, 1970). Tritium-labeled 7-methylbenz[*a*]anthracene was prepared by catalytic exchange at the Radiochemical Centre, Amersham, and purified in our laboratory (Duncan *et al.*, 1969).

Benz[a]anthryl-7-methylpyridinium[1] *Tosylate.* 7-Hydroxymethylbenz[*a*]anthracene (0.516 g) and *p*-toluenesulfonyl chloride (0.4 g) in pyridine (5 ml) were kept at room temperature overnight. Crude product was precipitated by water (50 ml) and crystallized from hot water, yielding pale yellow crystals which melted (with decomposition) at 215–216°.

Anal. Calcd for $C_{31}H_{25}NSO_3$: C, 75.73; H, 5.13; N, 2.83; S, 6.52. Found: C, 75.37; H, 5.35; N, 2.69; S, 6.58.

When this product (30 mg) in methanol was run through a small column of Dowex 50 (H^+), *p*-toluenesulfonic acid (11.7 mg estimated by uv spectroscopy) was found in the eluate. The proposed ionic structure requires 10.5 mg of this acid to be obtained from the sample used.

7-Aminomethylbenz[a]anthracene. A suspension of potassium phthalimide (2.5 g) in dimethylformamide (25 ml) was treated with an equimolar quantity of 7-bromomethylbenz[*a*]anthracene in dimethylformamide (20 ml) and kept at 45° for 15 min. After cooling, the slurry was poured into ice–water (100 ml), and the precipitated *N*-(benz[*a*]anthryl-7-methyl)phthalimide was collected (92% yield) and crystallized from glacial acetic acid, mp 237–239°.

Anal. Calcd for $C_{27}H_{17}NO_2$: C, 83.70; H, 4.42; N, 3.62. Found: C, 83.49; H, 4.47; N, 3.66.

A stirred suspension of the phthalimide derivative (4.8 g) in methanol (70 ml) was treated with hydrazine hydrate (0.65 ml) and kept under reflux overnight. The solvent was evaporated and the residue was extracted with hot benzene. The benzene soluble product was purified by elution from a silicic acid column with benzene–methanol (1:1). Crystallization from ethanol–water yielded yellow needles of 7-aminomethylbenz[*a*]anthracene which melted at 125–127°.

[1] Benz[*a*]anthracenyl-7-methylpyridinium; the contracted form (anthryl) is used throughout.

Anal. Calcd for $C_{19}H_{15}N$: C, 88.72; H, 5.84; N, 5.44. Found: C, 88.43; H, 5.89; N, 5.44.

3-(Benz[a]anthryl-7-methyl)cytidine Hydrobromide. Cytidine (0.1 g) and 7-bromomethylbenz[*a*]anthracene (0.15 g) were warmed with dimethylacetamide (2 ml) until solution was obtained. After 23 hr at 37° crude product was precipitated by the addition of acetone (4 ml) and ethyl acetate (40 ml). The gummy residue, after being washed with ethyl acetate and ether, was dissolved in methanol (1 ml), applied to a silicic acid column (made up in ethyl acetate), and eluted with 9% methanol in ethyl acetate. The desired product was eluted in fractions (10 ml) 22–60 and crystallized directly from these fractions after 4 days at room temperature, yielding 0.073 g of 3-(benz[*a*]anthryl-7-methyl)cytidine hydrobromide which melted at 177–179°.

Anal. Calcd for $C_{28}H_{25}N_3O_5 \cdot HBr$: C, 59.55; H, 4.65; N, 7.45; Br, 14.15. Found: C, 59.07; H, 4.72; N, 7.45; Br, 14.18.

Effect of Acid on 3-(Benz[a]anthryl-7-methyl)cytidine. The effects of concentrated HCl at room temperature overnight and 1 N HCl in 50% aqueous 1-propanol at 70° overnight were essentially the same. In both cases the reaction mixture was partitioned into aqueous and benzene phases. Cytidine plus traces of starting material were found in the aqueous phases by thin-layer chromatography and identified by elution and comparison of ultraviolet absorption spectra. The benzene phases contained only 7-hydroxymethylbenz[*a*]anthracene, identified by thin-layer chromatographic comparison in four solvent systems with an authentic marker (Badger and Cook, 1939).

2',3-O-Isopropylidene-7-(benz[a]anthryl-7-methyl)guanosine Hydrobromide. Isopropylideneguanosine (Sigma Chemical Co.) (0.5 g) and 7-bromomethylbenz[*a*]anthracene (1 g) were dissolved in dimethylacetamide (8 ml) and left at room temperature for 7 days. The solution was then poured into ether (250 ml) and the precipitate was collected, washed with ether, and crystallized from ethanol (5 ml), yielding 0.68 g of white crystalline material. An analytical specimen which did not melt below 290° was obtained by further recrystallization from ethanol–methanol (1:1) and from methanol–ethyl acetate.

Anal. Calcd for $C_{32}H_{29}N_5O_5 \cdot HBr$: C, 59.64; H, 4.69; N, 10.86. Found: C, 59.46; H, 4.50; N, 10.71.

7-(Benz[a]anthryl-7-methyl)guanine Hydrochloride. When the isopropylidene derivative was heated at 50° for 1 hr in aqueous 2 N HCl–methanol (1:1), the sugar residue was removed and the substituted guanine was deposited as a white crystalline material. This was collected, washed with water, and dried in *vacuo*, mp >280°.

