Interaction of Nucleic Acids. IV. The Physical Binding of 3,4-Benzpyrene to Nucleosides, Nucleotides, Nucleic Acids, and Nucleoprotein*

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ABSTRACT: Complexing of 3,4-benzpyrene (BP) with a series of nucleosides, 5′-nucleotides, native and denatured deoxyribonucleic acid (DNA), calf thymus nucleohistone, synthetic polyribonucleotides, and yeast ribonucleic acid (RNA) has been investigated using radiochemical assay methods. Physical binding to the polynucleotides and nucleohistone has been demonstrated by sucrose gradient electrophoresis and ultracentrifugation.

In the case of the nucleohistones only a small fraction of the bound BP can be extracted with the histone moiety. Spectral studies of the energies of the visible absorption bands of BP in a variety of solvents and solutions of other compounds have led to corroboration for the intercalation model of BP binding to nucleic acids. Comparison of binding to native DNA of carcinogenic hydrocarbons and one of their noncarcinogenic 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 member of each pair, although the binding constant for the two benzpyrenes exceeded by a factor of five that for the two dibenzanthracenes. Exchange experiments eliminated the possibility that both 3,4- and 1,2-benzpyrene bind to specific and identical sites along the DNA helix, although these studies could not distinguish between other possible binding models.

Solubilization of 3,4-benzpyrene (BP)[1] in aqueous solution of caffeine and other nitrogenous bases was first reported by Brock *et al.* (1938). Since then, many other investigators, notably Weil-Marlherbe (1946), Booth and Boyland (1953), Booth *et al.* (1954), and Boyland and Green (1962a) have confirmed this observation and shown that many purines and nucleosides exert this solubility effect toward different aromatic hydrocarbons. Booth *et al.* (1951), Liquori *et al.* (1962), Boyland and Green (1962b), and Ts'o and Lu (1964a) have noted that BP is soluble to a much greater extent in solutions of deoxyribonucleic acids than in solutions of simple purines. More recently, the problem of binding of BP to DNA has been further studied in various laboratories (Giovanella *et al.*, 1964; Ball *et al.*, 1965; Lerman, 1964; Kodama *et al.*, 1966; Nagata *et al.*, 1966; Liquori *et al.*, 1967; Isenberg *et al.*, 1967).

Previous work in our group (Ts'o *et al.*, 1962a,b, 1963a,b; Ts'o and Chan, 1964; Chan *et al.*, 1964; Ts'o and Lu, 1964a; Schweizer *et al.*, 1965a) has indicated that hydrophobic and stacking interactions are important in the association of purine bases and nucleosides in solution, as well as in the binding of purine nucleosides, their analogs, and aromatic hydrocarbons to nucleic acids. Recently, we have reported the interesting discovery that a chemical linkage between BP and DNA and other polynucleotides is induced by photoirradiation at wavelengths above 320 mμ and also by X-ray irradiation (Ts'o and Lu, 1964b; Ts'o *et al.*, 1964c; Rapaport and Ts'o, 1966).

As an extension to the early work, a detailed study was undertaken of the binding of BP to nucleosides, nucleotides, native and denatured DNA, polyadenylic acid (poly A), polycytidylic acid (poly C), polyuridylic acid (poly U), yeast RNA, and calf thymus nucleohistone (DNP), by the use of tritiated BP. Since the concentration of BP in aqueous media is very low because of the limited solubility, results obtained by the sensitive radiochemical method may be more reliable and more straightforward in interpretation than those obtained by spectrophotometric or fluorometric assays employed by others. Special attention has been given to providing conclusive evidence for the physical binding of BP to these biopolymers. Extensive spectral data which support the proposal of intercalation of BP between the base stacks in the nucleic acid are presented. We have also made a preliminary survey of the binding of other carcinogenic and noncarcinogenic polycyclic hydrocarbons to native DNA.

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[1] Abbreviations used: BP, 3,4-benzpyrene; DNP, calf thymus nucleohistone; [^{3}H]BP, tritiated, 3,4-benzpyrene; HMP, phosphate buffer (pH 6.8) consisting of disodium hydrogen phosphate (0.0025 M), sodium dihydrogen phosphate (0.005 M), and EDTA (0.001 M); TMP, phosphate buffer (pH 6.8) consisting of disodium hydrogen phosphate (0.00025 M) and sodium dihydrogen phosphate (0.0005 M); 5′-AMP, -CMP, -TMP, and -GMP, 5′-adenosine, -cytidine, -thymidine, and -guanosine monophosphates; TMS, tetramethylsilane; TMU, 1,3,7,9-tetramethyluric acid.

Materials

[^{3}H]BP with specific activities varying from 345 to 790 mc/mmole was purchased as dry powder from the Radiochemical Centre through the Nuclear-Chicago Corp. Radiochemical purity, based on chromatographic assay, was reported to be over 99% by the manufacturer. Chromatographic examination of two batches in our laboratory out of the several purchased confirmed this report (Ts'o and Lu, 1964b; Ts'o *et al.*, 1964). Generally labeled [^{3}H]1,2-benzpyrene (242 mc/mmole, purity >98%), [^{3}H]1,2,3,4-dibenzanthracene (306 mc/mmole, purity >96%), and [^{3}H]1,2,5,6-dibenzanthracene (270 mc/mmole, purity ≈95%) were also purchased from the Nuclear-Chicago Corp. Nonradioactive BP was obtained from Nutritional Biochemicals Corp. The nucleotides and nucleosides were purchased either from Sigma Chemical Corp. or Calbiochem and used without further purification. The highly polymerized calf thymus DNA was obtained from Sigma Chemical Corp. This commercial preparation of DNA has a $s_{20,50\%}$ value of 13.5 S and 35–40% hyperchromicity when heated above melting temperature after shaking for 10 days. Other characteristics of this DNA preparation, such as optical rotatory dispersion properties and density measured in CsCl gradient centrifugation, were reported earlier from our laboratories (Helmkamp and Ts'o, 1961; Ts'o *et al.*, 1962a,b, 1963a). Thymus DNA prepared from sheared nucleohistone by procedure of Ts'o and Squires (1959) has also been used, especially in experiments for comparative studies between DNA and nucleohistones. This DNA preparation has a $s_{20,50\%}$ value of 12.1 S and 37% hyperchromicity on melting. No difference in BP binding was found between the commercial and our own DNA preparation.

All solutions were made up in aqueous 0.01 M phosphate buffer (HMP) at pH 6.8, which consisted of disodium hydrogen phosphate (0.0025 M), sodium dihydrogen phosphate (0.005 M), and EDTA (0.001 M) unless otherwise noted.

