Interaction of Nucleic Acids. V. Chemical Linkage of 3,4-Benzpyrene to Deoxyribonucleic Acid in Aqueous Solution*

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ABSTRACT: Chemical reactions between the carcinogen, 3,4-benzpyrene, and deoxyribonucleic acid have been carried out in neutral, aqueous solution and at room temperature. Data presented are consistent with the formation of a covalent linkage between 3,4-benzpyrene and deoxyribonucleic acid in these mild conditions. This chemical linkage has been induced by reacting the physical complex with iodine, hydrogen peroxide in the presence and absence of ferrous ion, and with the ascorbic acid model hydroxylating system. Up to 40% of the physically bound 3,4-benzpyrene can be linked covalently to the deoxyribonucleic acid under appropriate conditions. In these same reactions, a noncarcinogenic isomer, 1,2-benzpyrene, is linked to deoxyribonucleic acid only to a very limited extent.

The covalently linked benzpyrene cannot be extracted by organic solvents which remove over 99% of the physically bound benzpyrene from deoxyribonucleic acid. The chemical linkage was further characterized by sucrose gradient electrophoresis and gel filtration of the chemical complex before and after enzymic hydrolysis. The present data suggest that benzpyrene free radicals (possibly cationic) may be involved as intermediates in the reactions.

Ever since the discovery of the carcinogenicity of certain polycyclic hydrocarbons, workers in the field have been confronted with the challenging question about the origin of the biological specificity of these compounds. For instance, 3,4-benzpyrene is a potent carcinogen while the isomeric 1,2-benzpyrene is not. This question must be related to the basic mechansim of carcinogenesis of these compounds which would in turn explain how these relatively inert polycyclic hydrocarbons exert their biological effect.

During the last few years several laboratories, including our own, have studied the physical binding of polycyclic hydrocarbons such as 3,4-benzyprene and 1,2-benzyprene to nucleic acids (Boyland and Green, 1962; Liquori *et al.*, 1962; Lerman, 1964; Ts'o and Lu, 1964; Ball *et al.*, 1965; Kodama *et al.*, 1966; Isenberg *et al.*, 1967; Lesko *et al.*, 1968). Our studies indicate that 3,4-benzyprene stacks with the bases of nucleic acid *via* a face-to-face mode (Lesko *et al.*, 1968). These findings corroborate the intercalation model of binding which has been proposed previously (Boyland and Green, 1962), and are supported also by other observations (Liquori *et al.*, 1962; Lerman, 1964; Nagata *et al.*, 1966; Green and McCarter, 1967). Comparison of binding with native DNA of carcinogenic hydrocarbons and one of their structural isomers (3,4-benzpyrene and 1,2-benzpyrene; 1,2,5,6-dibenzanthracene and 1,2-3,4-dibenzanthracene) led to nearly identical binding constants for the carcinogenic and noncarcinogenic members of each pair (Lesko *et al.*, 1968). Apparently, these polycyclic hydrocarbons show no specificity in their physical binding to DNA, and hence, this type of interaction cannot be correlated with carcinogenicity.

Chemical complexes of polycyclic carcinogens with DNA have been found in biological systems (Heidelberger and Davenport, 1961; Brookes and Lawley, 1964), and the available data indicate that the extent of complex formation of various compounds may be correlated with the carcinogenicity of these compounds (Brookes and Lawley, 1964). Ts'o and Lu (1964) have reported that a 3,4-benzpyrene–deoxyribonucleic acid chemical complex could be formed efficiently in aqueous solution at neutral pH and room temperature when the physical complex was photoirradiated at wavelengths above 320 mμ at the absorption band of benzpyrene. Rapaport and Ts'o (1966) have achieved a chemical linkage between 3,4-benzpyrene and DNA by X-ray irradiation of the physical complex. The success of this experiment suggested that the chemical reaction between 3,4-benzpyrene and DNA may be activated by hydroxyl radicals formed during X-ray irradiation. Rochlitz (1967) reported that a covalent linkage between 3,4-benzpyrene and pyridine had been induced by iodine vapor in a pyridine solid-phase system. Here, we wish to report the observations indicating the formation of 3,4-benzpyrene–DNA chemical linkage in an aqueous, neutral solution at 0–50° induced by peroxide, iodine, and the ascorbic acid model hydroxylating system of Udenfriend *et al.* (1954). Little or no 1,2-benzpyrene–DNA chemical complex formation was found under identical conditions. Thus, the chemical reactivity of this pair of isomers with DNA in the present aqueous systems can be correlated with their carcinogenicity. Preliminary results on certain parts of this work have previously been reported (Umans *et al.*, 1968, 1969).

Materials

[^{3}H]3,4-Benzpyrene with a specific activity of 500 mCi/mmole was purchased from Amersham–Searle Corp. Radiochemical purity, based on chromatographic assay, was reported to be 99% by the manufacturer. Chromatographic examination of two batches confirmed this report (Ts'o and Lu, 1964). 1,2-Benzpyrene (242 mCi/mmole purity greater than 98%) and [^{3}H]1,2,3,4-dibenzanthracene (306 mCi/mmole, pur-

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ity greater than 96%) were also purchased from Amersham–Searle. The hydrocarbons, received in benzene solution, were lyophilized and used dry. Highly polymerized calf thymus DNA was purchased from Sigma Chemical Corp. This preparation of DNA had a $s_{20,50\%}$ value of 13.5 S and a hyperchromicity of 35–40% when heated above the melting temperature after shaking for 10 days, the time required to maximize the physical binding of benzpyrene to DNA (Lesko *et al.*, 1968).

DNA solutions were made up in 1×10^{-2} M phosphate buffer at pH 6.8 which consisted of Na_2HPO_4 (0.0025 M) and NaH_2PO_4 (0.005 M). The DNA concentration was calculated from a molar extinction coefficient of 6.6×10^3, the absorbancy at 259 mμ of a solution containing 1 mole of DNA phosphorous/l. of phosphate buffer. DNA was denatured in phosphate buffer by heating in boiling water for 10 min, after which the solution was quick cooled in ice for 30 min.