Anal. Calcd for $C_{24}H_{17}N_5O \cdot HCl \cdot H_2O$: C, 64.71; H, 4.49; N, 15.73. Found: C, 64.67; H, 4.55; N, 15.35.

1-(Benz[a]anthryl-7-methyl)adenosine. Adenosine (1 g) and 7-bromomethylbenz[*a*]anthracene (2.4 g) were dissolved in dimethylacetamide (15 ml) and kept at room temperature for 3 days. Crude product was precipitated by the addition of ether, suspended in ether, and applied to a silicic acid column. The column was eluted with 1-butanol–ethanol–water (4:1:5), and after several days the desired product crystallized from fractions (5 ml) 5–35. After collection and washing with 1-butanol 0.56 g of crystalline material was obtained, mp 217–218° dec; mass spectrum (relative intensity) M^+ (7); 375 (8); 241 (38); 218 (42); 164 (42); 135 (100); 120 (3).

Anal. Calcd for $C_{29}H_{25}N_5O_4 \cdot H_2O$: C, 66.28; H, 5.18; N, 13.32. Found: C, 66.54; H, 5.12; N, 13.19.

N^6-(Benz[a]anthryl-7-methyl)adenosine. The 1-substituted adenosine (0.17 g) was heated at 50° for 48 hr in methanol

(25 ml) plus 1 N NaOH (5 ml). The crystalline solid which separated out was collected, washed with water, and dried, yielding 0.08 g of product. After recrystallization from methanol–ethyl acetate this material had mp 229–232°; mass spectrum M^+ (30); 375 (68); 241 (100); 229 (14); 135 (3); 120 (16).

Anal. Calcd for $C_{29}H_{25}N_5O_4 \cdot H_2O$: as above. Found: C, 66.76; H, 5.11; N, 13.26.

This product had the same ultraviolet absorption spectrum and thin-layer chromatographic properties (in several solvent systems) as N^6-(benz[*a*]anthryl-7-methyl)adenosine prepared through the following unambiguous route (Kissman and Weiss, 1956; Fleysher *et al.*, 1969).

6-Chloropurine riboside (7.9 mg) and 7-aminomethylbenz[*a*]anthracene (14 mg) dissolved in 2-ethoxyethanol (1 ml) were heated at 90° for 2 days. The reaction mixture was diluted to 5 ml with methanol, applied to a column of Sephadex LH-20 (75 cm × 1.5 cm diameter), and eluted with methanol, the N^6-(benz[*a*]anthryl-7-methyl)adenosine being eluted in fractions (5 ml) 61–66.

Conversion of N^6-(Benz[a]anthryl-7-methyl)adenosine to N^6-(Benz[a]anthryl-7-methyl)adenine by Acid. After heating the N^6-substituted nucleoside in MeOH–10 N HCl (9:1) at 80° overnight, the solution was neutralized with 1 N NaOH and evaporated to dryness. The residue was then extracted with methanol and the resulting solution contained a major uv-absorbing component which was chromatographically identical with N^6-(benz[*a*]anthryl-7-methyl)adenine prepared through the following unambiguous route (Bullock *et al.*, 1956).

6-Chloropurine (9 mg) and 7-aminomethylbenz[*a*]anthracene (30 mg) were dissolved in dimethyl sulfoxide (1 ml), and the solution was heated at 90° for 2 days. The solution was poured into water and the resulting precipitate was resolved into N^6-(benz[*a*]anthryl-7-methyl)adenine and unchanged 7-aminomethylbenz[*a*]anthracene by thin-layer chromatography on silica gel developed with acetone.

N^2-(Benz[a]anthryl-7-methyl)guanine. 2-Chloro-6-hydroxypurine (0.1 g) and 7-aminomethylbenz[*a*]anthracene (0.33 g) were heated in dimethyl sulfoxide (2 ml) at 80° for 4 days. The solution was then poured into ice-cold water (50 ml) and the precipitate was collected, washed with water, and dried. It was then dissolved in methanol, applied to a column of Sephadex LH-20 (64 cm × 3.2 cm diameter), and eluted with methanol in fractions (15 ml) 52–58. This yielded (65%) chromatographically homogeneous N^2-(benz[*a*]anthryl-7-methyl)guanine which did not melt below 260°.

Anal. Calcd for $C_{24}H_{17}N_5O \cdot H_2O$: C, 70.42; H, 4.65; N, 17.12. Found: C, 70.36; H, 4.59; N, 16.45.

Reaction of [^{3}H]7-Bromomethylbenz[a]anthracene with Nucleic Acids, Polynucleotides, and Nucleosides. Since variations in nucleotide or hydrocarbon concentration have been shown to lead to no qualitative changes in the nature of the products obtained, a typical experimental protocol may be summarized. Nucleosides, nucleic acids, or polynucleotides (in the range 4–20 mg/ml) in 0.01 M sodium acetate, pH 5.5, were separately treated with 0.1 volume of an acetone solution of [^{3}H]7-bromomethylbenz[*a*]anthracene such that the concentration of this agent in the final solution was in the range 20–50 μg/ml. After approximately 15 min polynucleotides and nucleic acids were precipitated by the addition of 1.5 volumes of ethanol saturated with sodium acetate, washed with acetone, ethanol, and ether, and dried. The excess hydrocarbon derivative was not removed from the nucleoside reaction mixtures. Specific reaction conditions for individual experiments are described in the legends to Figures 2 and 3.

