Chemical Linkage of Polycyclic Hydrocarbons to Deoxyribonucleic Acids and Polynucleotides in Aqueous Solution and in a Buffer–Ethanol Solvent System*

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ABSTRACT: The formation of a chemical linkage of polycyclic hydrocarbons with DNA in an iodine-induced reaction reported earlier (Lesko *et al.* (1969), *Biochemistry 8*, 2291) can be greatly increased by conducting the reaction in a solvent system of 1×10^{-2} M phosphate buffer (pH 6.8)–ethanol (2:1, v/v). The solubilities of the reactants (hydrocarbon and iodine) are increased in this system and the reaction is dependent on the concentration of these reagents. The yield of the chemical reaction can be further increased by a stepwise addition of the hydrocarbon, whose activated intermediate is short-lived, as well as by repeating the reaction after isolation of the hydrocarbon–DNA adduct. Using these procedures, we have introduced 1 3,4-benzpyrene molecule/300 bases of calf thymus DNA and 1/100 bases of *Bacillus subtilis* SB-19-transforming DNA. Under identical conditions, carcinogenic hydrocarbons (3,4-benzpyrene, 9,10-dimethyl-1,2-benzanthracene, and 20-methylcholanthrene) are manyfold (4–14-fold) more reactive than their respective noncarcinogenic isomers or analogs (1,2-benzpyrene and 1,2-benzanthracene). Reactions of 3,4-benzpyrene with ribosyl homopolynucleotides (poly A, poly G, poly C, poly U, poly I, poly X, poly A·poly U, and poly G·poly C) indicate that the hydrocarbon is linked principally to poly G (even in the poly G·poly C complex) in the iodine-induced reaction. The 3,4-benzpyrene–poly G adduct has been degraded to mononucleotides and examined by density gradient electrophoresis. When activated in a hydrogen peroxide–ferrous ion system (Lesko *et al.* (1969), *Biochemistry 8*, 2291), 3,4-benzpyrene is linked preferentially to purine polynucleotides with poly G being the most reactive. The present data support the suggestion that a 3,4-benzpyrene radical cation is the active intermediate in the iodine-induced reaction, and indicate that the active intermediate in the reaction induced by the hydrogen peroxide–ferrous ion system is most likely different from that in the iodine-induced reaction.

In the preceding paper of this series from our laboratories (Lesko *et al.*, 1969), we reported the formation of a chemical linkage of 3,4-benzpyrene to DNA in neutral, aqueous solution at low or room temperature. This chemical linkage was induced by reacting a physical complex of 3,4-benzpyrene and DNA with iodine, or with a H_2O_2–Fe^{2+} system, or with an ascorbic acid model hydroxylating system. Up to 40% of the physically bound 3,4-benzpyrene can be linked covalently to the DNA under appropriate conditions. In these same reactions, a noncarcinogenic isomer, 1,2-benzpyrene, is linked to DNA only to a very limited extent. Previously, our laboratories have reported the formation of a 3,4-benzpyrene–DNA complex in aqueous solution at neutral pH and room temperature by photoirradiation at wavelengths above 320 mμ at the absorption bands of benzpyrene (Ts'o and Lu, 1964) and by X-ray irradiation (Rapaport and Ts'o, 1966).

In this paper, we wish to report the improvement of reaction yield by 10–30-fold in the I_2-induced reaction, *i.e.*, to the extent of 1 benzpyrene molecule linked/100–300 bases in native DNA. This is accomplished in part by changing the reaction medium from an aqueous buffer solution to a buffer–ethanol (33%) system, and in part by continuously replenishing the hydrocarbon at intervals during the course of the reaction. Under these conditions, carcinogenic 3,4-benzpyrene, 9,10-dimethyl-1,2-benzanthracene, and 20-methylcholanthrene are comparably reactive, while the noncarcinogenic analogs, such as 1,2-benzpyrene and 1,2-benzanthracene are much less reactive (4–14-fold). Studies on the chemical linkage of 3,4-benzpyrene to ribosyl homopolynucleotides and their double-stranded complexes showed that poly G was much more preferred than other polynucleotides, especially in the I_2-induced reaction. These findings provide additional understanding about the specificity and the mechanism of these chemical reactions and, because of the high reaction yield, allow the direct examination of the biological effect of the chemical linkage of 3,4-benzpyrene on the transforming DNA (Lesko *et al.*, 1970).

Materials

The following radioactive compounds were purchased from Amersham-Searle Corp.: [^{3}H]-3,4-benzpyrene (specific activity 500 mCi/mmole), [^{3}H]-1,2-benzpyrene (4500 mCi/mmole), [^{3}H]-9,10-dimethyl-1,2-benzanthracene (500 mCi/mmole), [^{3}H]-1,2-benzanthracene (750 mCi/mmole), [^{3}H]-20-methylcholanthrene (500 mCi/mmole), and [^{14}C]-3,4-benzpyrene (7.7 mCi/mmole). Chromatographic examination of these compounds in systems A and B confirmed the radiochemical purity of 98–99% reported by the manufacturer. The hydrocarbons, received in benzene solution,

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were lyophilized and used dry, or redissolved in ethanol for the reaction mixtures containing ethanol. Highly polymerized calf thymus DNA was purchased from Sigma Chemical Corp. Ribosyl homopolynucleotides (poly A, poly U, poly G, poly C, poly X, and poly I) were purchased from Miles Laboratories. Ribonuclease A was obtained from Worthington and the ribonuclease T_1 was obtained from Sankyo Co., Tokyo.

The DNA and the polynucleotide solutions were made in HMP[1] or in a mixture of the same phosphate buffer and ethanol in 2:1 volume ratio. The following extinction coefficients ($\times 10^{-3}$) of the nucleic acids in dilute neutral buffer were used for concentration measurements: DNA (259 mμ, 6.6; Ts'o *et al.*, 1962), poly A (257 mμ, 10.5; Ts'o *et al.*, 1962), poly U (260 mμ, 9.2; Ts'o *et al.*, 1962), poly A·poly U (260 mμ, 6.8; Blake *et al.*, 1967), poly G (252 mμ, 10.1; Michelson and Pochon, 1969), poly C (268 mμ, 6.8; Ts'o *et al.*, 1962), poly G·poly C (258 mμ, 8.2; Michelson and Pochon, 1969), poly I (268 mμ, 10.0; Sarkar and Yang, 1965), poly I·polyC (248 mμ, 7.8; Chamberlin, 1965), and poly X (250 mμ, 8.2; Michelson and Monny, 1966).

Experimental Procedures

Studies on the Physical Binding of Hydrocarbons to Polynucleotides in Aqueous Solutions and in Buffer–Ethanol Systems. The conditions for studying the physical binding of hydrocarbons to polynucleotides in aqueous solutions are essentially the same as those used previously for the studies on binding of hydrocarbons to DNA (Lesko *et al.*, 1968). Solid [^{3}H]hydrocarbons in excess were added to polynucleotide solutions ($\approx$1 mg/ml or 3 $\pm$ 0.5 $\times 10^{-3}$ M) in phosphate buffer. After 3-weeks shaking in the dark at 5°, the solutions were filtered and assayed for radioactivity. For the physical binding studies in the buffer–ethanol system (2:1, v/v), only 12-hr shaking time were required to reach a saturation level of physical complex formation.

Studies on the Chemical Linkage of Hydrocarbons to DNA and Polynucleotides in Aqueous Solutions and in Buffer–Ethanol Systems. The procedure for conducting the chemical reaction of hydrocarbons with DNA and polynucleotides induced by I_2 or H_2O_2–Fe^{2+} is the same as that published previously (Lesko *et al.*, 1969). In the case of poly G or poly G·poly C a more extensive washing of the precipitated product was found to be necessary, *viz.*, six times with ethanol instead of three to four times.