The ascorbic acid hydroxylating system (Boyland *et al.*, 1964) was prepared as follows: 41.7 mg of $FeSO_4$, 280 mg Na_2EDTA, and 280 mg of ascorbic acid were suspended in 2 ml of 0.2 M KH_2PO_4 and then 25 ml of 0.2 M Na_2HPO_4 was added to a final pH of 6.6. Upon use, this solution was diluted 1:1 with a DNA–benzpyrene physical complex in 1×10^{-2} M phosphate buffer.

Experimental Procedures

Preparation and Treatment of Physical Complex. The [^{3}H]-hydrocarbon–DNA physical complexes were prepared in 1×10^{-2} M phosphate buffer and the radioactivity was measured as previously described (Lesko *et al.*, 1968). To induce chemical linkage, an aqueous solution of I_2 or H_2O_2 (with or without $FeCl_2$) was added to the physical complex. Final concentration of I_2 and H_2O_2 were 1×10^{-4} and 1.5×10^{-2} M, respectively; $FeCl_2$ was added to a final concentration of 1×10^{-3} M. To solutions without $FeCl_2$, sodium citrate (pH 7.0) was added to 1×10^{-2} M in order to chelate any trace metals. All work was conducted in the dark when possible. After incubation at varying lengths of time and desired temperature, the DNA was precipitated with 95% ethanol in the presence of $NaClO_4$ or NH_4Ac at 0° and the precipitate was washed exhaustively with 95% ethanol and ether as previously described (Ts'o and Lu, 1964). After removal of the ether *in vacuo*, the DNA precipitate was redissolved in 1×10^{-2} M phosphate buffer for measurement of radioactivity and concentration of DNA. The DNA precipitates from reaction mixtures containing $FeCl_2$ were redissolved in 1×10^{-2} M EDTA and allowed to stand overnight at room temperature. This DNA was then precipitated again as described above and washed three times with 95% ethanol and two times with ether, dried, and treated as above. When the chemical linkage was induced with ascorbic acid, equal volumes of hydroxylating system and physical complex were combined in a stoppered vessel with a long chimney and oxygen was bubbled through the solution. After incubation for varying lengths of time at room temperature, the DNA was precipitated with cold $HClO_4$ (final concentration was 0.2 N). The precipitate was washed once with 0.2 N $HClO_4$ and then three times with 95% ethanol and two times with ether. After drying *in vacuo*, the residue was redissolved in 1×10^{-2} M phosphate buffer and precipitated again with 95% ethanol at 0°. The precipitate was then treated as above in the I_2 reaction. This washing procedure removed over 99% of the tritium-labeled hydrocarbons from the physical complex (Ts'o and Lu, 1964). However, when the tritiated hydrocarbons were chemically linked to DNA, they could no longer be extracted by the above procedure. The extent of chemical reaction is expressed as the percentage of hydrocarbon physically bound converted into hydrocarbon chemically linked to DNA.

Procedure for Enzymic Hydrolysis. The [^{3}H]3,4-benzpyrene–DNA chemical complex was hydrolyzed to nucleotides with DNase I and snake venom phosphodiesterase and to nucleosides with bacterial alkaline phosphatase as previously described (Rapaport and Ts'o, 1966).

Sucrose Gradient Electrophoresis. The [^{3}H]3,4-benzpyrene–DNA chemical complex was subjected to sucrose gradient electrophoresis in 3.3×10^{-3} M phosphate buffer (pH 6.8). Nucleotides and nucleosides produced by enzymic hydrolysis of the chemical complex were subjected to electrophoresis in 5×10^{-3} M sodium citrate buffer (pH 3.5). The apparatus and procedure have been described previously (Lesko *et al.*, 1968).

Gel Filtration Chromatography. The [^{3}H]3,4-benzpyrene–DNA chemical complex and nucleotide hydrolysates of the chemical complex were examined by gel filtration through Sephadex G-200; 1 ml of complex (absorbance at 259 mμ about 10) was layered on a 1.4×32 cm column and eluted with 1×10^{-2} M phosphate buffer at a flow rate of 8 ml/hr; 2-ml fractions were collected and absorbance and radioactivity were measured.

Photoirradiation of Physical Complexes. The apparatus and procedure for photoirradiation have been described previously (Ts'o and Lu, 1964). In these experiments, the [^{3}H]hydrocarbon–DNA physical complexes were photoirradiated for 1 hr and cooled by circulation of ice–water through a jacketed vessel.

Results

Photoirradiation. Preliminary experiments have been done to determine if the extent of chemical linkage induced by photoirradiation can be correlated with the carcinogenicity of aromatic polycyclic hydrocarbons. For the same dosage of photoirradiation at wavelengths above 300 mμ, the percentage of conversion from a physical complex into a chemical linkage was about fourfold higher for 3,4-benzpyrene–DNA complexes than for 1,2-benzpyrene complexes. In the case of 1,2,3,4-dibenzanthracene–DNA complexes *vs.* 1,2,5,6-dibenzanthracene–DNA complexes, the percentage of conversion in both cases was similar to that of the 1,2-benzpyrene complex, about 20%, and less than that of the 3,4-benzpyrene complex. These results are not unexpected since the absorption of light at wavelengths above 320 mμ by 1,2-benzpyrene, 1,2,3,4-dibenzanthracene, and 1,2,5,6-dibenzanthracene is much less than that of 3,4-benzpyrene. More definite conclusions about the selective activation of these polycyclic hydrocarbons by photoirradiation must await quantitative experiments based on quantum yield calculations.