Degradative Procedures. Reaction products were examined only after conversion to either nucleosides or bases using the following procedures.

(A) Polyribonucleotides (4 mg/ml) were converted to mononucleotides by incubation at 37° overnight in 0.33 N KOH.

(B) Enzymic conversion of polyribonucleotides (4 mg/ml) to mononucleotides was effected by incubation at 37° overnight in 0.05 M sodium citrate buffer, pH 6: for RNA—ribonuclease A at 100 μg/ml and spleen phosphodiesterase at 100 μg/ml; for poly(A)—spleen phosphodiesterase at 100 μg/ml; for poly(G)—spleen phosphodiesterase at 100 μg/ml plus venom phosphodiesterase at 100 μg/ml.

(C) DNA (2 mg/ml) was enzymically converted to mononucleotides by incubation overnight at 37° in 0.01 M Tris buffer, pH 7, which was also in 0.01 M magnesium chloride together with deoxyribonuclease (100 μg/ml) and venom phosphodiesterase (100 μg/ml).

(D) Conversion of mononucleotides to nucleosides was effected by one of the two methods: (i) after adjusting the pH of the mononucleotide solution to 8.5, bacterial alkaline phosphatase was added to a concentration of 100 μg/ml and the solution was incubated at 37° for 3 hr; (ii) the mononucleotide solution in 0.05 M sodium citrate buffer, pH 6, was treated with crude wheat germ acid phosphatase (2 mg/ml) and incubated at 37° for a minimum of 3 hr.

(E) Deoxyribose derivatives were converted to bases by heating in 0.1 N HCl at 100° for 15 min.

(F) Ribose derivatives were converted to bases by heating in 1 N HCl at 100° for 1 hr.

Chromatography on Sephadex LH-20. The pH of the nucleoside or base solution (0.5–1.0 ml) was adjusted to pH 6 and 5 ml of methanol (containing the appropriate marker compounds) was added. Any precipitated salts were spun down, and the supernatant was applied to a column of Sephadex LH-20 (generally 75 cm × 1.5 cm diameter) in methanol. The column was eluted with methanol, and 100 fractions (*ca.* 5 ml) were collected. The optical density during elution was monitored by an LKB Uvicord and radioactivity eluted was followed by counting an aliquot (0.2–0.5 ml) of each fraction in a Packard liquid scintillation counter using a toluene–dioxane–ethanol based general purpose phosphor.

Where chromatography in this system suggested identity between a radioactive product and an added uv-absorbing marker, this identity was further established by checking that the ratio, counts per minute/optical density, was relatively constant throughout the peak. It was also established that the radioactivity and marker were not separable by thin-layer chromatography in a least three solvent systems.

Results

The basic objective of the present investigation was the elucidation of the chemistry of reaction of 7-bromomethylbenz[*a*]anthracene with nucleic acids. Since the low extent of reaction occurring under aqueous conditions precludes the isolation and subsequent characterization of the products from nucleic acids, reference compounds were prepared and compared with nucleic acid products which were detectable through the use of tritiated 7-bromomethylbenz[*a*]anthracene.

The reaction products of 7-bromomethylbenz[*a*]anthracene with cytidine, adenosine, and isopropylideneguanosine in dimethylacetamide were isolated. However, since the ultraviolet absorption of the benz[*a*]anthracene nucleus is many times more intense than that of a purine or pyrimidine, the

TABLE I: Properties of Benz[*a*]anthracene Derivatives

Compound	Ultraviolet λ_{max} (mμ) ($E_{max\ relative}$)[b,c]	R_F Values on Polygram SIL N-HR/UV (Camlab.)			
		Me_2CO	MeOH	Ethyl Acetate–MeOH (1:1)	C_6H_6–EtOH (1:1)
Benz[*a*]anthryl-7-methylpyridinium tosylate	i 325 (0.47); 339.5 (0.72); 355 (1.0); 371 (0.71); 389 (0.28)				
Benz[*a*]anthryl-7-methyl-[4-(-*p*-nitrobenzyl)]-pyridinium bromide (Dipple and Slade, 1970)	i 325 (0.48); 339.5 (0.80); 355 (1.0); 371 (0.74); 389 (0.27)				
1-(Benz[*a*]anthryl-7-methyl)adenosine hydrobromide[a]	i 327 (0.46); 340 (0.77); 357 (1.0); 374 (0.72); 392 (0.22)		0.5	0.7	
2′,3′-*O*-Isopropylidene-7-(benz[*a*]anthryl-7-methyl)guanosine hydrobromide	323 (0.45); 337 (0.78); 352 (1.0); 370 (0.74); 388 (0.20)		0.6		0.65
3-(Benz[*a*]anthryl-7-methyl)cytidine hydrobromide	i 323 (0.43); 337 (0.72); 352 (1.0); 370 (0.71); 388 (0.15)		0.55		0.3
Benz[*a*]anthryl-7-methylpiperidine (Badger and Cook, 1939)	320 (0.48); 355 (0.82); 350 (1.0); 369 (0.77); 387 (0.11)				
7-Aminomethylbenz[*a*]anthracene	321 (0.45); 335.5 (0.77); 351 (1.0); i 365 (0.62); 370 (0.66); 389 (0.09)	0.3	0.3		
N^6-(Benz[*a*]anthryl-7-methyl)adenosine (alkali-catalyzed rearrangement of 1-(benz[*a*]anthryl-7-methyl)adenosine)	321.5 (0.42); 335 (0.74); 350 (1.0); i 364 (0.64); 369 (0.72); 388 (0.11)	0.8	0.6	0.8	0.95
N^6-(Benz[*a*]anthryl-7-methyl)adenosine (7-aminomethylbenz[*a*]anthracene plus 6-chloropurine riboside)	321 (0.48); 355.5 (0.74); 350 (1.0); i 364 (0.67); 369 (0.73); 388 (0.12)	0.8	0.6	0.8	
N^6-(Benz[*a*]anthryl-7-methyl)adenine	320 (0.54); 334 (0.79); 349.5 (1.0); i 364 (0.66); 368 (0.72); 387 (0.15)	0.45	0.6	0.75	
N^2-(Benz[*a*]anthryl-7-methyl)guanine	320.5 (0.49); 335 (0.80); 350 (1.0); i 365 (0.72); 369 (0.77); 388 (0.11)	0.15	0.55		0.8