The concentrations of the nucleotide, nucleoside, and DNA solutions were calculated from ultraviolet absorption data. The molar extinction coefficient per nucleotide of native DNA at 260 mμ was taken as 6.6×10^3 (Ts'o *et al.*, 1963a) measured in 0.05 M Tris buffer (pH 7.5). Solutions of denatured DNA in HMP were prepared by heating the native material in boiling water for 10, 20, or 40 min, after which time the solutions were cooled in ice–water for 1 hr to prevent renaturation.

Poly A was purchased from the Miles Chemical Co. However, two batches were prepared in this laboratory using the procedure of Steiner and Beers (1961). One sample was prepared at room temperature and had a sedimentation coefficient of 14 S while the other sample, which was prepared at 5°, had a sedimentation coefficient of 19 S. The concentration of poly A solutions was calculated from the absorbancy at 257 mμ in 0.05 M Tris buffer (pH 7.5) using a molar extinction coefficient of 10.5×10^3/mole of bases (Helmkamp and Ts'o, 1962).

Poly C and poly U were both obtained from the Miles Chemical Co., the molar extinction coefficients being taken as 6.6×10^3/mole of bases at 267 mμ and 9.2×10^3/mole of bases at 260 mμ, respectively (Ts'o *et al.*, 1963b).

Purified yeast RNA, type XI (prepared by Crestfield procedure), was purchased from Sigma Chemical Co., and concentrations were calculated from the absorbancy at 259 mμ taking the molar extinction coefficient as 8.0×10^3/mole of bases.

Nucleohistone was extracted from calf thymus by the method of Zubay and Doty (1959). This procedure was followed up to the dissolution of the DNP in water, when at this stage the material was dispersed in 400 ml of water rather than 1 l. The viscous gel was dialyzed against 0.001 M phosphate (TMP) (pH 6.8) and then sheared in Virtis "45" homogenizer for 90 sec at 30 v using 80-ml lots. After the addition of 100 ml of TMP the sheared material was stirred for 1 hr. The DNP was centrifuged for 15 min at 30,000*g* in a Sorvall refrigerated centrifuge and the top three-fourths of the supernatant was decanted. This solution was then centrifuged at 40,000*g* for 1 hr and the supernatant was again carefully decanted. A typical preparation had an absorbancy at 259 mμ of approximately 10, contained approximately 40% DNA and 60% protein by weight, and had an average sedimentation coefficient of 30 S. DNA extracted from the sheared DNP had an average sedimentation coefficient of 12 S. The nucleotide concentration of a DNP solution was calculated from the absorbancy at 259 mμ using a molar extinction coefficient of 6.7×10^3/mole of bases (Fredericq, 1962) in TMP. Whole histone was extracted from DNP by the procedure of Luck *et al.* (1958).

Experimental Procedures

Preparation and Treatment of Substrate–Benzpyrene Solutions. A small amount of the solid [^{3}H]BP was added to volumetric tubes containing 1- or 2-ml samples of the solution under test. These tubes were covered with aluminum foil and shaken on a rotatory shaker at 5°. After varying intervals of time, aliquots were taken from each tube and the excess [^{3}H]BP solid was removed by filtration through a fine sintered-glass filter under a slight positive pressure of nitrogen. Since most nucleohistone preparations would not pass through fine sintered-glass filters, excess [^{3}H]BP was removed from these solutions by centrifugation, 15 min at 5000 rpm and then 1 hr at 15,000 rpm in a Sorvall RC-2B. Experimental data showed that the same results for BP binding to DNP were obtained by either filtration or centrifugation procedures.

The radioactivity in the filtrates was measured using a Packard Tri-Carb liquid scintillation spectrometer (Model 314 A). For counting tritium the glass counting vials contained either a 0.1- or 0.2-ml sample and a 10-ml counting solution prepared from naphthalene (120 g), 2,5-diphenyloxazole (4.0 g), and 1,4-bis[2-(5-phenyloxazolyl)]benzene (0.05 g) in *p*-dioxane (1000 ml). For purposes of comparison with spectrophotometric

methods, the binding of [^{3}H]BP to native DNA in two experiments was measured simultaneously by radioactivity and by ultraviolet absorption spectroscopy with a 10-cm cell. In both cases the agreement was within 5% as calculated from the provided specific activity of the [^{3}H]BP and the molar extinction of the intense visible band of BP in ethanol ($E_{384\ m\mu}$ 27,500). The possible presence of a radioactive impurity would affect primarily the aqueous solubility measurements which are used to calculate equilibrium constants but would have little influence on the molar ratio of base to bound hydrocarbon due to the extremely high affinity for DNA of these polycyclic hydrocarbons as compared with most other compounds.

The glass filters were chosen with care and selected as follows. A solution of a nucleotide was shaken with [^{3}H]BP for several days as described, and portions were filtered through each of the filters in turn. The radioactivity in each of the filtrates was measured and compared. The filter which yielded the lowest amount of radioactivity in the filtrate was selected as the standard, and any filter which passed 10% more radioactivity per milliliter than the standard was rejected.

A problem encountered was that of adsorption of [^{3}H]BP to various materials, especially to plastics, celluloses, Millipore filters, dialysis tubing, etc., a fact which has also been observed by Boyland and Green (1964a). Experiments showed that some slight adsorption of the hydrocarbon to glass occurred over a period of time, and that this effect was more noticeable at very low hydrocarbon concentrations. Hence, the time between filtration and counting the radioactivity was kept to a minimum (under 5 min), where these adsorption problems were found to be negligible.

Sucrose gradient electrophoresis experiments were made with an apparatus similar to that designed by Olivera *et al.* (1964). The important part of the apparatus consists of two concentric glass tubes about 40 cm long. The inner tube, with a diameter of 1.5 cm, is the electrophoresis column, and the outer is a cooling jacket through which water at 4° is circulated. A linear density gradient between 6 and 27% sucrose, with uniform supporting electrolyte (HMP) was placed through the top of the tube and 0.5 ml of sample solution containing 4% sucrose was layered on the top of the density gradient column. Finally, the sample solution was covered with the buffer solution and then 3–4 ma of current were applied through bridges which contained suitable concentrations of sucrose and electrolytes.

After several hours, the solution in the inner tube was collected through the bottom stopcock into 45–50 fractions each of which contained about 1 ml. These samples were assayed for optical density at 260 mμ and for radioactivity. When the histone–BP complex was subjected to sucrose electrophoresis, the protein content of each fraction was determined by the method of Lowry *et al.* (1951).

Sucrose Gradient Ultracentrifugation. The denatured DNA–BP samples were layered on a 5–25% sucrose gradient and centrifuged for 22 hr at 20,000 rpm in a Spinco Model L centrifuge with a SW 25 swinging-bucket rotor at 5°. After the run, 2-ml fractions were collected from the bottom and the absorbancy at 260 mμ together with the radioactivity in each fraction were measured.

The DNP–BP samples were layered on a 4.6-ml, 5–20% sucrose gradient and centrifuged at 30,000 rpm for 16 hr in a Spinco Model L centrifuge using a SW 39 swinging-bucket rotor at 5°. Ten drop fractions were collected into tubes containing 1 ml of TMP. The absorbancy and radioactivity of each fraction were measured.