Chemical Linkage Induced by Iodine. Figure 1 shows the rate of conversion of a physical complex of 3,4-benzpyrene–native DNA into a chemical complex in the presence of 1×10^{-4} M I_2 at room temperature in 1×10^{-2} M phosphate buffer. The reaction is almost over in 30 min and reaches a plateau at 2–6 hr. Prolonged incubation up to 24 hr results in a loss in the amount of [^{3}H]3,4-benzpyrene linked to DNA as assayed

TABLE I: Percentage of Physically Bound Benzpyrene that Becomes Chemically Linked to DNA When Induced by 1×10^{-4} M Iodine in Aqueous Solution.[a]

	Room Temp	0°
3,4-Benzpyrene		
Native	5.2, 6.2, 10.4	6.1, 5.7
DNA	9.8, 11.5	
Denatured	32.1, 31.5	29.2, 23.0
DNA	31.5	
1,2-Benzpyrene		
Native	0.7, 0.5, 1.3	
DNA	0.5	
Denatured	0.7, 0.4, 1.2	
DNA	0.6	

[a] In these experiments 0.05 ml of 10^{-3} M I_2 was added to 0.45 ml of DNA–benzpyrene physical complex dissolved in 1×10^{-2} M phosphate buffer. The reaction mixtures were incubated at indicated temperatures for 2 hr after which the DNA was precipitated by adding 0.05 ml of 4 M NH_4Ac and 1.5 ml of 95% ethanol followed by cooling for 1 hr in ice. The precipitate was washed three times with 95% ethanol and twice with ether. After vacuum drying the residue was dissolved in 1 ml of 1×10^{-2} M phosphate buffer and radioactivity and absorbance at 259 mμ were measured.

by organic solvent extraction, indicating instability of the products. Table I shows the specificity of chemical complex formation, the effect of temperature, and the influence of conformational states of DNA in the I_2 reaction. As shown, the I_2-induced linkage is very specific; 3,4-benzpyrene becomes chemically linked to DNA while 1,2-benzpyrene reacts only to a very limited extent. The values shown for 1,2-benzpyrene are about the same as background level values from incubation mixtures containing no I_2. The percentage of conversion of 3,4-benzpyrene from physically bound to chemically linked was much higher with denatured DNA (about 30%) than for native DNA (5–10%). The variation in the native DNA values may be due to differing degrees of denaturation in the native samples. The reaction appears to be relatively independent of temperature; the small effect observed with denatured DNA may be due to the influence of temperature on the conformation. Here, for the first time, is an effective procedure for linking 3,4-benzpyrene to DNA under mild conditions. The reaction is highly specific and the extent of linkage can be correlated with the carcinogenicity of this pair of isomers, *i.e.*, carcinogenic 3,4-benzpyrene is linked to DNA while noncarcinogenic 1,2-benzpyrene is not linked to any great extent. As shown below, essentially the same results were obtained for the reaction with H_2O_2.

Chemical Linkage Induced by H_2O_2. Figure 2 shows the rate of conversion of a physical complex of 3,4-benzpyrene–denatured DNA into a chemical complex in a reaction mixture containing 1.5×10^{-2} M H_2O_2 at 37° in the presence and in the absence of 1×10^{-3} M $FeCl_2$. As indicated, the presence of ferrous ion greatly enhances the rate and the extent of the reaction. The reaction proceeds at a higher rate for the first

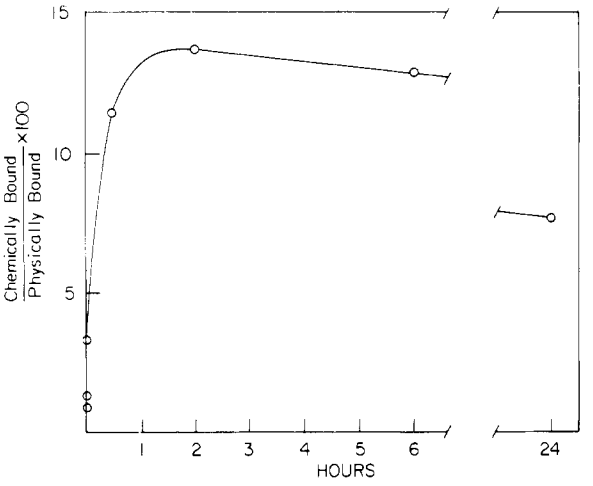

FIGURE 1: Kinetics of the I_2-induced chemical linkage of 3,4-benzpyrene to native DNA at room temperature in 1×10^{-4} M I_2.

3 hr and then gradually levels off leading to a 40% conversion after 24 hr. In Figure 3 is shown the percentage of physically bound 3,4-benzpyrene that becomes chemically linked to denatured DNA as a function of H_2O_2 concentration after incubation for 1 hr at 37° in the presence of 1×10^{-3} M $FeCl_2$. Analysis of these data led us to use a concentration of 1.5×10^{-2} M H_2O_2 in our experiments since this appears to be the saturation level. As shown in later paragraphs, H_2O_2 can lead to degradation of DNA and hence, the concentration of H_2O_2 should be kept as low as possible. The rate and the extent of the H_2O_2-induced reaction can be correlated with carcinogenicity as shown in Figure 4 for denatured DNA and Figure 5 for native DNA. The data clearly indicate that this H_2O_2-induced reaction is highly specific, *i.e.*, carcinogenic 3,4-benzpyrene becomes chemically linked to DNA while noncarcinogenic 1,2-benzpyrene is linked to only a limited extent. The reaction proceeds more rapidly and more extensively with denatured DNA than with native DNA. The effect of temperature on the H_2O_2-induced reaction is shown in Table II.