[a] Prepared as described in the Experimental Section for the hydrate but purified by chromatography in nonaqueous system as described for 3-(benz[*a*]anthryl-7-methyl)cytidine hydrobromide. [b] In methanol. [c] i indicates point of inflexion.

ultraviolet spectra of the products gave no clear confirmation of their expected structures. The spectra of all hydrocarbon-containing products were very similar to that of a 7-substituted benz[*a*]anthracene. The only region of this spectrum where differences were noted was in the 300–400-mμ range, and the spectral properties in this region are recorded in Table I, along with R_F values. Examination of these data, and those of the model compounds included, reveals differences in spectra between ring nitrogen substitution and amino group substitution. However, it can be seen from the spectrum of the piperidine derivative, which is similar to that for amino group substitution apart from the lack of a point of inflexion in the 365-mμ region, that the major spectral differences between amino group and ring nitrogen substitution are probably related to the positive charge carried on the nitrogen in the latter case.

Infrared and nmr spectra were similarly nondiagnostic, and therefore the structural assignments for the products obtained by reaction in dimethylacetamide are based largely on the method of preparation. Under the type of reaction conditions employed, Jones and Robins (1963) have shown that various methylating agents attack N-7 of guanosine and N-1 of adenosine, and Brookes and Lawley (1962) have shown that cytidine is methylated at N-3. Also, because of the difficulties encountered with the benz[*a*]anthracene derivative, we carried out a model study with benzyl bromide (Brookes *et al.*, 1968) and demonstrated that in dimethylacetamide this simple aralkyl halide did in fact attack N-7 of guanosine, N-1 of adenosine, and N-3 of cytidine. The structural assignments in the Experimental Section are reasonable, therefore, but in view of the lack of structural confirmation from spectroscopic data it seemed necessary to establish the structure of at least one of these products in a somewhat more rigorous fashion.

Accordingly the effect of alkali on the presumed 1-substituted adenosine was examined since alkali is known to convert 1-alkyladenosines into 6-alkylaminopurine ribosides (*e.g.*, Brookes and Lawley, 1960; Macon and Wolfenden, 1968; Fleysher *et al.*, 1969), and the results of this investigation are summarized in Figure 1. 1-(Benz[*a*]anthryl-7-methyl)-adenosine (I) was converted by alkali into II which was identical in composition with I but which differed from I in melting point, ultraviolet spectrum, R_F value on thin layer chromatograms, position of elution from Sephadex LH-20 (Figure 2b), and mass spectrum. The identification of II as N^6-(benz[*a*]anthryl-7-methyl)adenosine was confirmed by demonstrating that II was also obtained by reaction of 7-aminomethylbenz[*a*]anthracene with 6-chloropurine ribonucleoside. Fur-

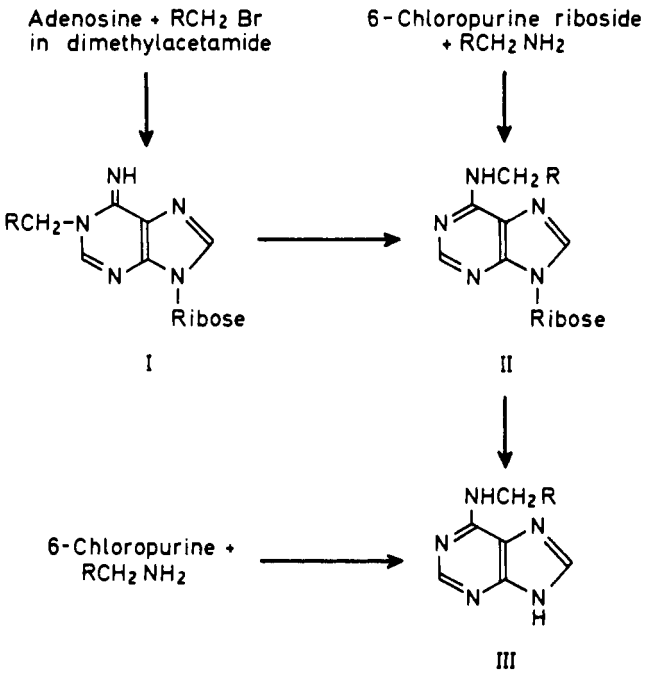

FIGURE 1: Chemistry of 7-bromomethylbenz[*a*]anthracene adenosine products (RCH_2 = benz[*a*]anthryl-7-methyl.