Absorption Spectra. Spectra of BP dissolved in solutions of DNA, poly A, 5′-AMP, and purine were taken with a Cary 14 or 15 spectrophotometer with cells of 10-cm optical path. In the cases of 5′-AMP and purine, difference spectra were taken. Reference solutions consisted of 5′-AMP or purine of concentrations identical with those in the substrate–BP solutions. All other BP spectra were taken with cells of 1-cm optical path.

Nuclear Magnetic Resonance Spectroscopy. The nuclear magnetic resonance data were obtained with a 100-Mcycle Varian HA-100 spectrometer with a temperature-control probe. Chemical shifts were measured from an external TMS capillary.

Results

Binding to Nucleosides and 5′-Nucleotides. The solubility of BP in HMP was determined by shaking the [^{3}H]BP in the dark at 5°. Aliquots were removed and filtered after various intervals of time. Maximum solubility was found to be reached in 2 days and a value of 0.017 μM was obtained by averaging ten experiments, a figure used throughout these investigations. (In some of these experiments a value as high as 0.06 μM has been obtained). By comparison a value of 0.016 μM in water was found by Davis *et al.* (1942) using a nephelometric technique; 0.024 μM was found by Weil-Marlherbe (1946) and 0.009 μM by Boyland and Green (1962b), both using a fluorescence procedure. From our own group, a slightly higher figure of 0.03 μM was observed previously.

Several determinations have been made of the solubility of [^{3}H]BP in solutions of nucleosides and nucleotides of both ribosyl and deoxyribosyl compounds in HMP, as well as the solubility in mixtures of nucleotides both in HMP and in 0.5 M NaCl. For each determination, three replicates were shaken separately with [^{3}H]BP and the average solubility was calculated. No measureable difference was encountered whether the samples were filtered at 5 or 20°. Only one filtering was found to be necessary, as a second filtration never removed more than an additional 5% of the [^{3}H]BP.

The kinetics for all the nucleotides and nucleosides were individually examined and, on the average, 5–10 days were required to achieve saturation. This contrasts with the finding of Weil-Marlherbe (1946) and Boyland and Green (1962a,b), who observed that saturation was complete in 16–24 hr. However, these investigators used a large excess of BP, whereas in the present

TABLE I: Binding of 3,4-Benzpyrene to Deoxy- and Ribonucleotide Mixtures at 5°.

Buffer		Concn (mM)	Molar Ratio of Base:BP $\times 10^{-3}$	Binding Constant K (M^{-1})
HMP–0.5 M NaCl	d-AMP	35	107	550
	TMP	46		
	d-CMP	34		
	d-GMP	47		
HMP	d-AMP	32	107	550
	TMP	45		
	d-CMP	33		
	d-GMP	43		
HMP–0.5 M NaCl	r-AMP	51	116	505
	r-UMP	48		
	r-CMP	55		
	r-GMP	57		
HMP	r-AMP	31	84	700
	r-UMP	47		
	r-CMP	57		
	r-GMP	57		

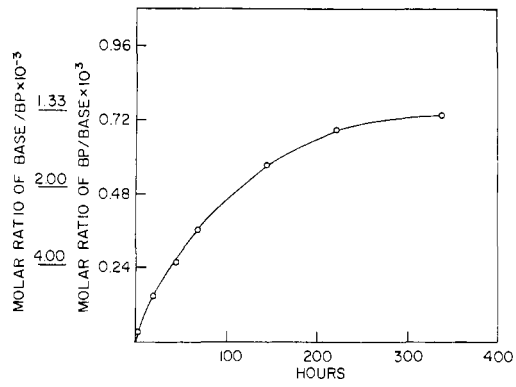

FIGURE 1: Kinetics of the binding of the [^{3}H]BP to native DNA in TMP (1:10 M HMP) at 5°.

experiments only a small amount of the [^{3}H]BP was added.

For each case, the solubility results were used to determine a binding constant K, defined as

$$K\ (M^{-1}) = \frac{\text{(BP solubilized)}}{\text{(substrate)(solubility of BP in HMP)}}$$

where the concentration of the nucleic acid (substrate) was expressed in term of monomeric units. Owing to the extremely low solubility of BP in aqueous buffer, the value determined (0.017 μM) probably has an uncertainty factor of two. Therefore, the true values of K (M^{-1}) may differ from the apparent values by the same factor. The problem of this uncertainty, however, is fortunately minimized for two reasons. First of all, since a given value for the solubility of BP has been adopted throughout this research, the different K values can be directly compared with one another without involving the uncertainly factor since the same denominator has been used for all these calculations. Secondly, the free-energy values obtained from these binding constants will be considerably more accurate since they involve log K, which is a very slowly varying function of K.

Because of the substantial variation of the data, only general conclusions can be reached. Adenosine, 5′-AMP, cytidine, 5′-CMP, thymidine, 5′-TMP, and 5′-GMP each tested in both the ribo and deoxyribo forms, all exhibited binding constants ranging from 100 to 600, clustering mainly around 400. Although the variation in the results was too great to permit conclusions to be drawn concerning differences in solubility of BP in deoxyribo *vs.* ribo compounds, or in purine *vs.* pyrimidines, it was quite clear that there are no great differences of solubility between BP solutions of nucleosides and nucleotides. Mixtures of nucleotides did not bring out any synergistic effect on the binding, and it appears that the average result was obtained (Table I). The binding constants, however, are 10–100-fold lower than those calculated for corresponding solutions of nucleic acids and polynucleotides (see later sections).

Binding to Native and Denatured DNA. SOLUBILITY MEASUREMENTS. For each determination, three or four replicates were shaken at 5° in the dark. Only one filtration was necessary as less than 5% of the [^{3}H]BP was removed by a second filtration. No difference was found whether the filtering was carried out at 5° or at room temperature. A period of 10–14 days was required for the solutions to become saturated with the hydrocarbon (Figure 1). This is a much longer time than the 1 day noted by others (Liquori *et al.*, 1962; Boyland and Green, 1962a,b) and may be attributed to the small amount of radioactive solids added.

The results are summarized in Table II. In this table, the denatured DNA was obtained by heating the native sample at 100° for 10 min. DNA heated for 20 and 40 min was also used. The results obtained from these samples (1100–1400 M ratio of bases to BP and binding constants (M^{-1}) 4.4–5.4 $\times$ 10^4) were slightly higher than those obtained from the denatured DNA heated for 10 min. There are certain variations in the results. For example, 12 determinations of BP binding to native DNA in HMP have been made, representative values of which are reported in Table II. The average value for the binding constant is 4.4 $\times$ 10^4 with a spread of 2.5–6.0 $\times$ 10^4.