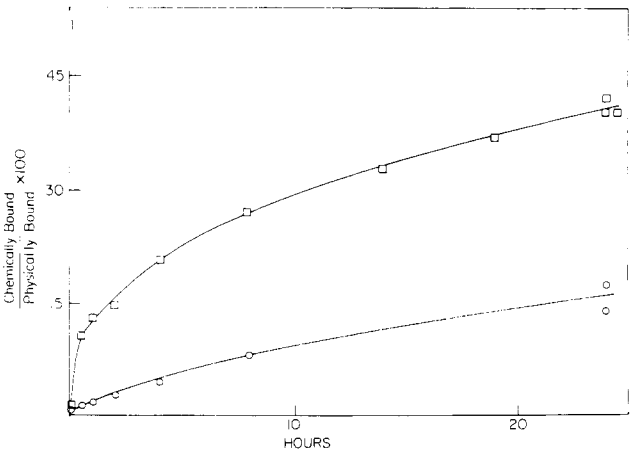

FIGURE 2: Kinetics of the H_2O_2-induced chemical linkage of 3,4-benzpyrene to denatured DNA at 37° in the presence and in the absence of 1×10^{-3} M $FeCl_2$ with 1.5×10^{-2} M H_2O_2. (□–□–□) 1×10^{-3} M $FeCl_2$ and (○–○–○) 1×10^{-2} M sodium citrate.

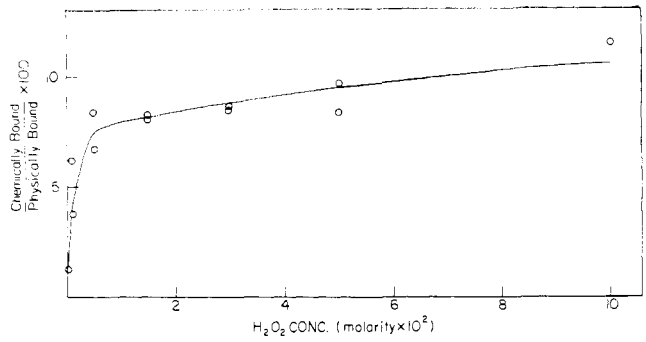

FIGURE 3: Effect of H_2O_2 concentration on the chemical linkage of 3,4-benzpyrene to denatured DNA in 1×10^{-2} M phosphate buffer. Reaction mixtures (1 ml) were run in duplicate and incubated 1 hr at 37° with 1×10^{-3} M $FeCl_2$.

The data suggest that the mild temperature effect is an indirect one, *i.e.*, the temperature variation exerts its effect on the conformational states of DNA which have a strong influence on the reaction.

Chemical Linkage Induced by Ascorbic Acid Model Hydroxylating System. Figure 6 shows the rates of conversion of physical complexes of 3,4-benzpyrene–denatured DNA and 1,2-benzpyrene–denatured DNA into chemical complexes in reaction mixtures which contained 6.75×10^{-3} M EDTA, 1.38×10^{-3} M $FeSO_4$, and 1.46×10^{-2} M ascorbic acid at room temperature. The rate of conversion of 3,4-benzpyrene starts slowly, proceeds at a rapid rate from 2 to 7 hr, and then levels off to a value of 42% after 24 hr. The reaction is also specific with 32% of the physically bound 3,4-benzpyrene being chemically linked while only 7% of the 1,2-benzpyrene becomes chemically linked after 7 hr. This value of 7% for 1,2-benzpyrene is significantly higher than that observed in the I_2- and H_2O_2-induced reactions (1–2%). Since the reaction in the standard model hydroxylating system was carried out at a higher ionic strength than that of the H_2O_2 system (0.1 M salt compared to 0.01 M salt), we studied the linkage of 1,2-benzpyrene to denatured DNA with the model hydroxylating system at a lower salt concentration (0.027 M). The results obtained in the low salt reaction were similar to those observed

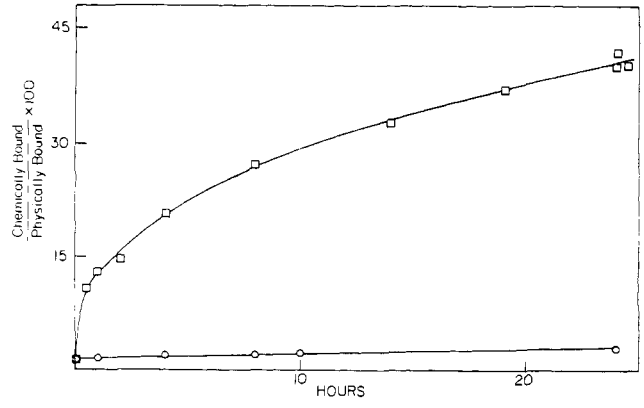

FIGURE 4: Comparison of the kinetics of the H_2O_2-induced chemical linkage of 1,2-benzpyrene and 3,4-benzpyrene with denatured DNA in 1.5×10^{-2} M H_2O_2–1×10^{-3} M $FeCl_2$ at 37°, 1×10^{-2} M phosphate buffer. (□–□–□) 3,4-Benzpyrene and (○—○–○) 1,2-benzpyrene).

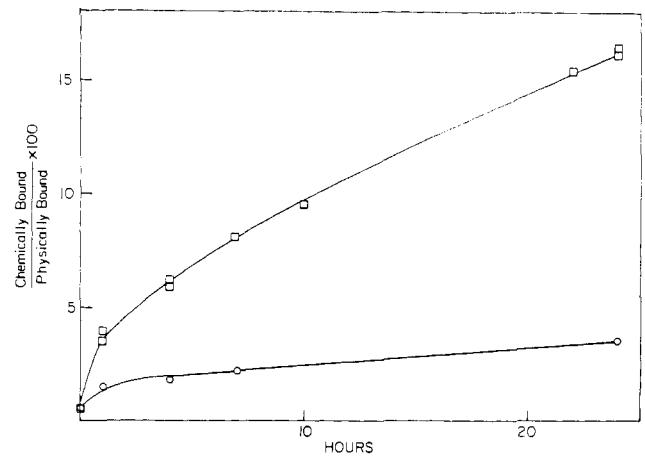

FIGURE 5: Comparison of the kinetics of the H_2O_2-induced chemical linkage of 1,2-benzpyrene and 3,4-benzpyrene with native DNA in 1.5×10^{-2} M H_2O_2–1×10^{-2} M $FeCl_2$ at 37°, 1×10^{-2} M phosphate buffer. (□–□–□) 3,4-Benzpyrene and (○–○–○) 1,2-benzpyrene.

at the higher ionic strength, therefore the effect of salt cannot be responsible for increased chemical linkage. In addition to the production of hydroxyl radicals as in the case of the H_2O_2 system, Grinstead (1960) has proposed the formation of perhydroxyl and ascorbate radicals as well in the ascorbic acid model hydroxylating system. At present, the reason for the increase in chemical linkage of 1,2-benzpyrene to denatured DNA in the model system is not obvious.