A typical incubation mixture for the reaction between DNA and the [^{3}H]hydrocarbons induced by I_2 in the HMP–ethanol (33%) solvent system was prepared as follows: a 2.5-ml reaction mixture contained DNA (4.2×10^{-4} M), [^{3}H]hydrocarbon (2.56×10^{-5} M), and I_2 (5×10^{-8} M). DNA was dissolved in HMP, and the [^{3}H]hydrocarbon and I_2 were dissolved in 100% ethanol. I_2 was always the last reactant to be added to the incubation mixture. After thorough mixing, the solution was incubated for 2 hr in the dark at room temperature. The DNA was precipitated with 0.42 ml of 5 M $NaClO_4$ and 4.2 ml of 95% ethanol. After standing 30 min in ice, the DNA precipitate was washed four times with 95% ethanol and once with ether. The precipitate was then redissolved in HMP for analysis. In other reactions the same amount of [^{3}H]hydrocarbon was added stepwise every 30 min. The stepwise reaction could be repeated to increase the amount of [^{3}H]hydrocarbon bound to DNA. The washing procedure removed over 99% of the [^{3}H]hydrocarbons from the physical complex (Lesko *et al.*, 1969). However, when the [^{3}H]hydrocarbons were chemically linked to DNA, they could no longer be extracted by this procedure (Ts'o and Lu, 1964; Rapaport and Ts'o, 1966; Lesko *et al.*, 1969). From the known specific activity of the [^{3}H]hydrocarbon and the data of counts per minute per optical density at 260 mμ, the number of [^{3}H]-hydrocarbon molecules linked per 1000 bases of nucleic acid was calculated.

Separation of Poly A and Poly U from the Poly A·Poly U Complex. The [^{3}H]-3,4-benzpyrene–poly A·poly U chemical complex, which was formed in an I_2-induced reaction, was subjected to sucrose gradient electrophoresis in 0.01 M sodium acetate buffer at pH 4.0 with a running time of 2.45 hr at 3 mA and a gradient of 50 V/cm. About 40 fractions (1.1 ml each) were collected at the end of the experiment and the absorbance at 260 mμ and the radioactivity of each fraction was assayed. The electrophoresis apparatus and the procedure have been described previously (Lesko *et al.*, 1968).

Formation of Poly G·Poly C Complex. Reports in the literature indicate that the formation of the poly G·poly C helix complex starting from the single-stranded poly G and poly C may not always occur because of the strong self-interaction of these single-stranded polynucleotides (Fresco *et al.*, 1963). However, it has also been established that resistance to nuclease attack is a reliable criterion for the formation of the complex (Haselkorn and Fox, 1965; Barnard, 1969). Therefore, resistance to pancreatic ribonuclease A or to RNase T_1 was used in the present work to detect formation of the complex. The enzymic hydrolysis was followed continuously by the increase of optical density at the uv range in a Beckman spectrophotometer. When dissolved separately in 0.2 M Tris-NaCl buffer (pH 7.5) the poly C is sensitive to pancreatic ribonuclease A and the poly G is sensitive to ribonuclease T_1. After being mixed in equal molar quantities (3.2×10^{-3} M), the poly C and poly G became much more resistant (slower in the rate of hydrolysis by at least 500-fold) to their respective nucleases. The complex is not inhibitory to the action of these nucleases, since the amount of each polynucleotide, when added in excess over the equimolar quantity of the complementary strand, is hydrolyzed by its respective enzyme. In other words, in the mixtures which contained poly G and poly C not in equivalent amounts, the polynucleotide in excess is hydrolyzed by the appropriate enzyme, so that the resultant mixtures after enzymic hydrolysis contain only a 1:1 poly G·poly C complex. These enzyme experiments clearly indicate that in the present mixture of poly G and poly C a complex of poly G·poly C was formed.

Separation of Poly G from the Poly G·Poly C Complex by the Alkaline Separation–Enzymic Hydrolysis Procedure. Various attempts to separate poly G from poly C in the poly G·poly C complex by sucrose gradient electrophoresis at pH 3.5–4.5 were unsuccessful. However, Haselkorn and Fox (1965) have shown that poly C is degradable by ribo-

[1] Abbreviation used is: HMP, phosphate buffer (pH 6.8) consisting of 0.0025 M disodium hydrogen phosphate and 0.005 M sodium dihydrogen phosphate.

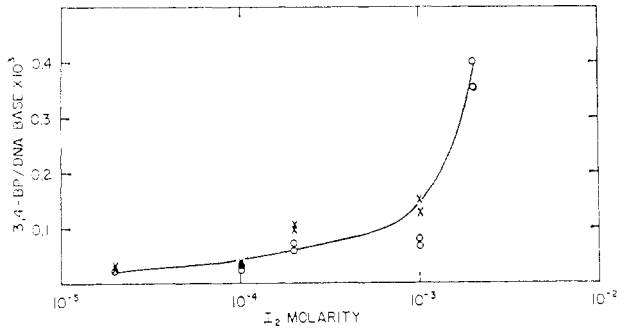

FIGURE 1: Dependence of the extent of covalent linkage of [^{3}H]-3,4-benzpyrene to native calf thymus DNA on the I_2 concentration. Reactions were run for 2 hr at 25° in HMP–ethanol (33%).

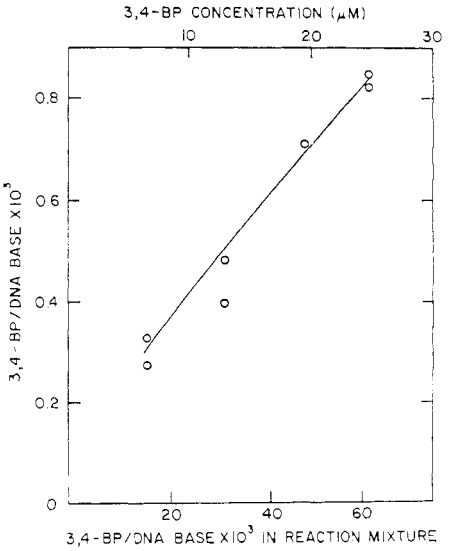

FIGURE 2: Dependence of the extent of covalent linkage of [^{3}H]-3,4-benzpyrene to native calf thymus DNA on the hydrocarbon concentration. Reactions were run in HMP–ethanol (33%) for 2 hr at 25° using 5×10^{-3} M I_2. The amount of hydrocarbon per DNA base in the reaction mixture is also shown.

nuclease A in the presence of poly G if the two-stranded complex if first separated by alkali, and the alkaline mixture is then neutralized with an acidic buffer solution (sodium acetate, pH 4.5) which contains a sufficient amount of ribonuclease A. Upon neutralization, the ribonuclease A rapidly hydrolyzes the pyrimidine strand before the reunification with the poly G takes place sufficiently to provide adequate protection. In such a procedure, the poly G can be separated from the degraded poly C. The [^{3}H]-3,4-benzpyrene–poly C·poly G chemical complex used in this experiment was formed in an I_2-induced reaction and was dissolved in HMP buffer (pH 6.8). The solution was mixed with 2 N NaOH to reach a pH of 12.8. After 5 min at room temperature, this alkaline solution was neutralized with 0.1 M sodium acetate buffer (pH 4.5) which contained ribonuclease A. The final mixture had a pH of 7.5 and was left for 15 min at 37°. The enzyme concentration was predetermined so that the hydrolysis of the poly C was 90% finished within the first 2 min. The unreacted poly G was precipitated with ethanol and washed exhaustively with ethanol and ether. After removal of the ether *in vacuo*, the precipitate was redissolved in HMP buffer for measurement of radioactivity and ultraviolet absorbance. In order to examine for possible contamination by cytosine mono- or oligonucleotides, a portion of the poly G sample was first hydrolyzed by ribonuclease T_1 and then treated again with ribonuclease A. Paper chromatography in system C of these enzyme hydrolysates revealed the presence of 3′-GMP and the absence of cytosine nucleotides.

Chromatographic Systems. System A contains petroleum ether–chloroform–benzene (70:20:10, v/v) on silica gel. System B contains ethanol–dichloromethane–H_2O (20:10:1, v/v) on acetylcellulose (20% acetylated, Brinkman, Inc.; Schaad *et al.*, 1969). System C contains 1-propanol–concentrated ammonia–H_2O (60:30:10) on paper (Schleicher & Schuell 2043).