One interesting effect, *i.e.*, the influence of salts on the binding of BP to DNA, is strongly dependent on the DNA's being in the native or denatured form (Table II). Addition of salts decreases the binding in the case of native DNA but increases the binding in the case of denatured DNA. Further proof for the first part of this

TABLE II: Binding of Benzpyrene to Native and Denatured DNA at 5°.

DNA	Nucleotide Concn (mM)	Salt Concn	Molar Ratio of Base:BP	Binding Constant, K (M^{-1})
Native	2.2[a]	HMP	2,400	25,000
	2.8	HMP	1,200	50,000
	4.1	HMP	1,700	35,000
	5.0	HMP	1,000	55,000
	2.2	TMP[b]	1,800	33,000
	2.2	HMP + 0.5 M NaCl	6,700	9,000
Denatured	2.2	HMP	2,400	25,000
	3.7	HMP	2,500	23,000
	6.0	HMP	2,100	27,000
	9.2	HMP	3,200	18,000
	2.2	TMP	2,100	27,000
	2.2	HMP + 0.5 M NaCl	900	60,000

[a] Triplicates were run for experiments with nucleotide concentration at 2.2 mM. Quadruplicates were run for all other sets of nucleotide concentration. [b] TMP is one-tenth the concentration of HMP.

statement can be obtained from Table III. In this experiment, salts were introduced to the BP–DNA mixture after 2 weeks of shaking at 5°. Then, the samples were allowed to shake for an additional 20 hr. Comparison of the solubility of BP determined on the same tube before and after the increase of ionic strength (Table III) indicated that addition of salt up to 0.2–0.5 M did decrease the binding of BP for the native DNA sample shown in Table II. The decrease was not noticeable for the native DNA when the ionic strength was changed from TMP to HMP, again in agreement with Table II. The increase in binding of BP to denatured DNA at higher ionic strength (0.5 M NaCl) (Table II) was not evident in Table III. We attribute this difference to a kinetic effect. It is not unreasonable to believe that the time required for the DNA to lose an excess amount of bound BP in an unfavorable condition is much shorter than the time required for the DNA to bind an additional amount of BP from the solution in a favorable condition. Therefore, the period of 20 hr apparently is sufficient for the demonstration of the first process (decrease in binding) but not for the second process (increase in binding).

TABLE III: Effect of Increase in Ionic Strength on the Binding of BP to the DNA at 5°.[a]

Increase		Solubility before Addn of Salts: Solubility after Addn of Salts	
From	To	Native	Denatured DNA
TMP	HMP[b]	1.0	1.0
HMP	HMP–0.2 M[c] NaCl	0.5	1.0
HMP	HMP–0.5 M[b] NaCl	0.5	1.0

[a] Solubility of BP was redetermined after the addition of salts and further shaking for 20 hr. [b] Triplicates—the absolute value of the control (without the addition of salts) is reprinted in Table II, data from experiments done with nucleotide concentration at 2.2 mM. [c] Quadruplicates.

The binding constant of BP for denatured DNA reported in Table II is comparable to that found previously by us for DNA heated for 30 min (5.4×10^{-4} in HMP; Ts'o and Lu, 1964b). However, the binding constant for the native DNA is about five times higher than that obtained earlier (Ts'o and Lu, 1964b). The low value obtained previously was most likely due to inadequate shaking in the early experiments because of the high viscosity of native DNA solutions. The values of molar ratio of base to BP reported in Table II are in the same range as that published by Boyland and Green (1964b) or by Kodama *et al.* (1966), and that for native DNA published by Liquori *et al.* (1962, 1967). The values in Table II are about 10–20-fold lower than that published by Ball *et al.* (1965), Lerman (1964), and for denatured DNA by Liquori *et al.* (1962). This discrepancy will be discussed in later sections.

Since 2 weeks of shaking were required for maximal solubilization of the hydrocarbon in these experiments, it was imperative to determine the state of the nucleic acids and [^{3}H]BP after this period. Helix–coil transition profiles of the native and denatured DNA were obtained by optical density measurements before and

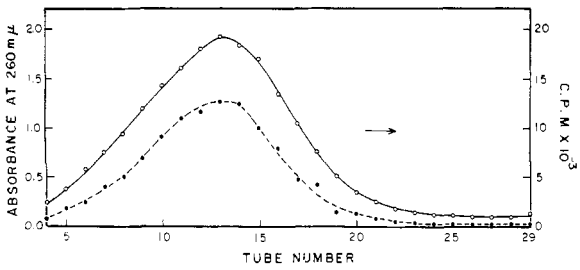

FIGURE 2: Sucrose gradient centrifugation pattern of the BP-denatured DNA physical complex. SW 25 rotor 20,000 rpm; 22 hr in HMP, pH 6.8. Samples of 2 ml were collected and optical density (O——O) and radioactivity (●——●) were measured.

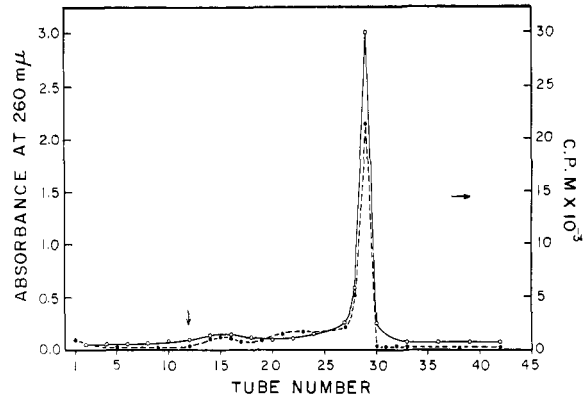

FIGURE 3: Sucrose gradient electrophoretic pattern of BP-native DNA physical complex in HMP (pH 6.8); 1000 v, 4 ma, 2.5 hr. Samples of collected and optical density (O——O) and radioactivity (●- - - -●) were measured.

after a 21-day period of shaking. The hyperchromicity and shape of the profiles were found to be unchanged and in agreement with previous experiments (Ts'o *et al.*, 1962a,b, 1963a). Therefore, it was assumed that native and denatured DNA maintained their conformation after this period of shaking in HMP at 5°.

The state of the physically bound [^{3}H]BP was checked by the following experiments. DNA (2 ml) previously saturated with [^{3}H]BP and filtered was extracted twice with *n*-hexane (2 ml). The extraction removed 95% of the radioactivity from the aqueous layer. Nonradioactive BP was added as carrier and the mixture was spotted on an Eastman Silica chromatogram sheet. After development in *n*-hexane, it was found that over 85% of the original radioactivity was contained in the small fluorescent spot of the carrier BP. No other spots of radioactivity were found. This experiment indicated that the radioactivity bound to DNA after shaking and filtration is indeed due to BP and not to impurities or degradation products. It should also be noted that after ethanol precipitation and washing, virtually all the [^{3}H]BP (99.8%) could be removed from the DNA, indicating that no chemical linkage was formed between [^{3}H]BP and DNA.