Sucrose Gradient Electrophoresis and Gel Filtration Chromatography. The 3,4-benzpyrene chemical complex was also characterized by sucrose gradient electrophoresis and molecular sieve chromatography in order to show that the complex was not contaminated by any 3,4-benzpyrene reaction prod-

TABLE II: Effect of Temperature on the Formation of DNA–Benzpyrene Chemical Complexes Induced by 1.5×10^{-2} M H_2O_2–1×10^{-3} M $FeCl_2$ in Aqueous Solution.[a]

	Temp (°C)	Chemically Linked/(%) Physically Bound
Native DNA (4-hr incubation)	0	2.0
	30	3.8
	37	5.5
	50	14.5
Denatured DNA (1-hr incubation)	0	3.1
	30	10.4
	37	13.3
	50	14.7

[a] Reaction mixtures (1 ml) were run in duplicate and incubated at indicated temperatures. Reaction was performed in 1×10^{-2} M phosphate buffer.

TABLE III: Effect of Various Conditions on the Stability of DNA–Benzpyrene Chemical Linkage.[a]

Experimental Condition	% Decrease in Sp Act. (cpm/OD at 259 mμ)
100° for 10 min, pH 6.8	9
1×10^{-3} N NaOH for 1 hr, pH 11.0	9
24 hr at 37°, pH 6.8	4
18 hr at 37°, pH 6.6, and 6 hr at 37°, pH 9.2	6
12 days at 5°, pH 6.8[b]	13

[a] After experiments, the DNA–benzpyrene chemical complex was reprecipitated by adding 0.25 ml of 5 M $NaClO_4$ and 3 ml of 95% ethanol/ml of DNA solution. This was allowed to stand for 1 hr in ice. After centrifugation, the precipitate was washed three times with 95% ethanol and twice with ether and then vacuum dried. Residue was dissolved in 1×10^{-2} M phosphate buffer and radioactivity and absorbance at 259 mμ were measured. [b] Native DNA, other experiments done with denatured DNA.

ucts insoluble in organic solvents. A sucrose gradient electrophoretic pattern of 3,4-benzpyrene–denatured DNA chemical complex induced by H_2O_2 is shown in Figure 7. This chemical complex had been precipitated and washed exhaustively with alcohol and ether as previously described. It can be seen that the [^{3}H]3,4-benzpyrene has the same electrophoretic mobility as denatured DNA, thus indicating an attachment between 3,4-benzpyrene and DNA even after organic solvent extraction. The results of a study by molecular sieve column chromatography are shown in Figure 8. A 3,4-benzpyrene–native DNA chemical complex induced by I_2 was placed on a column of Sephadex G-200 after precipitation and extraction with organic solvents. As shown in Figure 8a, [^{3}H]3,4-benzpyrene and DNA are eluted together from the column at the void volume. The 3,4-benzpyrene–native DNA chemical complex was next hydrolyzed to mononucleotides by DNase I and snake venom phosphodiesterase. The hydrolysate was then placed on the same column of Sephadex G-200. The [^{3}H]3,4-benzpyrene was eluted from the column with the same effluent volume as the hydrolytic products (Figure 8b). The data indicate that [^{3}H]3,4-benzpyrene eluted at void volume before enzymic hydrolysis was attached to native DNA by an I_2-induced reaction, and after enzymic hydrolysis of the DNA, the [^{3}H]3,4-benzpyrene was then eluted together with the resultant mononucleotides.

The hydrolytic products were further examined by sucrose gradient electrophoresis at pH 3.5 and the pattern is shown in Figure 9a; 95% of the radioactivity, [^{3}H]3,4-benzpyrene–nucleotides, left the origin and migrated toward the anode producing definite and reproducible peaks. However, the mobility of the radioactivity was not the same as that of the free nucleotides. This result was not unexpected since chemical attachment of benzpyrene may cause the pK_a's of the bases to vary or increase the size of the nucleotides substantially. The nucleotide hydrolysate was next treated with alkaline phosphatase to produce nucleosides. The majority of the radioactivity remained at the origin with the nucleosides when the alkaline phosphatase digest was analyzed electrophoretically (Figure 9b). One may conclude from these results that the migration of about 95% of the radioactivity, representing the [^{3}H]3,4-benzpyrene, toward the anode shown in Figure 9a must result from attachment to nucleotides. The small amount of radioactivity migrating toward the anode in Figure 9b could be due to [^{3}H]3,4-benzpyrene–dinucleoside monophosphates or oligonucleotides. Therefore, the [^{3}H]-3,4-benzpyrene must be chemically coupled to the base or sugar moiety of DNA in the H_2O_2 reaction. The possibility that the radioactivity represents merely an exchange of tritium between [^{3}H]3,4-benzpyrene and DNA was excluded since the radioactivity and the ultraviolet absorbance do not