Results

Chemical Reactions of [^{3}H]Polycyclic Hydrocarbons with Native DNA Induced by Iodine in a HMP–Ethanol (33%) System. As described in the introduction, a substantial increase in the yield of the chemical reaction of carcinogenic polycyclic hydrocarbons with native DNA is highly desirable. This increase in yield is especially important for the iodine-induced reaction, since the DNA was found not to be degraded under this reaction condition (Lesko *et al.*, 1969). Such an enhancement of the chemical reaction may be obtained by increasing the concentration of the hydrocarbon (such as 3,4-benzpyrene) and the I_2 in the reaction mixture. The concentration of these reactants, however, are limited by their low solubility in the aqueous solution. Therefore, the reaction was conducted in a solvent system containing 33% ethanol which increases substantially the solubilities of I_2 and of the hydrocarbons. On the other hand, the concentration of ethanol in the solvent system cannot be increased much beyond 33% since a higher concentration of ethanol may bring about the precipitation of high molecular weight DNA. Experiments were done first to examine the conditions which may lead to a large increase in the yield of the chemical reaction of [^{3}H]-3,4-benzpyrene with native DNA induced by iodine. After the establishment of favorable reaction conditions of [^{3}H]-3,4-benzpyrene with DNA, other polycyclic hydrocarbons were used for comparison in examining the specificity of the reaction.

Figure 1 shows the dependence of the extent of chemical linkage of [^{3}H]-3,4-benzpyrene to DNA on the I_2 concentration at room temperature. There is a sharp increase in the number of [^{3}H]-3,4-benzpyrene molecules linked per 10^3 bases of DNA when the I_2 concentration in the mixture is increased from 1 to 5 mM, which is near the solubility limit of I_2 in this solvent system. In Figure 2, the dependence of the extent of chemical linkage of [^{3}H]-3,4-benzpyrene to DNA on the concentration of [^{3}H]-3,4-benzpyrene is shown (5 mM I_2, 2 hr at room temperature). A linear dependence of the reaction yield on the concentration of the [^{3}H]-3,4-benzpyrene was found in this experiment which contained a constant concentration of DNA. Figure 3 shows the inverse dependence of the extent of chemical linkage of [^{3}H]-3,4-benzpyrene to DNA on the concentration of DNA as measured by the amount of benzpyrene linked/10^3 bases when the

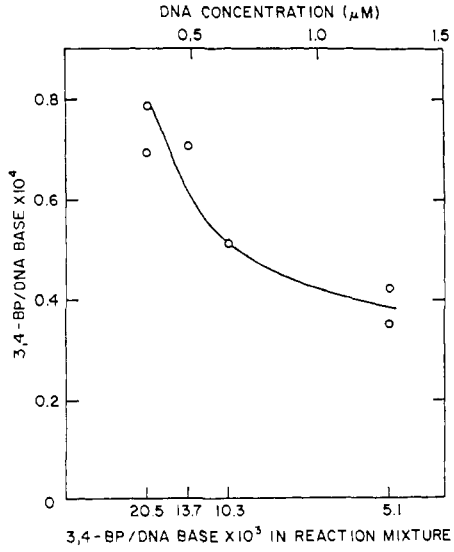

FIGURE 3: Dependence of the extent of covalent linkage of [^{3}H]-3,4-benzpyrene to native calf thymus DNA on the DNA concentration. Reactions were run in HMP–ethanol (33%) for 2 hr at 25° using 5×10^{-3} M I_2. The amount of hydrocarbon per DNA base in the reaction mixtures is also shown.

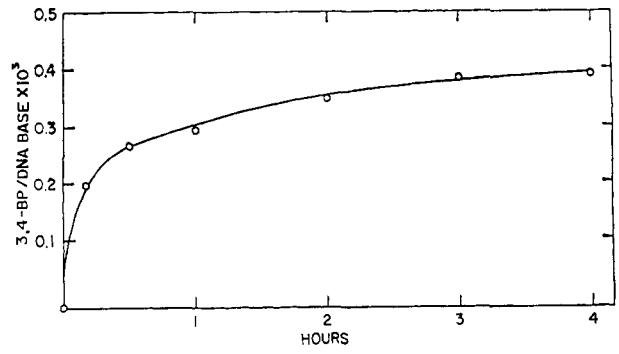

FIGURE 4: Kinetics of the covalent linkage of [^{3}H]-3,4-benzpyrene to native calf thymus DNA in HMP–ethanol (33%). Reactions were run at 25° using 5×10^{-3} M I_2.

reaction is induced at an invariant concentration of [^{3}H]-3,4-benzpyrene. Under this condition, the total amount of [^{3}H]-3,4-benzpyrene reacted was about the same for each reaction mixture containing varying amounts of DNA. In other words, in this DNA concentration range and with a constant amount of benzpyrene in the reaction mixture, the yield of addition products cannot be increased just by adding more DNA. Obviously, therefore, the larger the amount of DNA added the smaller is the ratio of 3,4-benzpyrene reacted:DNA base as shown in Figure 3. Generally, the interest is to have more hydrocarbon reacted per a given amount of DNA. Therefore, the input of [^{3}H]hydrocarbon:DNA base ratio is of importance and these ratios are also shown in the abscissa of Figures 2 and 3. Figure 4 illustrates the kinetics of this reaction of [^{3}H]-3,4-benzpyrene with DNA at room temperature in the presence of 5 mM I_2. The reaction proceeds rapidly for the first 30 min, then levels off to a value of 0.30 [^{3}H]-3,4-benzpyrene/10^3 bases after 1 hr, to a value of 0.39 after 4 hr (Figure 4), and finally to a value of 0.58 after 24 hr. This reaction is also dependent on temperature. A threefold decrease in the extent of chemical linkage was observed in a 2-hr reaction when the temperature was reduced from room temperature to 0°.

The kinetic data in Figure 4 suggest that the [^{3}H]-3,4-benzpyrene is being used up rapidly in the present reaction mixture. This conclusion was confirmed by a comparative study of two experiments. In one experiment, a given amount of [^{3}H]-3,4-benzpyrene was added initially and the reaction was allowed to proceed in 5 mM I_2 at room temperature for 2 hr. In the other experiment, one-fourth of the same amount of [^{3}H]-3,4-benzpyrene was added to the reaction mixture at four intervals, *i.e.*, initial time, 0.5, 1, and 1.5 hr, and the reaction was terminated at 2 hr. In other words, the solutions in both experiments at the end contained the same amount of [^{3}H]-3,4-benzpyrene, DNA, and iodine and both were allowed to react for the same period of 2 hr. In the first experiment, however, the total amount of [^{3}H]-3,4-benzpyrene was added initially, while in the second experiment the same amount of [^{3}H]-3,4-benzpyrene was divided into four portions and one portion was added initially and the other three portions were added in a stepwise fashion afterward at 0.5-hr intervals. The results showed that the extent of the [^{3}H]-3,4-benzpyrene reaction with DNA in the first experiment, in which all the [^{3}H]-3,4-benzpyrene was added at once initially, was only 60% of that in the second experiment in which the [^{3}H]-3,4-benzpyrene was added in a stepwise fashion. These data indicated that the [^{3}H]-3,4-benzpyrene was being used up in various side reactions rapidly and that the introduction of [^{3}H]-3,4-benzpyrene in a stepwise fashion can be adopted to increase significantly the extent of the reaction for the same amount of hydrocarbon at the same period of reaction time.

When the comparative experiments were done at 0°, no difference in the extent of the reaction was found between the experiment in which all the [^{3}H]-3,4-benzpyrene was added at once and the experiment in which the [^{3}H]-3,4-benzpyrene was added in a stepwise fashion. Presumably at 0°, the lifetime of [^{3}H]-3,4-benzpyrene itself or its activated species in the reaction mixture is longer than at 25°.

Table I shows the results of experiments in which [^{3}H]-3,4-benzpyrene was added in a stepwise fashion to the incubation mixtures at 0, 0.5, 1, and 1.5 hr at room temperature in 5 mM iodine. At the end of 2 hr, the DNA was precipitated and washed five times with 95% ethanol. After washing, the precipitate of [^{3}H]-3,4-benzpyrene–DNA was redissolved for measurements of radioactivity and optical density. The results show that in this reaction, about 1.8 benzpyrene molecules were linked per 10^3 bases of DNA. Such a stepwise reaction was repeated twice with the same DNA sample. At the end of three reaction sequences, about 3.2 benzpyrene molecules linked/10^3 bases of native thymus DNA were found. In reactions with *B. subtilis* transforming DNA (Lesko *et al.*, 1970), a yield of 10 benzpyrene molecules linked/10^3 bases was obtained from a similar procedure.