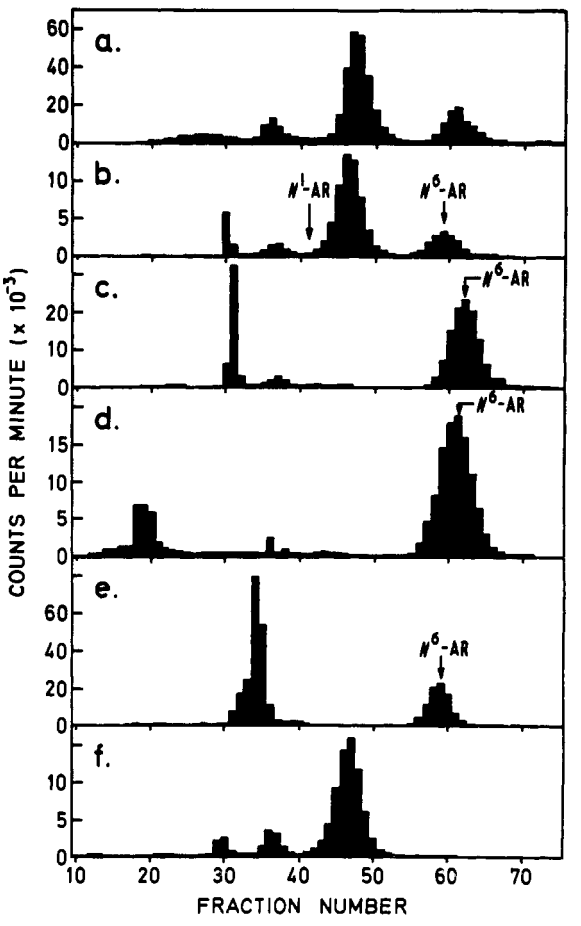

FIGURE 2: Column chromatography on Sephadex LH-20 eluted with methanol of reaction products of [^{3}H]7-bromomethylbenz[*a*]anthracene with polynucleotides and their constituents. (a) Purified commercial yeast RNA (20 mg/ml) was treated with [^{3}H]7-bromomethylbenz[*a*]anthracene (0.05 mg/ml) and then converted to nucleosides by degradative procedures A and Di (see Experimental Section); (b) RNA products converted to nucleosides by degradative procedures B and Dii; (c) sodium polyadenylate (10 mg/ml) was treated with [^{3}H]7-bromomethylbenz[*a*]anthracene (0.02 mg/ml) and converted to nucleosides by procedures B and Dii; (d) poly(A) products were converted to nucleosides by procedures A and Di; (e) adenosine (4 mg/ml) was treated with [^{3}H]7-bromomethylbenz[*a*]anthracene (0.05 mg/ml), no degradation necessary; (f) sodium polyguanylate (5 mg/ml) was treated with [^{3}H]7-bromomethylbenz[*a*]anthracene (0.02 mg/ml) and converted to nucleosides by procedures B and Dii. The arrows represent the position of maximum ultraviolet absorption of added reference compounds. N^1-AR designates 1-(benz[*a*]anthryl-7-methyl)adenosine, N^6-AR designates N^6-(benz[*a*]anthryl-7-methyl)adenosine.

thermore, acid hydrolysis converted II into III, and III could also be obtained by reaction of 7-aminomethylbenz[*a*]anthracene with 6-chloropurine and, therefore, would be expected to have the structure designated in Figure 1, *i.e.*, N^6-(benz[*a*]anthryl-7-methyl)adenine.

Since this investigation convincingly showed that, in dimethylacetamide, 7-bromomethylbenz[*a*]anthracene reacted with adenosine at the same site as did benzyl bromide and other alkylating agents, it is not unreasonable to assume that a similar situation obtained for the reaction with cytidine and isopropylideneguanosine.

The isopropylideneguanosine product of itself was of no value as a marker for the nucleic acid studies but was smoothly converted by acid into the 7-substituted guanine derivative. An interesting chemical observation was the conversion of the cytidine product by acid into cytidine and 7-hydroxymethylbenz[*a*]anthracene. This type of decomposition does not appear to have been observed previously for other alkylated cytidines, although an analogous cleavage of 1-(3-methylbut-2-enyl)adenine has been reported (Martin and Reese, 1968).

Somewhat surprisingly, none of the reference compounds, prepared by reaction of the nucleosides in dimethylacetamide with 7-bromomethylbenz[*a*]anthracene, appeared to correspond to the products obtained when nucleic acids, polynucleotides, or even nucleosides were treated with [^{3}H]7-bromomethylbenz[*a*]anthracene in aqueous solution. For example, the position of elution from Sephadex LH-20 of 1-(benz[*a*]anthryl-7-methyl)adenosine is illustrated in Figure 2b, and 3-(benz[*a*]anthryl-7-methyl)cytidine was eluted in a similar position while 7-(benz[*a*]anthryl-7-methyl)guanine was not eluted in the first 100 fractions. The latter should be compared to the elution of radioactivity in acid-treated DNA or deoxyguanosine (Figures 3b and 3c).

Figure 2a shows that reaction of [^{3}H]7-bromomethylbenz[*a*]anthracene with RNA gave rise to several radioactive products, separable by chromatography on Sephadex LH-20. We centered our attention on the last three radioactive peaks observed, since these were always reproducibly obtained, while this was not the case for radioactivity eluted before these peaks. In fact, it was shown that the sharp peak eluted near fraction 30 (*e.g.*, in Figures 2b and 2c) could be recovered and converted by further enzymic digestion into the products eluting in fractions 35–65.