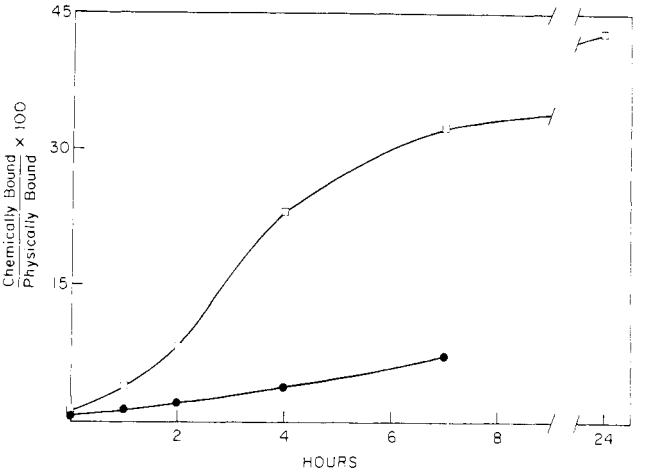

FIGURE 6: Comparison of the kinetics of the ascorbic acid induced chemical linkage of 1,2-benzpyrene and 3,4-benzpyrene with denatured DNA at room temperature in 6.8×10^{-3} M EDTA, 1.4×10^{-3} M $FeSO_4$, 1.46×10^{-2} M ascorbic acid, and 0.1 M phosphate buffer (pH 6.6). (□-□-□) 3,4-Benzpyrene and (●-●-●) 1,2-benzpyrene.

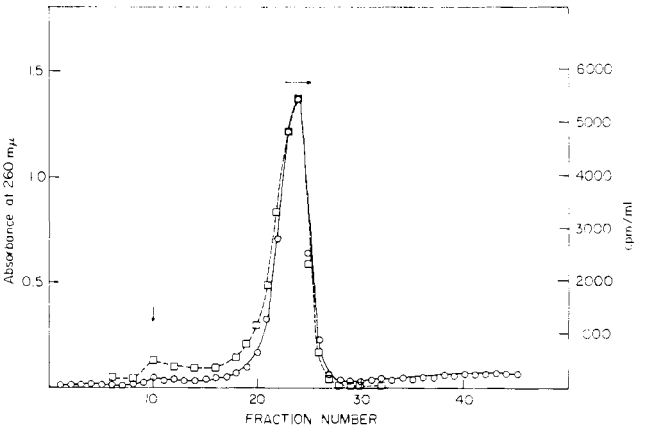

FIGURE 7: Sucrose gradient electrophoresis pattern of the denatured DNA–benzpyrene chemical complex induced by H_2O_2. Electrophoresis ran for 1 hr at 4 mA, 3150 V, using 3.3×10^{-3} M phosphate buffer (pH 6.8). Fractions (1 ml) were collected and absorbance at 260 mμ (○-○-○) and radioactivity (□-□-□) were measured.

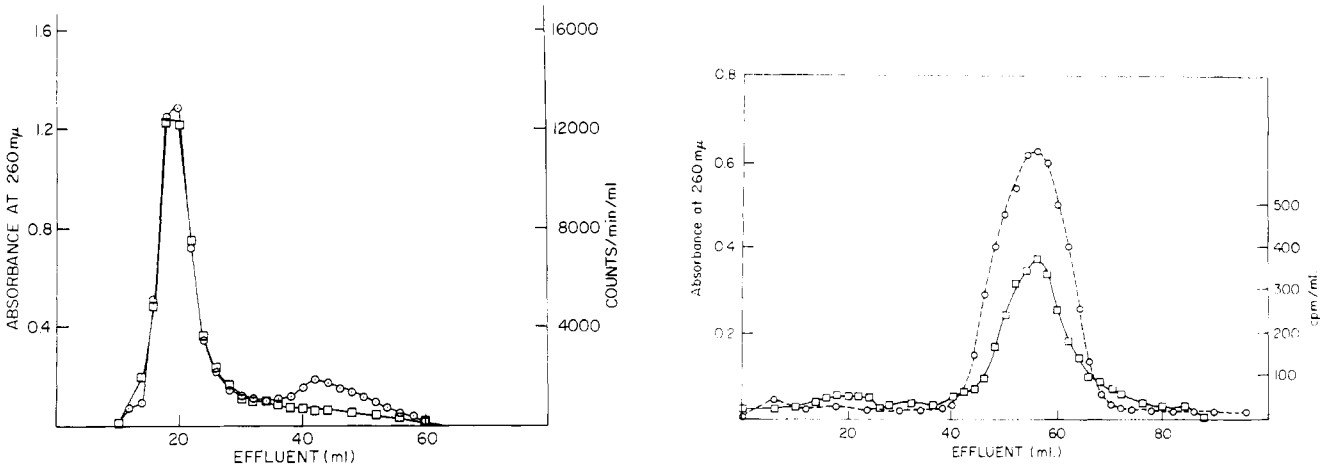

FIGURE 8: Gel filtration patterns on Sephadex G-200. (a, left) Of the native DNA–benzpyrene chemical complex induced by I_2. Fractions (2 ml) were collected and absorbance at 260 mμ (⊙-⊙-⊙) and radioactivity (□-□-□) were measured. (b, right) Of the hydrolytic products of the native DNA–benzpyrene chemical complex induced by I_2. The DNA–benzpyrene chemical complex was hydrolyzed as described by Rapaport and Ts'o (1966) with DNase I for 17 hr at 37°, pH 6.6, and snake venom phosphodiesterase for 6 hr at 37°, pH 9.2. (○- - -○- - -○) Absorbance at 260 mμ and (□-□-□) radioactivity.