The specificity of this chemical reaction, which has been reported previously for the same reaction in HMP, is maintained in this HMP–ethanol (33%) solvent system. Table II compares the results obtained with several carcinogenic and noncarcinogenic polycyclic hydrocarbons under the same reaction conditions. The extent of chemical linkage

TABLE I: Extent of Covalent Linkage of [^{3}H]-3,4-Benzpyrene to Native DNA Obtained in Reaction in a HMP–Ethanol (33%) Solvent System by a Stepwise Addition of Hydrocarbon.[a]

	[^{3}H]-3,4-Benzpyrene/DNA Base × 10^3		
	Expt A	Expt B	Av
First reaction	1.8	1.8	1.8
Second reaction	2.5	2.5	2.5
Third reaction	3.0	3.5	3.2

[a] In each reaction an equal portion of [^{3}H]-3,4-benzpyrene was added to the reaction mixtures at 0, 0.5, 1, and 1.5 hr. After 2-hr incubation at room temperature, the DNA was precipitated and washed five times with 95% ethanol. The [^{3}H]-3,4-benzpyrene–DNA complex was redissolved and the stepwise addition reaction repeated as indicated. I_2 concentration was 5.6×10^{-3} M at the first stepwise addition and 5.0×10^{-3} M after the final stepwise addition in each reaction. The DNA concentration in the first reaction was 0.42 mM and in the second and third reactions was about 0.3 mM. The 3,4-benzpyrene at the end of the fourth stepwise addition in each reaction was about 0.06–0.08 mM.

with native DNA of carcinogenic 3,4-benzpyrene is 13.5-fold higher than that of noncarcinogenic 1,2-benzpyrene; and the extent of chemical linkage of carcinogenic 9,10-dimethyl-1,2-benzanthracene is about 3.5-fold higher than noncarcinogenic 1,2-benzanthracene. Another carcinogenic polycyclic hydrocarbon, [^{3}H]-20-methylcholanthrene, also becomes covalently linked to DNA under these same reaction conditions. The value of chemical linkage for [^{3}H]-20-methylcholanthrene, 0.36 molecule of [^{3}H]hydrocarbon/10^3 bases, lies between the values for [^{3}H]-3,4-benzpyrene and for [^{3}H]-9,10-dimethyl-1,2-benzanthracene. The solubility of [^{3}H]-9,10-dimethyl-1,2-benzanthracene in the 33% ethanol–HMP solvent system, however, is much higher than that of [^{3}H]-3,4-benzpyrene. By increasing the concentration of dimethyl-1,2-benzanthracene (0.1 mM or 1 dimethyl-1,2-benzanthracene/3 bases) about twofold higher than that of [^{3}H]-3,4-benzpyrene in reaction mixtures, we were able to obtain a value of 2.6 molecules of [^{3}H]-9,10-dimethyl-1,2-benzanthracene linked/10^3 DNA bases after only one stepwise reaction under the condition shown in Table I. This higher yield should be compared with a value of 1.8 molecules of [^{3}H]-3,4-benzpyrene bound per 1000 bases which was also obtained in one stepwise reaction (Table I).

TABLE II: Extent of Covalent Linkage of Various [^{3}H]Hydrocarbons to Native Calf Thymus DNA in HMP–Ethanol (33%) Induced by 5×10^{-3} M I_2.[a]

Hydrocarbon	Carcinogenicity	[^{3}H]Hydrocarbon/DNA Base × 10^3
3,4-Benzpyrene	+	0.87
1,2-Benzpyrene	−	0.065
9,10-Dimethyl-1,2-benzanthracene	+	0.30
1,2-Benzanthracene	−	0.09
20-Methylcholanthrene	+	0.36

[a] The reaction mixtures were incubated 2 hr at room temperature. All reaction mixtures contained the same concentration of hydrocarbon (0.026 mM) and of DNA (0.42 mM) with DNA base/hydrocarbon of 16.3.

TABLE III: Physical Binding (Hydrocarbon/Base × 10^3) of [^{3}H]-3,4-Benzpyrene and [^{3}H]-1,2-Benzpyrene to Various Ribosyl Homopolynucleotides in HMP–Buffer (pH 6.8).[a]

Polymer	3,4-Benzpyrene at 5°	1,2-Benzpyrene at 5°	3,4-Benzpyrene at 37°
A	0.34	0.25	0.16
U	0.05	0.04	0.05
A + U	0.42	0.29	0.29
G	3.70	2.50	2.50
C	0.10	0.09	0.09
G + C	1.20	1.24	1.25
A (helical)	3.30		

[a] The polynucleotide solutions ($3.0 \pm 0.5 \times 10^{-3}$ M) were mixed with solid hydrocarbon and shaken for 2 weeks at 5° in the dark. Undissolved hydrocarbon was removed with a fine sintered glass filter. For the experiments at 37°, aliquots were incubated for 24 hr at 37° before filtration.

Physical Binding and Chemical Linkage of [^{3}H]-3,4-Benzpyrene and [^{3}H]-1,2-Benzpyrene to Ribosyl Homopolynucleotides in Aqueous Buffer System. The progress on the chemical reaction of [^{3}H]-3,4-benzpyrene with DNA in aqueous buffer reported previously suggests that the base specificity of this reaction should now be examined. In order to provide the background information, and perhaps the model compounds for the research on nucleic acids, the interaction of [^{3}H]-3,4-benzpyrene and [^{3}H]-1,2-benzpyrene with ribosyl homopolynucleotides was investigated.

In order to supply the hydrocarbons for the reaction in an aqueous solution, in which these compounds have a very low solubility, the hydrocarbons first have to be bound physically to nucleic acids. The formation of the physical complex increases the solubilities of the hydrocarbons in aqueous solution manyfold, and allows the hydrocarbons in close contact with the nucleic acids. However, the additional amount of hydrocarbons solubilized is dependent on the type of bases in these homopolynucleotides. To compensate for differences in the amount of hydrocarbons available for chemical reaction in solutions of various homopolynucleotides, the reaction yields are expressed in terms of per cent of physically bound hydrocarbons converted to chemically linked hydrocarbons (see Table IV). Only when the results are normalized in such a manner, can the reactivity of hydrocarbons to various bases of the

TABLE IV: Percentage of Physically Bound [^{3}H]-3,4-Benzpyrene or [^{3}H]-1,2-Benzpyrene That Becomes Chemically Linked Induced by I_2 (5°) or H_2O_2–Fe^{2+} (37°) in HMP–Buffer (pH 6.8).[a]

Polynucleotide	[^{3}H]-3,4-Benzpyrene Induced by I_2 (5°)	[^{3}H]-1,2-Benzpyrene Induced by I_2 (5°)	[^{3}H]-3,4-Benzyprene Induced by H_2O_2–Fe^{2+} (37°)
A	3.0	0.3	28.0
U	3.2	0.1	6.2
A + U	2.8	0.2	21.4
G	26.8	11.0	32.4
C	3.7	0.8	7.5
G + C	39.0	10.0	24.7
X			20.4

[a] The reaction mixtures were incubated 2 hr for the I_2-induced reactions and 24 hr for the H_2O_2–Fe^{2+}-induced reactions. The concentrations of the polynucleotides were 3.0 $\pm$ 0.5 $\times$ 10^{-3} M.

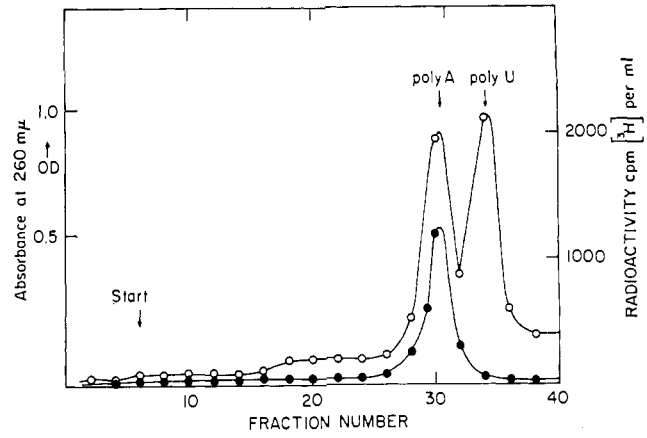

FIGURE 5: Sucrose gradient electrophoretic separation of poly U and poly A from a (poly A·poly U)–[^{3}H]-3,4-benzpyrene complex induced by I_2. Electrophoresis ran for 2.45 hr at 3.5 mA using a gradient of 50 V/cm in 1 $\times$ 10^{-3} M sodium acetate (pH 4.0). Fractions (1 ml) were collected and absorbance at 260 mμ (O—O—O) and radioactivity (●—●—●) were measured.