Addition of excess nonradioactive BP solid to solutions of [^{3}H]BP–DNA at 5° (both native and denatured) caused a rapid loss of radioactivity upon a second filtration. After 4 hr of shaking, 50–60% of the radioactivity was removed, while after 24 hr, 75% was removed. These results again indicate that this binding process is a reversible one.

SUCROSE GRADIENT ULTRACENTRIFUGATION AND ELECTROPHORESIS. A [^{3}H]BP-denatured DNA solution, after filtration, was analyzed by sucrose gradient centrifugation in order to obtain conclusive evidence for physical binding. The results (Figure 2) show that the radioactivity from the [^{3}H]BP followed exactly the denatured DNA as measured by absorption at 260 mμ. In the absence of DNA, BP did not sediment (Ts'o and Lu, 1964b). This result indicated that the BP cannot be separated from DNA by centrifugal force, thus demonstrating the binding between these two species of molecules. In this experiment, the total optical density recovered was identical with that layered on the gradient, whereas only 50% of the radioactivity was recovered. The following experiment showed that this loss was due to adsorption of [^{3}H]BP on the polypropylene centrifuge tube. A 10-ml solution of [^{3}H]BP-denatured DNA containing 44,000 cpm was allowed to stand in a 13.5-ml polypropylene centrifuge tube at 5°. After 20 hr, the solution was shaken briefly to ensure uniform dispersion and the radioactivity was remeasured. A 40% loss of radioactivity was observed indicating a strong adsorption of BP to the polypropylene tube.

The [^{3}H]BP–native DNA solutions and [^{3}H]BP–denatured DNA solutions were also analyzed by sucrose gradient electrophoresis. The results, presented in Figures 3 and 4, clearly indicate that the radioactivity from [^{3}H]BP followed exactly the 260-mμ-absorbing material, thus further demonstrating the existence of the BP–DNA physical complex. Free BP, which is uncharged, does not migrate in an electrical field. In both

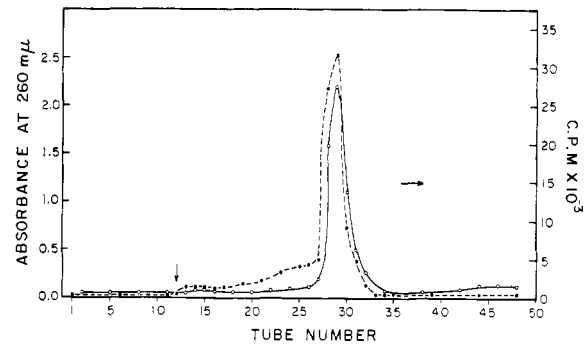

FIGURE 4: Sucrose gradient electrophoretic pattern of BP-denatured DNA physical complex in HMP (pH 6.8); 1000 v, 3 ma, 2.5 hr. Samples of 1 ml were collected and optical density (O——O) and radioactivity (●- - - -●) were measured.

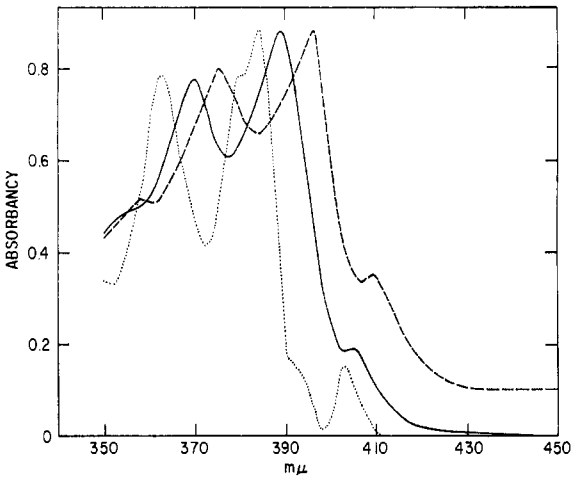

FIGURE 5: Spectra of BP in ethanol (. . . ., 1-cm path), 0.3 M purine solution–HMP (pH 6.8) (——, 10-cm path), and 0.1% poly A solution–0.05 M acetate (pH 5.0) (- - - -, 10-cm path). Absorbance of the most intense peak of BP–ethanol solution and of BP–poly A solution are normalized to the most intense peak of BP in purine solution.

TABLE IV: Spectral Maxima of Benzpyrene in Various Solvents and Solutions.

Solvent or Solution	λ_{max}, mμ			
50% aqueous ethanol	403	384	363	347
Ethanol	403	384	363	347
Heptane	403	383	362	347
Cyclohexane	403	384	363	347
Benzene	405	389	369	351
Pyridine	405	390	370	352
0.2% denatured DNA	407	393	373	354
0.2% native DNA	408	394	374	355
0.1% poly A–0.05 M acetate (pH 5.0)	409	396	376	358
0.2 M 5′-AMP (pH 6.8)–HMP	406	390	370	
0.3 M purine (pH 6.8)–HMP	405	389	370	
0.3 M purine–ethanol	403	384	363	

of these electrophoresis experiments the recovery of the DNA layered on the gradient was quantitative, but again the recovery of radioacivity was only 48–52%. Careful studies showed that this was definitely not due to quenching of the counts by sucrose. It was observed that [^{3}H]BP was slightly adsorbed on the glass, an effect especially noticeable in dilute solutions. A solution of [^{3}H]BP–DNA containing 3000 cpm/ml was observed to lose 20–30% of the counts over a 3-hr standing period in a test tube. Therefore, it was concluded that adsorption of [^{3}H]BP to the glass column of the apparatus is most likely the prime reason for the loss of counts.

SPECTRAL STUDIES. It has been previously noted (Liquori *et al.*, 1962; Boyland and Green, 1962b; Ts'o and Lu, 1964b) that the absorption spectrum of BP in DNA undergoes a pronounced bathochromic shift of 10 mμ. The spectral maxima of BP in a variety of solvents and solutions are given in Table IV. No shift of λ_{max} was observed for BP dissolved in a number of solvents possessing a wide variation in dielectric constants and hydrogen-bonding properties, such as heptane or 50% aqueous ethanol. This red shift is detectable when BP is dissolved in benzene or pyridine (4–7 mμ) and becomes very significant when BP is complexed with DNA and helical poly A (9–13 mμ). This spectral information is pertinent to the mode of binding of BP to nucleic acids and will be discussed further in later sections.

Spectra for BP dissolved in 5′-AMP and in purine solutions were measured by difference techniques. 5′-AMP (0.2 M) and purine (0.3 M) were chosen for this work in that they exhibited absorbances in HMP no higher than 0.1 (or 1 with 10-cm path) in the range of 450–360 mμ. They could thus be expected to yield reliable difference spectra in this range, using BP–5′-AMP or BP–purine as samples and 5′-AMP or purine alone as references. The 5′-AMP and purine solutions were prepared by shaking overnight an appropriate amount of material, and then filtering and centrifuging to remove all undissolved particles. An initial base line was obtained by dividing each solution in half, and running one half against the other in the Cary 15. A large amount of BP was then added to one of the halves of each solution, and all solutions were shaken for 3 days at 5° and 1 day at room temperature.