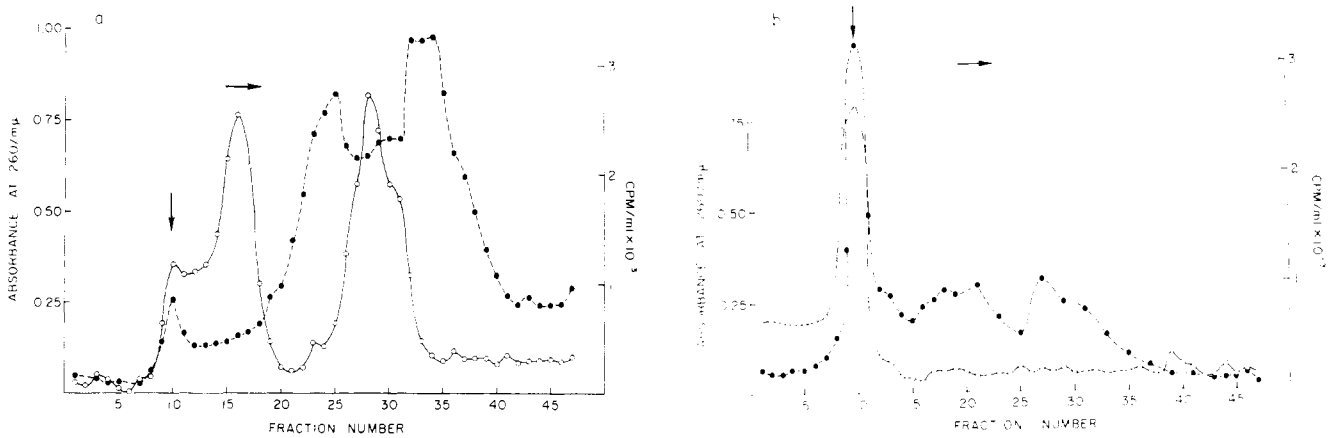

FIGURE 9: Sucrose gradient electrophoresis patterns of nucleotide and nucleoside hydrolysates resulting from enzymic digestion of a denatured DNA–benzpyrene chemical complex induced by H_2O_2. (a) Nucleotides (see Figure 8b for details of hydrolysis). Electrophoresis ran for 5 hr at 4.5 mA, 2250 V, using 5×10^{-3} M sodium citrate buffer (pH 3.5); 1-ml fractions were collected and absorbance at 260 mμ (○-○-○) and radioactivity (●-●-●) were measured. (b) Nucleosides which were obtained by hydrolysis of the nucleotide mixture with alkane phosphatase for 3 hr at 37° at pH 9.2. Conditions for electrophoresis are the same as in a. (○-○-○) Absorbance at 260 mμ and (●-●-●) radioactivity.

exactly coincide in the electrophoretic pattern of the nucleotides (Figure 9a).

Stability of Chemical Linkage. The chemical linkage between [^{3}H]3,4-benzpyrene and DNA induced in these reactions tends to be somewhat unstable. Table III shows the effect of various conditions on the stability of the [^{3}H]3,4-benzpyrene–DNA chemical linkage. After the incubation period for enzymic hydrolysis (37°, 18 hr at pH 6.6 and 6 hr at pH 9.0), a certain amount of the radioactivity (5–10%) was extractable from this aqueous hydrolysate by cyclohexane. This material presumably represents the breakdown product of 3,4-benzpyrene–nucleotides, since this material if not removed by cyclohexane extraction, remained at the origin during electrophoresis.

Extent of DNA Degradation. The extent of DNA degradation produced by these three chemical reactions has been examined. No diminution in the sedimentation coefficient of native DNA was found after the I_2 reaction (1×10^{-4} M I_2, 2 hr at room temperature). Oxidation of DNA by iodine takes place only under much more drastic conditions (Jones *et al.*, 1966). There was, however, a reduction in the sedimentation coefficient of native DNA after reaction with 1.5×10^{-2} M H_2O_2–1×10^{-3} M $FeCl_2$ at 37° after 24 hr. Under the same conditions of H_2O_2–$FeCl_2$, denatured DNA showed a reduction of 30% in *s* value after 1 hr and a 44% reduction after 24 hr. No detectable change in optical density was observed when denatured DNA (concentration 1 mg/ml) was incubated with 1.5×10^{-2} M H_2O_2–1×10^{-3} M $FeCl_2$ at 37° for 24 hr. In the absence of ferrous ion, the reduction in *s* value of denatured DNA was negligible (only 3%) when incubated in the presence of 1×10^{-2} M sodium citrate at 37°. These data indicate that there was degradation of DNA leading to chain scission in the H_2O_2-induced reaction. The effect of H_2O_2 on DNA has recently been studied in several laboratories (Yam-

afuji and Uchida, 1966; Rhaese and Freese, 1968a,b; Rhaese, 1968). There also appears to be DNA degradation in the ascorbic acid reaction since the amount of DNA that can be recovered decreases as the reaction proceeds. It should also be noted that hydroxyl or perhydroxyl radicals are probably involved in the ascorbic acid system and therefore it should be similar to the H_2O_2–$FeCl_2$ system (Udenfriend *et al.*, 1954; Grinstead, 1960).

Discussion

It is too early to speculate on the precise mechanism of these interesting reactions for the formation of DNA–benzpyrene chemical complexes, especially in view of the fact that active research toward this goal is now in progress. The present data implicate benzpyrene free radicals (possibly cationic) as intermediates. Rochlitz (1967) has proposed that the cationic radicals are involved in the formation of benzpyrene–pyridine salt with a C–N linkage. Recently, one-electron transfer oxidation of the carcinogen 7,12-dimethylbenz(a)anthracene by MnO_2, Fe^{III} Fe^{III} $(CN)_6$, and $(NH_4)_2$ Ce^{IV} $(NO_3)_6$ has been reported by Fried and Schumm (1967), a radical cation having been suggested as the intermediate in the reaction. Marcoux *et al.* (1967), have reported on the one-electron oxidation of a whole class of polycyclic hydrocarbons. By the use of cyclic voltammetry, they have demonstrated that electrochemical oxidation of these compounds goes through an activated stage involving a one-electron oxidation process.