polynucleotides be appropriately compared. Therefore, the physical binding of [^{3}H]-3,4-benzpyrene and [^{3}H]-1,2-benzpyrene with the polynucleotides and the polynucleotide complexes was investigated. In this experiment, the polynucleotides and the solid [^{3}H]hydrocarbons were shaken together for 14 days at 5° in HMP, and then filtered for measurement of radioactivity. The results in Table III show that the poly G, helical poly A (formed at pH 5), and poly G·poly C have a much higher affinity for [^{3}H]-3,4-benzpyrene than the neutral poly A, poly C, and poly U. The data from the present investigation agree reasonably well with the binding data reported from our laboratories previously on poly U, poly C, acidic helical poly A, and neutral poly A (Lesko *et al.*, 1968). This observation shows that purine polynucleotides and polynucleotides which have a considerable amount of secondary structure have higher affinity for [^{3}H]-3,4-benzpyrene. The affinity of all these polynucleotides to [^{3}H]-1,2-benzpyrene, the noncarcinogenic analog, ranges from about the same within experimental error to slightly less than the affinity to [^{3}H]-3,4-benzpyrene (Table III). Since the solubility of [^{3}H]-1,2-benzpyrene and [^{3}H]-3,4-benzpyrene in water is about the same (Lesko *et al.*, 1968), these data suggest that the binding constants of these polynucleotides to [^{3}H]-1,2-benzpyrene and [^{3}H]-3,4-benzpyrene are very similar. The same results were observed from the comparative study on the physical binding of [^{3}H]-1,2-benzpyrene and [^{3}H]-3,4-benzpyrene to DNA. Therefore, the physical binding of this pair of analogous polycyclic hydrocarbons (3,4-benzpyrene *vs.* 1,2-benzpyrene) to nucleic acids is about the same and shows no specificity in relation to carcinogenic activity. Portions of each of the [^{3}H]-3,4-benzpyrene–polynucleotide mixtures shaken at 5° for 14 days were exposed to 37° for 24 hr before filtration. These conditions are identical with those used to induce covalent linkage of hydrocarbons to DNA with H_2O_2. The data from these samples (Table III) show that elevation of temperature to 37° for such a period of time has only a small effect in reducing the amount of physical complex formed previously at 5° for 14 days.

The chemical reactions of [^{3}H]-3,4-benzpyrene with polynucleotides in HMP induced by iodine were conducted at 5° with 0.1 mM iodine for a period of 2 hr, a condition which has been used previously for native and denatured DNA in aqueous solution (Lesko *et al.*, 1969). The extent of the reaction is evaluated by the per cent of conversion of [^{3}H]-3,4-benzpyrene–polynucleotide physical complex into a chemical complex as indicated by the amount of radioactivity remaining after precipitation and washing. This method of evaluation was also adopted previously (Lesko *et al.*, 1969) since the amount of [^{3}H]-3,4-benzpyrene in each sample varied with different polynucleotides solutions (Table III). In Table IV, the chemical reactions of [^{3}H]-3,4-benzpyrene and [^{3}H]-1,2-benzpyrene as measured by the conversion percentages are compared. The data clearly indicate that the I_2-induced reaction yields for poly G and poly G·poly C are about 10-fold higher than reaction yields for other polynucleotides. Also, the reaction yields with [^{3}H]-1,2-benzpyrene are about 10–35% of the yields with [^{3}H]-3,4-benzpyrene. This observation supports previous data on DNA that the chemical linkage of this pair of analogous polycyclic hydrocarbons (3,4-benzpyrene *vs.* 1,2-benzpyrene) to nucleic acids induced by I_2 is highly specific; furthermore, the specificity of this chemical reaction parallels their carcinogenic activity.

The chemical reactions of [^{3}H]-3,4-benzpyrene with polynucleotides in HMP as induced by the H_2O_2–Fe^{2+} system (1.5 $\times$ 10^{-2} M H_2O_2 and 1 $\times$ 10^{-3} M $FeSO_4$) at 37° for 24 hr have also been studied (Table IV). Again, this reaction condition has been used previously for the DNA studies (Lesko *et al.*, 1969). As shown in Table IV, this reaction is specific for the purine polynucleotides, *i.e.*, the yields with poly A, poly G, and poly X are much higher than those with poly C and poly U. The difference in yields between poly A and poly G is not significant (Table IV). Therefore, the chemical reaction of [^{3}H]-3,4-benzpyrene with the poly-

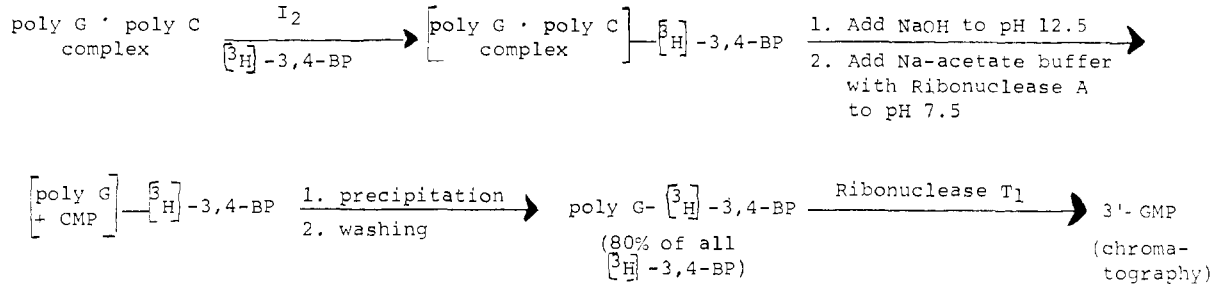

FIGURE 6: Scheme for the separation of poly G from a poly G·poly C complex by the alkaline separation–enzymic hydrolysis procedure.

nucleotides induced by I_2 appears to react predominantly with G, while the reaction induced by the H_2O_2–Fe^{2+} system reacts both with G and A.

The data in Table IV show that [^{3}H]-3,4-benzpyrene reacted with the double-stranded poly A·poly U complex and with the double-stranded poly G·poly C complex in the presence of iodine. It is of interest to know under this circumstance whether the [^{3}H]-3,4-benzpyrene interacts with both strands in the complex or only one strand (which one?) in the complex. The poly A strand and the poly U strand in the [^{3}H]-3,4-benzpyrene–poly A·poly U complex were separated from each other in a sucrose gradient electrophoretic experiment performed at pH 4.0, 5°. Under this condition, the partially protonated poly A, as shown by its ultraviolet absorption spectrum, was separated from the poly U and migrated with a lower mobility toward the anode (Figure 5). About 95% of the radioactivity of the [^{3}H]-3,4-benzpyrene was found to be coincident with the poly A peak and the poly U fraction was found to be completely free of radioactivity (Figure 5). In one experiment, all the poly A fractions were pooled together and the poly A was precipitated by ethanol with added nonradioactive poly A as carrier. After the standard washing procedure, within the experimental error, all the [^{3}H]-3,4-benzpyrene was found to be with the poly A. This experiment confirmed that the migrating [^{3}H]-3,4-benzpyrene was linked to the poly A. Thus, these observations show that when [^{3}H]-3,4-benzpyrene reacts with the double-stranded complex of poly A·poly U, the [^{3}H]hydrocarbon reacts only with the poly A strand, not with the poly U strand.

Attempts to separate the poly G from the poly C in the poly G·poly C complex by low pH sucrose electrophoresis experiments were not successful. Finally, the procedure of Haselkorn and Fox (1965) was adopted, involving separation at alkaline pH and treatment with pancreatic ribonuclease A. In this procedure, the poly C is hydrolyzed by pancreatic ribonuclease and poly G can be reisolated by alcohol as shown in the scheme depicted in Figure 6. A sample of [^{3}H]-3,4-benzpyrene–poly G·poly C formed in the iodine reaction was treated according to the scheme in Figure 6. After the ethanol precipitation, 80% of the [^{3}H]-3,4-benzpyrene originally found associated with poly G·poly C was now located with the poly G. This polymer material was degraded with RNAse T_1. Examination of the enzymic hydrolysate indicated the presence of 3′-GMP and the absence of cytosine nucleotides (paper chromatography in system C). Therefore, at least 80% of the [^{3}H]-3,4-benzpyrene linked to the poly G·poly C complex was with the poly G strand. The 20% [^{3}H]-3,4-benzpyrene not recovered with the poly G probably represents loss during precipitation and washing after alkaline treatment and not radioactivity previously located with the poly C. Therefore, the preference in the chemical reaction of [^{3}H]-3,4-benzpyrene for the poly G over the poly C in the two-stranded complex is likely to be much higher than 4 to 1.