When authentic markers of possible adenosine products were run in the same chromatogram as the radioactive RNA products (Figure 2b), it was seen that, while the elution of the last radioactive peak was exactly coincident with that of the ultraviolet absorption of the N^6-(benz[*a*]anthryl-7-methyl)-adenosine, no radioactivity was eluted together with the 1-substituted adenosine. It is known that N^6-substituted adenosines are readily obtained by treatment of 1-substituted adenosines with alkali (Brookes and Lawley, 1960), but the pH

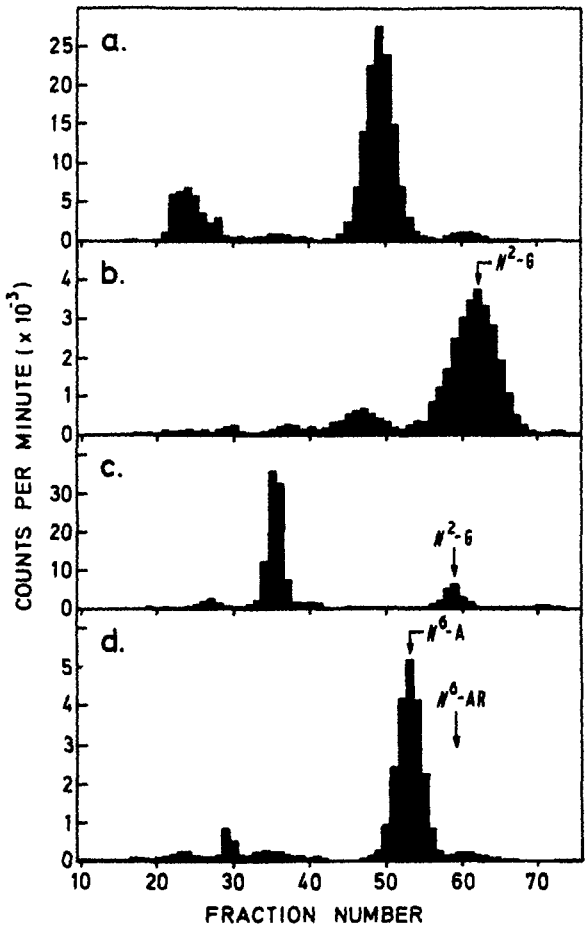

FIGURE 3: Column chromatography on Sephadex LH-20 eluted with methanol of reaction products of [^{3}H]7-bromomethylbenz[*a*]anthracene with polynucleotides and their constituents. (a) Commercial salmon sperm DNA (5 mg/ml) was treated with [^{3}H]7-bromomethylbenz[*a*]anthracene (0.02 mg/ml) and converted to nucleosides by procedures C and Di (see Experimental Section); (b) the main radioactive peak from chromatogram 2a was evaporated to dryness, treated with acid to remove the sugar residue (procedure E), and rechromatographed; (c) deoxyguanosine (4 mg/ml) was treated with [^{3}H]7-bromomethylbenz[*a*]anthracene (0.05 mg/ml) and then treated with acid to remove the sugar residue (procedure E); (d) the fractions comprising the radioactive peak eluted at fraction 62 in Figure 2c were pooled, evaporated to dryness, and treated with acid to remove the sugar residue (procedure F). N^6-(Benz[*a*]anthryl-7-methyl)adenosine reference compound was added again to this acid digest after neutralization. The arrows indicate the position of maximum ultraviolet absorption of added reference compounds. N^2-G designates N^2-(benz[*a*]anthryl-7-methyl)guanine, N^6-AR designates N^6-(benz[*a*]anthryl-7-methyl)-adenosine, and N^6-A designates N^6-(benz[*a*]anthryl-7-methyl)-adenine.

of the solution containing the radioactive products of Figure 2b was never higher than pH 6.

It was similarly shown that the major product of reaction of 7-bromomethylbenz[*a*]anthracene with poly(A) was the N^6-substituted adenosine derivative (Figure 2c and 2d) and that this did not depend upon the pH maintained during the degradative procedures. Also, reaction with adenosine in aqueous solution yielded this same product (Figure 2e), in complete contrast to the fact that reaction of 7-bromomethylbenz[*a*]anthracene with adenosine in dimethylacetamide yielded the 1-substituted adenosine as the major product. The major radioactive peak in Figure 2e corresponds to 7-hydroxymethylbenz[*a*]anthracene (arising from hydrolysis of 7-bromomethylbenz[*a*]anthracene) since in the reactions with nucleosides no steps were taken to purify the radioactive products prior to chromatography.

Figure 2f illustrates the elution profile of the nucleoside products obtained from poly(G) reacted with [^{3}H]7-bromomethylbenz[*a*]anthracene. Although an appropriate authentic marker was not available, it can be seen that the major radioactive product from poly(G) was eluted in a position similar to the major radioactive product from RNA, which strongly suggests identity between these two products.

Furthermore, when the nucleoside products from DNA reacted with [^{3}H]7-bromomethylbenz[*a*]anthracene were examined by chromatography on Sephadex LH-20, the major radioactive product was again eluted in the same region of the chromatogram (Figure 3a) as the guanosine products above, even though the sugar residue in the two cases was of course different.