These *in vitro* studies suggest that the relatively inert polycyclic hydrocarbons can be transformed into active free-radical intermediates in an aqueous solution, neutral pH, and at room temperature. This type of reaction could well be the general mechanism for the activation of polycyclic hydrocarbons in living organisms. When provided with an opportunity, these activated immediates of polycyclic hydrocarbons can chemically react with the DNA. Free-radical intermediates offer two distinct advantages in reactions with highly structured biopolymer systems. (1) Free-radical forms of large unsaturated hydrocarbon systems are often relatively long lived due to extensive delocalization of the free electron within the π system. In fact, electron spin resonance spectra of a number of large polycyclic hydrocarbon radicals (Lewis and Singer, 1965), including 3,4-benzpyrene (Kon and Blois, 1958), have been reported. The relatively long lifetimes of these species provide the time necessary to maximize the number of orientations which the hydrocarbon can assume with respect to the sterically restricted environment of the biopolymer. This, in turn, maximizes the possibility that the appropriate relative orientation will be reached; (2) the extensive delocalization of the free electron and the associated positive "hole" provide multiple sites for possible reactivity throughout the electronic system of the hydrocarbon. This is important in the sterically restricted biopolymer system where, even considering the relatively long lifetimes of the reactive states, potentially reactive sites on the biopolymer may overlap only with limited portions of the complexed hydrocarbon.

The importance of the geometric relationship between the 3,4-benzpyrene and the DNA in the physical complex is illustrated by the observation that the formation of chemical linkage is four- to sixfold more with the denatured DNA than with native DNA. The extent of physical binding of 3,4-benzpyrene, however, is about the same to both native and denatured DNA and our way of expression (per cent conversion of the physically bound) has already taken into account the small variation in physical binding. The decreased chemical linkage with native DNA can be due to the stereochemical barrier between the activating compounds (such as $\cdot OH$ and I_2) and the physically bound benzpyrene, or the stereochemical barrier between the activated benzpyrene and the DNA. The latter case is more likely to be the real reason since the same disparity between native and denatured DNA was observed in the photoinduced chemical reaction. In addition, the sizes of $\cdot OH$ and I_2 are greatly different, yet both have the same disparity with native and with denatured DNA. The inability of 1,2-benzpyrene to react under the experimental conditions might be explained by the following alternative reasons: (1) the compound will not form a radical intermediate; (2) the radical produced will not react with DNA. Experiments are now in progress to distinguish between these two possibilities.

The formation of a chemical linkage between 3,4-benzpyrene and DNA in the ascorbic acid model hydroxylating system is of considerable interest. This system was so named by Udenfriend and coworkers (Udenfriend *et al.*, 1954) because of its ability under certain conditions to catalyze oxidation and hydroxylation of aromatic compounds very similar to those catalyzed by the *in vivo* enzymic systems. Boyland *et al.* (1964) have compared the reaction products of naphthalene, anthracene, phenanthrene, pyrene, and benz(a)anthracene in both the model hydroxylating system and in the rat liver microsomal system. There were a lot of similarities between the products obtained from these two systems, especially for the simpler hydrocarbons. These observations suggest that the chemical reaction between 3,4-benzpyrene and DNA reported here could occur in a biological system.

What is the effect of chemical linkage of benzpyrene on the biochemical function of nucleic acids? The most likely effect should be on the replication and transcription processes involving DNA polymerase and RNA polymerase. One obvious answer is that linkage of benzpyrene to DNA may lead to stoppage of the replication or transcription process at that point. Another more interesting answer to the question is that attachment of benzpyrene to DNA may introduce errors in the replication or transcription process. Such errors are likely to produce serious biological consequences.

During the completion of this manuscript, Morreal *et al.* (1968) have reported the induction of a chemical linkage of the polycyclic hydrocarbons 3,4-benzpyrene, 3-methylcholanthrene, and 9,10-dimethyl-1,2-benzanthracene to calf thymus DNA with dilute solutions of H_2O_2.

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The Preparation, Preservation, and Properties of High Molecular Weight Polyadenylic Acid*

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ABSTRACT: Polyadenylic acid of a high degree of polymerization ($s_{20,w}$ = 20 S) was synthesized by use of polynucleotide phosphorylase from *Micrococcus lysodeikticus*. A simple "falling-object" viscometer was developed for assaying the polymerization reaction at low shear stress with adequate accuracy. During purification of the polymer it was noticed that extensive dialysis against glass-distilled water caused degradation of the polymer. We demonstrated that the polymer is not degraded if dialysis is carried out against a neutral electrolyte solution or against a 0.1 M buffer solution of pH 4.0, approximately the pH established inside the membrane when the polymer is dialyzed against water. In agreement with the sedimentation data of Fresco and Doty (Fresco, J. R., and Doty, P. (1957), *J. Am. Chem. Soc. 79, 3928*), spectral measurements indicate that polyadenylic acid is in the protonated helical form following water dialysis. We tentatively conclude that the electrostatic interactions are sufficiently large in the absence of salt to promote dramatically the chemical hydrolysis of the backbone chain. Melting curves were measured for the helix–coil transition of the protonated double-helical form present at acid pH. It was found that the transition curve for the high molecular weight sample ($s_{20,w}$ = 20 S) is considerably sharper than that for a commercial sample with $s_{20,w}$ = 8.2 S. This latter sample is therefore of a size range where equilibrium melting properties are not yet independent of molecular length.

Polyadenylic acid is commercially available only with rather low molecular weight. Since we are interested in studying the thermodynamic and kinetic properties of the helix–coil transition of this polymer in a size range where the thermodynamic properties are chain length independent, we set out to synthesize poly A with a molecular weight of at least five million (Crothers *et al.*, 1965). The reaction conditions for the synthesis were based on published conditions (Grunberg-Manago, 1963) with modifications to enhance the production of long-chain-length molecules (Peller and Barnett, 1962). In the assay and purification of the poly A we discovered and circumvented two major procedural steps which degrade the polymer.

The progress of the polymerization was assayed by taking periodic viscosity measurements of the reaction mixture. The high shear stress inherent in the use of a standard capillary

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