Chemical Linkage of [^{3}H]-3,4-Benzpyrene to Ribosyl Homopolynucleotides in the HMP–Ethanol (33%) System. Chemical reactions of [^{3}H]-3,4-benzpyrene with homopolynucleotides as induced by iodine were also conducted in the HMP–ethanol (33%) solvent system. In this experiment, solid [^{3}H]-3,4-benzpyrene and the polynucleotides were shaken together at 5° for 12 hr. Afterward, the suspensions were centrifuged in a refrigerated Sorvall centrifuge at 15,000 rpm for 15 min to remove the undissolved [^{3}H]-3,4-benzpyrene. The amount of [^{3}H]-3,4-benzpyrene dissolved in this solution of HMP–ethanol (33%) varied to some extent depending on the type of polynucleotide in solution. The amount of [^{3}H]-3,4-benzpyrene dissolved per base of polynucleotide among the five polynucleotides can be listed in an increasing order as follows: poly U (2.5), poly A (3.00), poly I (3.9), poly G (4.9), and poly G·poly C (6.3) (benzpyrene/10^3 bases). Thus the difference in the amount of [^{3}H]-3,4-benzpyrene dissolved among these solutions was less than threefold.

Table V shows the extent of the chemical reaction of [^{3}H]-3,4-benzpyrene with the polynucleotides as induced by iodine or by H_2O_2–Fe^{2+} in the HMP–ethanol (33%) solvent system. These reactions were carried out under the same conditions as the aqueous system so that the absolute yield (amount of [^{3}H]-3,4-benzpyrene per 10^3 bases of the polynucleotides) of the reaction in these two solvent systems can be compared. The data show that the reaction yield with poly G is much higher than that of the other polynucleotides in both solvent systems (Table V). In the I_2-induced reaction, the yield with poly G was about 35–40-fold higher than with the poly A or poly I, and was about 90–200-fold higher than with the poly C or poly U (Table V). A similar situation was found also for the H_2O_2–Fe^{2+}-induced reaction, even though the difference between the yield of poly G and that of poly A was smaller, *i.e.*, about fivefold in the HMP–ethanol system and about 16-fold in the HMP aqueous system. Generally, the absolute yields in the HMP–ethanol system are substantially higher than those in HMP aqueous system because of the higher concentration of [^{3}H]-3,4-benzpyrene dissolved in the HMP–ethanol solvent. Nevertheless, as shown in Table V, the specificity is the same in both solvent

systems, *i.e.*, an overwhelming preference for poly G in the chemical reaction.

The chemical linkage of 3,4-benzpyrene with DNA induced by photoirradiation, X-ray irradiation, and by chemical reagents has been examined by electrophoresis, chromatography, together with enzymic treatments (Ts'o and Lu, 1964; Rapaport and Ts'o, 1966; Lesko *et al.*, 1969). In every instance, the procedure of precipitation and washing with organic solvents (such as ethanol and ether) has been shown to be a reliable method of isolating the chemical complex from the physically bound [^{3}H]hydrocarbons. The general approach is the following: After the precipitation-washing procedure, the amount of radioactivity remaining with the DNA, which should represent the linked hydrocarbon fragment(s), was found to travel with the DNA in density gradient electrophoresis and Sephadex molecular sieve column chromatography. This indicates that the remaining [^{3}H]benzpyrene has the same charge and size as the DNA. After the nuclease hydrolysis, the radioactivity has electrophoretic mobility similar to (but not identical with) those of the nucleotides. The nucleotide digest was further hydrolyzed by alkaline phosphatase; now, a large portion of the radioactivity no longer migrates in the electrophoretic field. This observation indicates that this portion of [^{3}H]-benzpyrene was attached to the nucleosides. The remaining radioactivity continues to migrate as an anion after the alkaline phosphatase treatment. This represents [^{3}H]-3,4-benzpyrene linked to oligonucleotides which are resistant to the nucleases and whose phosphate groups cannot be totally removed by alkaline phosphatase (Lesko *et al.*, 1969).

In following this general approach, the chemical linkage of [^{3}H,^{14}C]-3,4-benzpyrene with poly G was investigated. In the density gradient electrophoresis experiments, it has been found that the [^{3}H]-3,4-benzpyrene in the chemical complex migrates with the polynucleotides after the precipitation-washing procedure. (The experiments with the poly A·poly U complex are shown in Figure 5; the experiments with poly G are not shown.) In addition, in order to exclude the possibility of tritium exchange in the reaction and to demonstrate the linkage of 3,4-benzpyrene to the guanosine residue, the following experiment has been performed. The poly G was reacted with 3,4-benzpyrene in an iodine-induced reaction in the HMP–ethanol (33%) system for 2 hr at room temperature. The 3,4-benzpyrene contained a mixture of [^{3}H]-3,4-benzpyrene and [^{14}C]-3,4-benzpyrene in a ratio of 18:1. After the precipitation-washing procedure, the isolated 3,4-benzpyrene–poly G complex was found to contain ^{3}H and ^{14}C in a ratio of 15:1. This finding completely excluded the possibility that the radioactivity remaining with the nucleic acids after washing in the [^{3}H]-3,4-benzpyrene experiments might have originated from an exchange of tritium. The reduction of the ^{3}H:^{14}C ratio is equivalent to a loss of about 16% of the tritium of 3,4-benzpyrene in reacting with poly G. Formation of a covalent bond between 3,4-benzpyrene and the poly G should remove at least one hydrogen atom (therefore, also the corresponding amount of tritium) from the hydrocarbon. If the 3,4-benzpyrene is labeled randomly with tritium, then the loss of the ^{3}H should be 8.3%. However, the commercially available [^{3}H]-3,4-benzpyrene is prepared as "generally labeled" therefore, certain position(s) of the 3,4-benzpyrene could be more easily activated than others in the ^{3}H-labeling process and in the chemical reaction with poly G. This situation can lead to a greater loss in per cent of tritium as found experimentally (16%). On the other hand, the covalently bound 3,4-benzpyrene fragments can suffer a further loss of tritium in the reaction such as by oxidation to account for the observed loss of ^{3}H, *i.e.*, the loss of about two hydrogen atoms. At present, there is no way to decide between these two alternatives.

TABLE V: Chemical Yields (3,4-Benzpyrene/Base × 10^3) between the [^{3}H]-3,4-Benzpyrene and Ribosyl Homopolynucleotides in Reactions Induced by Iodine (1×10^{-4} M, 2 hr) and by H_2O_2–Fe^{2+} (1.5×10^{-2} M H_2O_2–1×10^{-3} M $FeSO_4$, 24 hr) in HMP Buffer or in HMP–Ethanol (33%).

Polynucleotide	I_2 Reaction in HMP Buffer at 5°	H_2O_2–Fe^{2+} Reaction in HMP Buffer at 37°
A	0.01	0.05
U	0.0017	0.0028
A + U	0.0084	0.0081
G	1.0	0.8
C	0.0037	0.0067
G + C	0.47	0.31
I		
I + C		

	I_2 Reaction in HMP–Ethanol (33%) at 5°	H_2O_2–Fe^{2+} Reaction in HMP–Ethanol (33%) at 37°
A	0.05	0.21
U	0.007	0.008
A + U	0.013	0.029
G	1.8	1.5
C	0.019	0.03
G + C	0.11	0.09
I	0.04	0.035
I + C	0.021	0.07

The [^{3}H,^{14}C]-3,4-benzpyrene–poly G complex was degraded first by ribonuclease T_1 (0.002 M Tris, pH 7.5) and then by alkaline phosphatase (0.02 M Tris–NH_4OH, pH 8.5). After each hydrolytic step, an aliquot of the enzymic hydrolysate was analyzed by sucrose gradient electrophoresis in 0.01 M sodium acetate buffer (pH 4.7). The electrophoretic patterns of the hydrolytic products after each enzyme treatment are shown in Figure 7a,b. As shown in Figure 7a, most of the poly G was degraded to 3′-GMP by T_1 ribonuclease, but the pattern also indicates the presence of a component which has a higher electrophoretic mobility and is believed to be an oligonucleotide fraction. Most of the radioactivity from [^{3}H]-3,4-benzpyrene is associated with this oligonucleotide component. After the incubation with alkaline phosphatase, the 3′-GMP was converted into guanosine which remains at the origin during electrophoresis (Figure 7). The "oligonucleotide fraction" continues to move ahead of the added 5′-GMP marker and contains most of the radioactivity (Figure 7b). These observations are consistent