Although the amino groups of the heterocyclic bases are not usually reactive toward alkylating agents, they are attacked by acylating agents (*e.g.*, Schaller and Khorana, 1963; Shapiro *et al.*, 1969) and by phenyl isocyanate (Jones and Warren, 1970). Since 7-bromomethylbenz[*a*]anthracene resembles these reagents in its attack on adenine derivatives under aqueous conditions, it seemed plausible that it would also attack the amino groups of guanine and cytosine residues under aqueous conditions. Therefore, a synthesis of N^2-(benz[*a*]anthryl-7-methyl)guanine, from 2-chloro-6-hydroxypurine and 7-aminomethylbenz[*a*]anthracene (see Shaprio *et al.*, 1969), was undertaken and the product was compared with the major radioactive product from DNA. Thus, when the major peak from DNA (Figure 3a) was recovered and treated with acid to remove the deoxyribose residue it was found (Figure 3b) that the resultant radioactive product was eluted in coincidence with authentic N^2-(benz[*a*]anthryl-7-methyl)guanine. The same product also resulted from acid treatment of the product of reaction of 2′-deoxyguanosine with [^{3}H]7-bromomethylbenz[*a*]anthracene in aqueous solution (Figure 3c), again in sharp contrast to the main product obtained by reaction in dimethylacetamide. The early radioactive peak in Figure 3c is again 7-hydroxymethylbenz[*a*]anthracene. Figure 3d presents the elution profile obtained when the N^6-substituted adenosine peak from Figure 2c was treated with acid to remove the sugar and subsequently rechromatographed. Again, coincidence between the radioactivity and ultraviolet absorption from the marker was observed.

Essentially the same result as that depicted in Figure 3d was obtained when the last peak from a DNA, digested to nucleosides, was recovered and treated with mild acid (Experimental Section E). Thus, it was shown that reaction with both DNA and RNA resulted in attack on the 6-amino group of adenine residues.

In all cases where the elution from Sephadex LH-20 chromatograms of radioactive products and ultraviolet-absorbing reference compounds were compared, the ratio of radioactivity to optical density at 290 mμ in each fraction throughout the peak was routinely determined. The variation of this ratio was never greater than that expected from estimated experimental error. The actual values recorded for the N^6-(benz[*a*]anthryl-7-methyl)adenosine of Figure 1b, the N^2-(benz[*a*]anthryl-7-methyl)guanine of Figure 2b, and the N^6-(benz[*a*]anthryl-7-methyl)adenine of Figure 2d are presented in Table II.

Radioactive products together with reference compounds eluted in coincidence from Sephadex LH-20 were also examined by thin-layer chromatography in at least three solvent

systems, and the radioactivity (determined by cutting the plastic-coated strips into 1-cm bands and counting these in general purpose phosphor) and uv-absorbing areas were coincident in all cases.

The major radioactive nucleoside product from the reaction of poly(C) with [^{3}H]7-bromomethylbenz[*a*]anthracene did not correspond with the 3-substituted cytidine marker and was in fact eluted in fractions 35–40, together with the minor RNA product. This product is as yet unidentified, but might well be expected to have arisen from attack on the amino group of cytosine.

It would appear, therefore, that the major site of reaction of 7-bromomethylbenz[*a*]anthracene on nucleic acids in aqueous solution is the 2-amino group of guanine. This is accompanied by a less extensive attack on the 6-amino group of adenine and possibly by a small extent of reaction on the 4-amino group of cytosine.

TABLE II: Comparison of the Elution of Radioactive Products and Authentic Reference Compounds.

Reference Compound	Fraction No.	cpm/OD_{290} $\times 10^{-3}$
N^6-(Benz[*a*]anthryl-7-methyl)adenosine (from Figure 1b)	57	2.04
	58	1.95
	59	2.01
	60	1.90
	61	2.10
	62	2.02
N^2-(Benz[*a*]anthryl-7-methyl)guanine (from Figure 2b)	58	3.00
	59	3.28
	60	3.27
	61	3.27
	62	3.29
	63	3.15
N^6-(Benz[*a*]anthryl-7-methyl)adenine (from Figure 2d)	50	10.6
	51	10.1
	52	10.1
	53	9.96
	54	10.0
	55	9.54

Discussion

A number of interesting points are raised by these findings. The first of these is that the reaction of 7-bromomethylbenz[*a*]anthracene with nucleosides in dimethylacetamide leads mainly to attack on the ring nitrogens of the heterocyclic bases, while in aqueous solution the major sites of attack are the extranuclear amino groups. This solvent-mediated change in reaction path is not general for all alkylating agents. For example, dimethyl sulfate attacks guanosine derivatives mainly on N-7 in both dimethylacetamide (Jones and Robins, 1963) and water (Lawley, 1957). However, the chemistry of dimethyl sulfate and 7-bromomethylbenz[*a*]anthracene is quite different. The former compound typifies alkylating agents which tend to react through kinetically second-order processes, while the latter can undergo a first-order hydrolysis many times more readily even than benzyl bromide (Dipple and Slade, 1970) presumably because of the ease with which it can form an ionized intermediate. It seems reasonable, therefore, that the change of solvent from dimethylacetamide to water has little effect on the structure of the dimethyl sulfate reactive intermediate (either covalent substrate or close ion pair (Sneen and Larsen, 1969)) and that in either solvent the overall reaction could be represented by a nucleophilic attack of, say, guanosine on relatively covalent dimethyl sulfate, leading to 7-substitution. While the reaction of 7-bromomethylbenz[*a*]anthracene with guanosine derivatives in dimethylacetamide could be similarly described, it would appear that this reaction in water involves an intermediate of greater ionic character and that the overall reaction in this case could be better represented by an electrophilic attack of this intermediate on the guanosine derivative. This apparently leads to attack on the amino group in preference to the ring nitrogen, as is found in the reactions of acylating agents with guanine (Shapiro *et al.*, 1969). The substitution on the amino group of course leads to an electrically neutral species through the loss of a proton, and this, which is clearly important in acylation, could also be of significance in determining the reaction site for 7-bromomethylbenz[*a*]anthracene. This type of rationalization of the experimental data is far removed from a complete understanding of the detailed mechanisms involved but, nevertheless, has a certain appeal in that it would be consistent with Miller's (1970) generalization that all chemical carcinogens may be (or may be metabolically converted to) strong electrophilic reactants.