The difference spectrum for BP–purine *vs.* purine is shown in Figure 5. Analogous spectra for free BP in EtOH and BP–poly A complex, with absorbance of the most intense peak normalized to the most intense peak of BP in purine solution, are also given in Figure 5 for comparison. The spectrum for BP–5′-AMP *vs.* 5′-AMP yields virtually the same shifts as BP–purine (Table IV), though the intensity in this difference spectrum is considerably less than that for the BP–purine solution. More BP is dissolved in 0.3 M purine solution than in 0.2 M AMP solution.

The spectrum of BP dissolved in a 0.3 M solution of purine in ethanol was also obtained. Under these conditions, the spectrum of BP showed no red shift when compared with the spectrum of BP dissolved in ethanol alone. Further, proton magnetic resonance studies of purine in ethanol solution were performed, and indicated that the chemical shifts of the H-6, H-8, and H-2 protons show no downfield shift upon decreasing the concentration from 0.3 to 0.05 M in contrast to the concentration-dependent downfield shifts observed in aqueous solutions of purine (Chan *et al.*, 1964).

An additional experiment has been done to preclude the possibility that this red shift is due to the interaction of BP molecules with themselves in colloidal particles and not due to the interaction of BP with the bases in DNA. A saturated BP–denatured DNA solution,

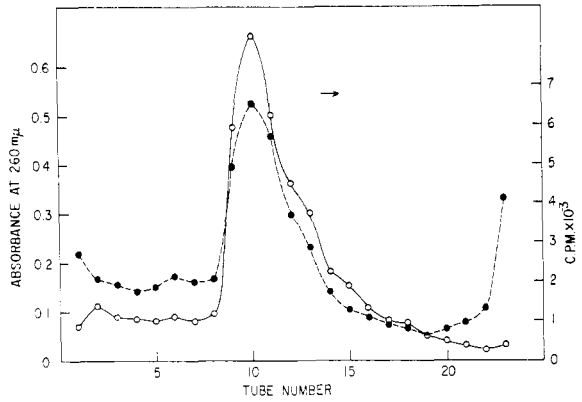

FIGURE 6: Sucrose gradient centrifugation pattern of the BP–DNP physical complex in 1:3 HMP (pH 6.8), SW 39 rotor, 34,000 rpm, 16 hr. Ten drop fractions were collected and after addition of 1 ml of 1:3 HMP, optical density (○——○) and radioactivity (●- - - -●) were measured.

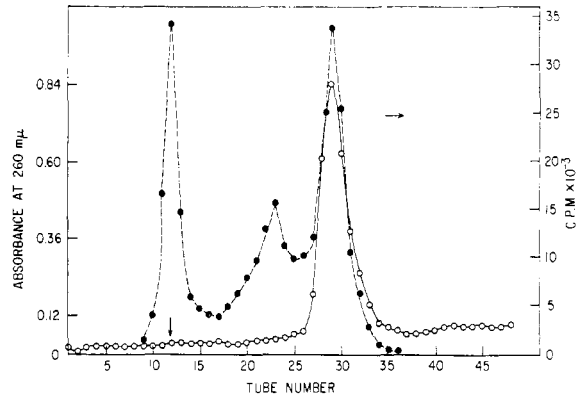

FIGURE 7: Sucrose gradient electrophoretic pattern of the BP–DNP physical complex, 1:3 HMP (pH 6.8); 2900 v, 4 ma, 2 hr. Samples of 1.1 ml were collected and optical density (○——○) and radioactivity (●- - - -●) were measured.

after filtration, exhibited an absorbance of 0.017 at 450 mμ (or 0.17 with 10-cm path length), the absorption being due to scattering. The solution was then centrifuged at 39,000 rpm with a SW 39 swinging-bucket rotor for 2 hr. After the run, the contents of the centrifuge tube were separated into the upper half and lower half. The spectrum of the upper half had an absorbance less than 0.01 at 450 mμ while the spectrum of the lower half had an absorbance of 0.03. Identical spectral positions for the three peaks were observed for the solution before centrifugation and for the two fractions obtained after centrifugation as described. This result strongly suggests that the red shift in the spectrum is due to the interaction of BP with DNA and not the self-interaction of BP in colloidal particles. Further evidence for the absence of BP self-interaction can be found in the emission studies of Isenberg *et al.* (1967).

Binding to Calf Thymus Nucleohistones. Solid [^{3}H]BP was added to nucleohistone in TMP and shaken at 5° in the dark for 12–14 days. After this period, the excess BP was removed by centrifugation or filtration, if possible (see procedure). The results for the binding of BP to DNP are reported in the first row in Table V.

The [^{3}H]BP–DNP physical complex was further examined by sucrose density gradient centrifugation and electrophoresis. In Figure 6, the pattern of distribution of [^{3}H]BP in the centrifuge tube followed closely the pattern of the DNP represented by the absorbance at 260 mμ. This is a strong indication of the existence of the BP–DNP complex. Some [^{3}H]BP was found at the bottom of the tube not accompanied by 260-mμ-absorbing material, indicating that a small number of free BP particles were left in solution. In Figure 7 most of the [^{3}H]BP migrates with the DNP on electrophoresis, again indicating the existence of the BP–DNP complex. Some free BP was left at the origin. Also, a certain amount of dissociation of BP from the complex can be detected, which was not found in the electrophoretic patterns of BP–DNA complexes (Figures 3 and 4). It should be noted that the rate of migration, as well as the distance travelled by the complex was much larger in electrophoresis (10 cm/hr) than in ultracentrifugation (0.15 cm/hr), and this may be the reason why dissociation was not detected in Figure 6. The base per BP ratio of the fractions collected at the peak position of the 260-mμ-absorbing material (28–30 in Figure 7) is reported in the second row of Table V. This is comparable to that of the whole sample (the first row in Table V). The base per BP ratio of the BP–DNP complex is about twice that of the BP–DNA complex in TMP (Table II). On the other hand, the base per BP ratio for the DNP is almost the same as that for the DNA solution, when the BP binding experiment was done in solution of 1.5 M NaCl (Table V). This high concen-

TABLE V: Binding of Benzpyrene to Thymus Nucleohistone.