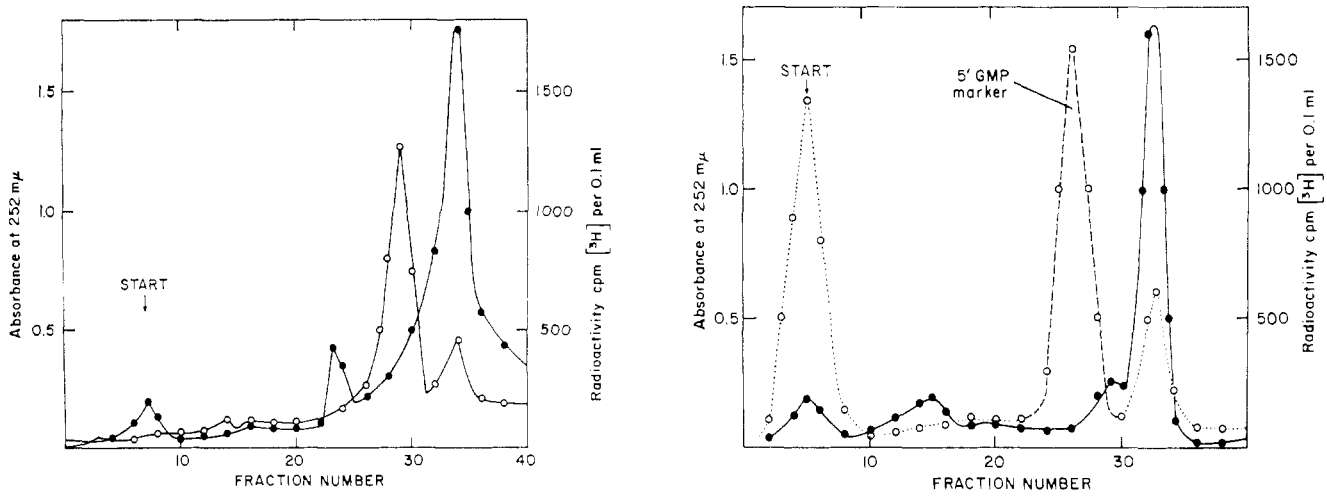

FIGURE 7: Sucrose gradient electrophoresis patterns of nucleotide and nucleoside hydrolysates resulting from enzymic digestion of a poly G–3,4-benzpyrene complex induced by I_2. Fractions (1 ml) were collected and absorbance at 260 mμ (O—O—O) and radioactivity (●—●—●) were measured. (a, left) Nucleotides obtained by treatment with RNase T_1 in 2×10^{-2} M Tris buffer (pH 7.5) at 37° for 24 hr. Electrophoresis ran 4.5 hr at 50 V/cm using 5 mA in 1×10^{-2} M sodium acetate buffer (pH 4.7). (b, right) Nucleosides obtained by treatment of the nucleotide hydrolysate with alkaline phosphatase in 2×10^{-2} M Tris (pH 8.5) at 37° for 24 hr. Conditions for electrophoresis are the same as in a. After removal of alkaline phosphatase, 5'-GMP was added as a marker (O------O).

with the idea that the attachment of [^{3}H]-3,4-benzpyrene to poly G has inhibited the action of the T_1 enzyme which has base specificity. Therefore, after T_1 digestion, most of the [^{3}H]-3,4-benzpyrene is found attached to oligonucleotide fragments; and the phosphate groups of the oligonucleotides are resistant to the alkaline phosphatase. The average size of the oligonucleotides must be bigger than a dimer since the calculated nucleotide:hydrocarbon ratio is much greater than two. Preliminary experiments indicate that these [^{3}H]-3,4-benzpyrene oligo G fragments are also rather resistant to the action of venom diesterase.

Discussion

The first objective of these studies was to increase the reaction yield of the chemically linked hydrocarbons to native DNA significantly, so that the reaction product(s) with the base(s) can be adequately characterized and the biological effect of the hydrocarbon–DNA chemical complex can be conveniently examined. To this end, three approaches have been adopted: (1) The solubilities (therefore, the availabilities) of the hydrocarbon and the iodine have been substantially increased by changing the reaction medium from an aqueous solution to a buffer–ethanol (33%) system. The DNA concentration is reduced so that the ratio of hydrocarbon:DNA in the reaction mixture becomes as high as possible. (2) Fresh hydrocarbon is introduced into the reaction mixture to set up a stepwise reaction procedure without reisolation of DNA. This approach is based on the knowledge of the reaction kinetics which indicates that the hydrocarbon is almost exhausted within 0.5 hr. (3) Finally, the once reacted DNA is reisolated and is used again in the next reaction under identical conditions. We have performed the reaction three times on the same DNA sample. With a combination of these approaches, we have introduced one benzpyrene molecule to about 300 bases for thymus DNA and to about 100 bases for *B. subtilis* transforming DNA (Lesko *et al.*, 1970). This is a 10–30-fold increase in reaction yield compared with the previous procedure in the aqueous system (Lesko *et al.*, 1969). However, the second and third approaches should be used with caution and moderation. When the reaction is being repeated several times, the probability of side reactions with the already attached hydrocarbon fragment(s) increases. The attached hydrocarbon fragments can be further affected by the activating agent such as oxidation or dimerization, etc. There is also loss in the step of reisolation of DNA from the reaction mixture.

The second objective of these studies was to investigate further the reactivity of various polycyclic hydrocarbons with native DNA under these reactions conditions. As shown in Table V, the two carcinogenic hydrocarbons, 3,4-benzpyrene and 9,10-dimethyl-1,2-benzanthracene, are manyfold (4–14-fold) more reactive than their respective noncarcinogenic analogs, 1,2-benzpyrene and 1,2-benzanthracene. The carcinogenic 20-methylcholanthrene is also comparably reactive in this iodine-induced reaction. Owing to the much higher solubility of 9,10-dimethyl-1,2-benzanthracene as compared with that of 3,4-benzpyrene, in the HMP–ethanol system, the reaction yield with saturating concentration of 9,10-dimethyl-1,2-benzanthracene can be 50% higher than that with 3,4-benzpyrene, even though the 3,4-benzpyrene is more reactive than the 9,10-dimethyl-1,2-benzanthracene when both hydrocarbons are present in the same concentration (Table II).

The third objective was the investigation of the base specificity of the reaction, since this is actually the first step to elucidate the precise chemical mechanism of the reactions. For this purpose, the homopolynucleotides and their double-stranded complexes were used as model systems. The results indicate that the reaction with 3,4-benzpyrene is indeed base specific and the degree of specificity depends on the activation system. In the I_2-induced reaction, the poly G, even in a double helix with poly C, is preferred overwhelmingly over other polynucleotides (Tables IV and V),

indicating the I_2-induced reaction is highly specific for guanine derivatives. In the H_2O_2–Fe^{2+}-induced system, the poly G is still much preferred over other polynucleotides if expressed in terms of the absolute basis, *i.e.*, benzpyrene/bases, especially in the complete aqueous solution (Table IV). However, if the reaction yield in the H_2O_2–Fe^{2+} system is expressed in terms of "per cent of physically bound converted into chemically linked" (Table IV), then the poly G is only about 15% more reactive than the poly A, while both purine polynucleotides are much more preferred than poly U and poly C.

While the chemical mechanisms in the I_2-induced and the H_2O_2–Fe^{2+}-induced reactions are not known in detail, there exists a considerable amount of pertinent information. A strong interaction between iodine and 3,4-benzpyrene is indicated by the formation of black "charge-transfer" complexes (Szent-Györgyi *et al.*, 1960; Epstein *et al.*, 1964) and the appearance of an electron paramagnetic resonance signal (Szent-Györgyi *et al.*, 1960). Formation of 5,5′ dimers of 3,4-benzpyrene and various quinones of 3,4-benzpyrene, as well as the formation of N-substituted 5-benzopyrenylpyridinium salts have been reported when 3,4-benzpyrene alone or with pyridine absorbed in a silica gel surface was exposed to iodine vapor (Wilk *et al.*, 1966; Rochlitz, 1967). Reaction of 3,4-benzpyrene with various bases and nucleosides in the silica gel–iodine vapor system has been shown by chromatography (Wilk and Girke, 1969). These findings led Wilk and coworkers to propose that the "radical cation" of 3,4-benzpyrene is the active intermediate in these 3,4-benzpyrene reactions induced by iodine vapor (for review, see Wilk and Girke, 1969). In our preliminary experiments, the 5-benzopyrenylpyridinium salt was also found as the reaction product in the 3,4-benzpyrene–pyridine (50%)–HMP buffer–I_2 system (Hoffman *et al.*, 1969). This finding suggests that when the reaction is carried out in a solution of water and organic solvents, the radical cation of 3,4-benzpyrene also is likely to be the active intermediate.