The possible biological implications of our data are also of interest. Certainly it would appear that the amino groups of the heterocyclic bases in nucleic acids should receive more consideration than has been given in the past as receptor sites for carcinogenic, mutagenic, and cytotoxic chemicals. Loveless (1969) has already introduced the O-6 of guanine as a possible receptor site for mutagenic and carcinogenic alkylating agents, and while it is conceivable that further investigations may link the mechanisms of action of various chemical carcinogens even more closely together, there is no good reason at present to assume that the same receptor sites need be involved in all cases.

Acknowledgments

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Corn and Potato α-1,4-Glucan:α-1,4-Glucan 6-Glycosyltransferase: Evidence for Separate Hydrolytic and Branching Components*

H. L. Griffin† and Y. Victor Wu

ABSTRACT: The hydrolytic and branching activities of branching enzyme preparations separated on DEAE-cellulose result from the functions of two separate enzymes. Two fractions can be separated from the DEAE-cellulose preparations by zonal ultracentrifugation or by membrane ultrafiltration. A high molecular weight fraction (M = 70,000) hydrolyzes amylose. The nature of this enzyme depends on its source. That from potato branching enzyme, like β-amylase, stops near branch points. That from dent and waxy corn, like α-amylase, can bypass branch points. A lower molecular weight fraction ($M \approx$ 20,000) introduces barriers to β-amylolysis in amylose without apparent hydrolysis. Composites of the two enzyme fractions will reproduce the effect of the original DEAE-cellulose branching enzymes on the structure of amylose.

The branching enzyme (α-1,4-glucan:α-1,4-glucan 6-glucosyltransferase, EC 2.4.1.18) belongs to a group of enzymes involved in the formation of storage polyglucans. This group of enzymes "branches" linear maltodextrins. Amylose, the exclusively α-1,4-glucosyl-linked polymer, is converted to amylopectin, an α-1,6-glucosyl-branched polymer, by the branching enzyme of the potato (Peat *et al.*, 1953; Barker *et al.*, 1950). The branching enzyme consists of an α-1,4-glucosidic hydrolytic activity and an α-1,6-glucosidic branching activity which either consecutively (Manners, 1962) or independently (Bourne and Peat, 1945; Barker *et al.*, 1950; Geddes and Greenwood, 1969) accomplish this conversion. Our work has supported the independent conversion.

We examined the physical nature of the associated hydrolytic and branching activities of potato and corn DEAE-cellulose branching enzyme preparations (Griffin and Wu, 1968), that is, whether they are separate functions of one two-headed enzyme or separate enzymes of a functional complex. To accomplish this study, branching enzyme preparations have been isolated from immature potato, dent, and waxy corn by the DEAE-cellulose method of Griffin and Wu (1968). The molecular weight, enzymic action on amylose, and chromatographic behavior of the corn preparations are compared with each other and with those of the DEAE-cellulose potato branching enzyme. Finally, the DEAE-cellulose branching enzymes were fractionated by zonal ultracentrifugation in a linear sucrose gradient and by Diaflo[1] ultrafiltration. We isolated two distinct components. Their effect on the structure of amylose is described and compared to that of the original DEAE-cellulose branching enzyme preparations.

Experimental Section

Isolation of Corn Juice Solubles. At 21 days after hand pollination kernels of hybrid waxy and ordinary dent corn (grown by Bear Hybrid Corn Co., Inc.) were cut from cobs immediately after snapping and husking the ear. The kernels were ground to a pulp in a hand corn mill. Cellular debris was removed from the juice by filtering it through a nylon bolting cloth. Starch was removed by centrifuging in an International table top centrifuge. The clarified juice was immediately frozen in a Dry Ice–acetone mixture and stored in Dry Ice while being transported to in-house lyophilization equipment. There the clarified juice was lyophilized at less than 0.1 mm Hg. The lyophilized juice was stored at −18° for subsequent column chromatography. About 1.0-g portions of the stored corn juice solubles were suspended in 10 ml of aqueous 0.20

* From the Northern Regional Research Laboratory, Agricultural Research Service, U. S. Department of Agriculture, Peoria, Illinois 61604. *Received March 15, 1971.*
† To whom correspondence should be addressed.

[1] Mention of firm names or trade products does not imply that they are endorsed or recommended by the Department of Agriculture over other firms or similar products not mentioned.