	Nucleotide Concn (mM)	Molar Ratio of Base:BP	Binding Constant K (M^{-1})
DNP in TMP	1.50	3,000	22,600[a]
	1.5	3,200	18,000[b]
DNP fraction collected	0.05	3,200	20,000[b]
after electrophoresis	0.12	2,000	27,000
DNP in 1.5 M NaCl	0.63	9,700	6,100[a]
	0.63	11,000	5,500
	0.63	8,700	6,800
DNA in 1.5 M NaCl	2.5	7,000	8,800[a]
	2.0	9,600	6,500

[a] Removal of the excess BP by filtration. [b] Removal of the excess BP by centrifugation.

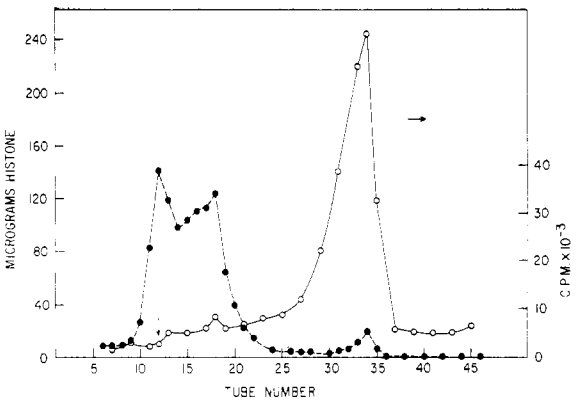

FIGURE 8: Sucrose gradient electrophoretic pattern of the BP–histone physical complex; 1:3 HMP (pH 6.8), 2700 v, 4 ma, 3.5 hr. Samples of 1.1 ml were collected and protein (○——○) and radioactivity (●- - - -●) were measured.

tration of salt would cause an almost complete dissociation of the histones from the DNA.

The proteins (75%), mainly the histones, can be solubilized and extracted from the DNP by addition of H_2SO_4 to a final concentration of 0.5 N for 15 min in the cold. When the [^{3}H]BP–DNP complex was so treated with H_2SO_4, 97% of the radioactivity was found remaining in the precipitate after centrifugation at 3000 rpm for 10 min in a clinical centrifuge. Treatment of the [^{3}H]BP–DNA complex with 0.5 N H_2SO_4 caused the precipitation of 99% of the counts. Together these experiments indicate that very little of the [^{3}H]BP originally bound to the DNP remains in association with the histones after the histones are extracted from the DNP. This conclusion received further support from the following experiment with the isolated histones. Preparations of isolated but unfractionated histones were shaken with [^{3}H]BP in HMP for 14 days at 5°. After filtration through a sintered-glass filter, BP was found to be solubilized to the extent of 0.23 mμmole of BP/mg of histones or about one-sixth of the amount solubilized by 1 mg of DNA. However, as shown in Figure 8, only a small fraction of the [^{3}H]BP migrated with the histones toward the cathode in the electric field, suggesting a rather loose binding of [^{3}H]BP to the histones.

Binding to Polyribonucleotides. Binding of [^{3}H]BP to poly A, poly C, poly U, and yeast RNA (Cresfield's type of preparation) in HMP at 5° is reported in Table VI. For poly A and RNA solutions, 10–14 days of shaking were required for the saturation whereas only 7–10 days were necessary for poly C and poly U. Losses of [^{3}H]BP between the first and the second filtration and the losses between the second and the third filtration for [^{3}H]BP–DNA solution (both native and denatured) were usually only about 1–3%; therefore only one filtration was necessary to remove excess BP from DNA solutions. However, such losses for [^{3}H]BP–poly A solutions were found to be around 5% between each successive filtration. [^{3}H]BP–poly C, –poly U, or –RNA solutions were found to lose about 20% of their counts between the first and the second filtration, and about 10% between the second and third filtration. The reason for these larger losses in solutions of BP–polyribonucleotides has not been further investigated, though this may reflect more rapid equilibrium in the binding of BP to these ribose polymers. Addition of excess nonradioactive BP solid to a solution of [^{3}H]BP–poly A caused a loss of radioactivity with a rate higher than that for the [^{3}H]BP–DNA solutions described in a previous section. After 4 hr of shaking at 5°, 80–85% of radioactivity was removed; and after 24 hr of shaking, 90% of the radioactivity was removed.

A most interesting observation is that when poly A was put into a double helical form at pH 5.0, 0.05 M acetate buffer (Ts'o *et al.*, 1962a; Rich *et al.*, 1961), the binding capacity of poly A for BP was increased 10–20 times over that of poly A in form of stacking coil at neutral pH. The base per BP ratio was found to be around 200–600, and the binding constant was found to be 1–2 × 10^5 M^{-1}. This extensive binding is also reflected by a pronounced bathochromic shift of the λ_{max} of BP in the region of 350–410 mμ for an acidic solution of poly A (Table IV). The sucrose gradient electrophoretic pattern of [^{3}H]BP–poly A in acidic buffer is given in Figure 9. It can be seen that all the [^{3}H]BP migrates with the ultraviolet-absorbing poly A, providing evidence for the existence of a tightly bound [^{3}H]BP–poly A complex in acidic solution.

A Comparison with Binding of Other Polycyclic Hydrocarbons. Binding of 1,2-benzpyrene (noncarcinogenic), 1,2,5,6-dibenzanthracene (carcinogenic), and 1,2,3,4-dibenzanthracene (noncarcinogenic) by native DNA has been studied in comparison with the binding

TABLE VI: Binding of Benzpyrene to Polyribonucleotides in HMP at 5°.[a]

Polymer[b]	Nucleotide Concn (mM)	Molar Ratio of, Base:BP	Binding Constant K (M^{-1})
Poly A	5.8	6,200	9,400
	2.6	4,000	14,400
	1.3	6,600	9,000
Poly C	4.0	13,500	4,300
	3.0	16,800	3,500
Poly U	4.1	30,000	1,950
	4.5	29,800	1,980
Yeast RNA	5.3	1,000	59,000
	5.4	2,800	21,300
	5.6	1,500	38,500

[a] Quadruplicates were run for each determination. [b] Poly A solution was filtered once, poly U and RNA solutions were filtered twice, and poly C solution was filtered three times. See text for reason.

of 3,4-benzpyrene. A DNA solution in HMP was prepared (*ca.* 1 mg/ml, pH 6.8) and divided into four batches. To each batch, a tritiated sample of one of the hydrocarbons was added, and the solutions were shaken in the dark at 5° for 3 weeks. Undissolved hydrocarbons were then removed by filtration through sintered-glass funnels, and the remaining radioactivity was assayed.

The ratio of nucleotide to hydrocarbon for both bound 1,2- and 3,4-benzpyrene was found to average 1100:1, ±10%. For 1,2,3,4- and 1,2,5,6-dibenzanthracene, this ratio fell between 4000:1 and 5000:1 (the solubilities in buffer for all of the four hydrocarbons were found to be identical within ±10%, and for this experiment ran as high as 0.06 μM). These values lead to nearly identical binding constants for 1,2- and 3,4-benzpyrene, and for 1,2,3,4- and 1,2,5,6-dibenzanthracene, the latter being about 20% of the former.