The chemical mechanism in the reaction induced by H_2O_2–Fe^{2+} is unlikely to be identical with that induced by I_2. As shown in Table IV, 3,4-benzpyrene reacts with both poly G and poly A to about the same extent in the reaction induced by H_2O_2–Fe^{2+}, while the 3,4-benzpyrene reacts mainly with poly G in the I_2-induced reaction. In our preliminary experiment on the 3,4-benzpyrene–pyridine (50%)–HMP–H_2O_2–Fe^{2+} system, a product other than the N-substituted 5-benzopyrenylpyridinium salt was found (Hoffmann *et al.*, 1969). It is tempting to speculate that the active intermediate of 3,4-benzpyrene in the H_2O_2–Fe^{2+} reaction is a neutral radical, either a neutral radical of the hydrocarbon itself generated by the abstraction of an H atom from the hydrocarbon by an OH radical or a neutral phenoxy radical which can be formed from the hydroxylated hydrocarbon by further oxidation. The latter type of radical was proposed by Nagata *et al.* (1967) to exist in the incubation mixture of 3,4-benzpyrene with skin homogenates based on electron paramagnetic resonance experiments. Hoffmann and Müller (1969) report that the product of 3,4-benzpyrene with guanine nucleotides formed by X-ray irradiation is an adduct of benzpyrene to the C-8 position of guanine residues. The neutral aryl radicals are known to be less specific in their reaction with the bases and they react with the C-8 atom of both guanine and adenine derivatives.

The electrochemical oxidation of 3,4-benzpyrene has been studied by Pysh and Yang (1963) and more recently by Jeftic and Adams (1970) in an extensive and thorough manner. The initial oxidation of 3,4-benzpyrene is a one-electron process to the radical cation which can form a neutral radical by reacting with H_2O and by removal of a proton. This neutral radical can be further oxidized to a hydroxyl hydrocarbon. The hydroxyl 3,4-benzpyrene upon further oxidation produces a neutral phenoxy radical which then becomes a quinonoid derivative. The results of Jeftic and Adams (1970) are very helpful in providing an understanding about the mechanism of the reactions reported here. A comprehensive and thoughtful review on the theoretical aspects of the formation of radical cations of polycyclic hydrocarbons and the possible relevance of such a reaction to the mechanism of chemical carcinogenesis has been written recently by the Pullmans and coworkers (1969). This article is of particular interest to the present paper, since the authors have attempted to calculate the stereochemical interaction of the 3,4-benzpyrene cation radical to the G·C base pair.

Chemical complexes of polycyclic carcinogens with DNA have been found in biological systems (Heidelberger and Davenport, 1961; Brookes and Lawley, 1964; Goshman and Heidelberger, 1967; Brookes and Heidelberger, 1969). More recently, a chemical linkage of 3,4-benzpyrene to nucleic acids through the action of microsomal enzyme systems involving NADPH has been reported (Grover and Sims, 1968; Gelboin, 1969). The yield of this enzymic reaction is about 1 benzpyrene/50,000–500,000 nucleotides. The amount of covalent complex of carcinogenic hydrocarbons and nucleic acids formed in biological or enzymic systems is likely to be very minute, and the characterization of the fragment(s) and the linkage(s) would be very difficult without the aid of model systems and model compounds. The reactions and the products described here may be helpful for this purpose. In addition, the high reaction yield (such as 1 benzpyrene/100 bases in nucleic acids) allows us to examine the biological effect of the linkage of the carcinogenic hydrocarbon to DNA in model systems. In our preliminary experiments, we have found that chemical linkage of 3,4-benzpyrene to *B. subtilis* SB 19 transforming DNA formed in an I_2-induced reaction has a mutagenic effect (Lesko *et al.*, 1970) when assayed with the linked genes in the tryptophan operon system (Maher *et al.*, 1968).

References

Barnard, E. A. (1969), *Ann. Rev. Biochem. 38*, 690.

Blake, R. D., Massoulie, J., and Fresco, J. R. (1967), *J. Mol. Biol. 30*, 296.

Brookes, P., and Heidelberger, C. (1969), *Cancer Res. 29*, 157.

Brookes, P., and Lawley, P. D. (1964), *Nature 202*, 781.

Chamberlin, M. J. (1965), *Fed. Proc. 24*, 1446.

Epstein, S., Bular, I., Kaplan, J., Small, M., and Mantel, N. (1964), *Nature 204*, 750.

Fresco, J. R., Klotz, L., and Richards, E. (1963), *Cold Spring Harbour Symp. Quant. Biol. 28*, 83.

Gelboin, H. V. (1969), *Cancer. Res. 29*, 1272.

Goshman, L. M., and Heidelberger, C. (1967), *Cancer Res. 27*, 1678.

Grover, P. L., and Sims, P. (1968), *Biochem. J. 110*, 159.

Haselkorn, R., and Fox, C. F. (1965), *J. Mol. Biol. 13*, 780.
Heidelberger, C., and Davenport, G. R. (1961), *Acta Unio Int. Contra Cancrum 17*, 55.
Hoffmann, H. D., Lesko, S. A., and Ts'o, P. O. P. (1969), 158th National Meeting of American Chemical Society, New York, N. Y., Abstract MEdI 074.
Hoffmann, H. D., and Müller, W. (1969), *Jerusalem Symp. Quantum Chem. Biochem. 1*, 183.
Jeftic, L., and Adams, R. N. (1970), *J. Am. Chem. Soc. 92*, 1332.
Lesko, S., Hoffmann, H., Ts'o, P. O. P., and Maher, V. (1970), *Biophys. J. 10*, 171a.
Lesko, S. A., Jr., Smith, A., Ts'o, P. O. P., and Umans, R. S. (1968), *Biochemistry 7*, 434.
Lesko, S. A., Jr., Ts'o, P. O. P., and Umans, R. S. (1969), *Biochemistry 8*, 2291.
Maher, V. M., Miller E. C., Miller, J. A., and Szybalski, W. (1968), *Mol. Pharm. 4*, 411.
Michelson, A. M., and Monny, C. (1966), *Biochim. Biophys. Acta 129*, 460.
Michelson, A. M., and Pochon, F. (1969), *Biochim. Biophys. Acta 174*, 604.
Nagata, C., Kodama, M., and Tagashira, Y. (1967), *Gann 58*, 493.
Pullman, A., Pullman, B., Umans, R., and Maigret, B. (1969), *Jerusalem Symp. Quantum Chem. Biochem. 1*, 325.
Pysh, E. S., and Yang, N. C. (1963), *J. Am. Chem. Soc. 85*, 2124.
Rapaport, S. A., and Ts'o, P. O. P. (1966), *Proc. Natl. Acad. Sci. U. S. 55*, 381.
Rochlitz, J. (1967), *Tetrahedron 23*, 3043.
Sarkar, P. K., and Yang, J. T. (1965), *Biochemistry 4*, 1238.
Schaad R. Bachmann, R., and Gilgen, A. (1969), *J. Chromatog. 41*, 120.
Szent-Györgyi, A., Isenberg, I., and Baird, S. L. (1960), *Proc. Natl. Acad. Sci. U. S. 46*, 1444.
Ts'o, P. O. P., Helmkamp, G. K., and Sander, C. (1962), *Biochim. Biophys. Acta 55*, 584.
Ts'o, P. O. P., and Lu, P. (1964), *Proc. Natl. Acad. Sci. U. S. 51*, 272.
Wilk, M., Bez, W., and Rochlitz, J. (1966), *Tetrahedron 22*, 2599.
Wilk, M., and Girke, W. (1969), *Jerusalem Symp. Quantum Chem. Biochem. 1*, 91.