In order to determine whether physical binding to DNA involved specific and identical sites for both the carcinogens and noncarcinogens, the following exchange experiment was performed. Complexes of tritium-labeled 3,4-benzpyrene with DNA and tritium-labeled 1,2-benzpyrene with DNA in HMP were prepared by the usual procedure, and the amount of bound hydrocarbon was measured. To half the 3,4-benzpyrene samples a large amount of unlabeled 1,2-benzpyrene was added, and to half the 1,2-benzpyrene samples a similar amount of unlabeled 3,4-benzpyrene was added. The other half of each batch served as control. All solutions were shaken for a total of 2 weeks at 5°, small samples being withdrawn after 5 days for filtration and counting. After 2 weeks, the solutions were removed from the cold, filtered, and counted.

After 5 days, the 1,2-benzpyrene–DNA solutions to which 3,4-benzpyrene had been added exhibited binding of 1,2-benzpyrene corresponding to 80% of the control, while the 3,4-benzpyrene–DNA solutions to which 1,2-benzpyrene had been added exhibited binding corresponding to 75% of control. After 2 weeks, the figures for the percentage of counts remaining (with respect to control) were 70% for 1,2-benzpyrene–DNA solutions and 60% for 3,4-benzpyrene–DNA solutions.

An important assumption implicit in the above experiment is the absence of cocrystallization of 1,2- and 3,4-benzpyrene. To test this assumption, the following experiment was performed. Saturated solutions of [^{3}H]1,2-benzpyrene and [^{3}H]3,4-benzpyrene in HMP were prepared by shaking for 5 days at 5°. After filtration a large amount of unlabeled 3,4-benzpyrene was added to half the 1,2-benzpyrene samples, and a similar amount of unlabeled 1,2-benzpyrene was added to half the 3,4-benzpyrene samples. The other half of each batch served as control. The solutions were shaken for 1 week at 5°. They were then filtered and the remaining radioactivity was measured.

The 1,2-benzpyrene solutions to which 3,4-benzpyrene had been added exhibited residual radioactivity corresponding to 90% of the control. The 3,4-benzpyrene solutions to which 1,2-benzpyrene had been added exhibited residual radioactivity corresponding to 60% of the control. The greater tendency of 3,4-benzpyrene to cocrystallize with 1,2-benzpyrene as compared with the reverse process, may have led in the competitive binding experiment to the observed slightly greater replacement of bound 3,4-benzpyrene by 1,2-benzpyrene as compared with replacement of bound 1,2-benzpyrene by 3,4-benzpyrene.

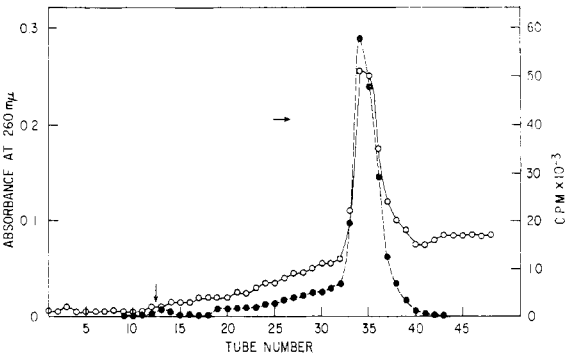

FIGURE 9: Sucrose gradient electrophoretic pattern of the BP–poly A (helical) physical complex in 0.0025 M acetate (pH 5.0), about 3000 v, 3 ma, 2 hr. Samples of 1.1 ml were collected and optical density (O——O) and radioactivity (●- - - -●) were measured.

Discussion

The first objective of this investigation was to provide unambiguous evidence for the physical binding of BP to nucleic acids. This appears to have been accomplished by the use of sucrose gradient electrophoresis and ultracentrifugation together with the radiochemical assay. The radiochemical method is more sensitive and more straightforward in the interpretation of its results than spectrometric or fluorometric assays once the radiochemical purity of the compound has been assured (Ts'o and Lu, 1964b; see Materials and Results). Thus, the physical binding of BP, regardless of its mechanism, to native and denatured DNA, poly A, and nucleohistones has been clearly demonstrated. The magnitude of binding of BP to DNA (about 1 BP/1000–3000 bases), as stated in the results, is comparable to those obtained by Boyland and Green (1964a,b), by Kodama *et al.* (1966), and by Liquori *et al.* (1962, 1967), but is definitely lower than that published by Ball *et al.* (1965) and Lerman (1964). The data of Ball *et al.* in the aqueous system agreed with that published by Boyland and Green in 1962b, but was about four to five times higher than that published by the same authors in 1964b at 10^{-3} M salt. Ball *et al.* also reported a "formation of BP–DNA complex which was stable for long periods of time" with a ratio of 1 BP/60–80 nucleotides. This was achieved by adding BP in methanol to the DNA solution. The final solution of DNA and BP thus contained 1–3% methanol, and the solubility of BP in buffer containing 1–3% methanol was not reported. Lerman has reported a ratio of 1 BP/100–120 nucleotides by his interesting technique of

zone-crossing chromatography. In his experiment, it was tacitly assumed that there was no interaction between the DNA and caffeine which was used to solubilize the BP in aqueous buffer. However, caffeine has been shown to be a powerful denaturant of DNA (Ts'o *et al.*, 1962a) and its binding to native DNA, though it is substantially lower than that to the denatured DNA, is not totally negligible (Ts'o and Lu, 1964a). Thus, it appears reasonable to conclude that when DNA is in equilibrium in an aqueous buffer saturated with BP there is generally about 1 BP bound/1000–3000 nucleotides, depending on ionic strength and temperature.

The second objective was to shed more light on the question of mechanism of the physical binding, especially with regard to the model of intercalation (Boyland and Green, 1962b). This model was originally proposed on the basis of stereochemical feasibility and its analogy to the acridine derivatives (Lerman, 1964). Only recently, there were some preliminary data from flow dichrosim (Nagata *et al.*, 1966) and from viscosity (Lerman, 1964) in support of this point of view. The spectral measurements in this paper are very pertinent to this question and constitute a very strong support, though indirect, for the model of intercalation. The argument from the spectral data comes in three parts. (a) The spectral maxima of BP from 350 to 410 mμ are the same in a variety of solvents ranging from 50% aqueous ethanol to hexane, but are shifted 4–7 mμ to longer wavelengths when dissolved in benzene or pyridine (Table IV). This suggests that the preferential stabilization of the excited state over the ground state of BP occurs when BP interacts with a π-electron system, and is not easily induced through changes in the dielectric constant or hydrogen-bonding properties of the solvent. Further, the equivalence of the BP spectrum in benzene and in pyridine indicates that the nitrogen lone-pair electrons play no significant role in the shift. (b) That this interaction arises from vertical stacking is strongly suggested by the spectral data for BP in aqueous solutions of 5′-AMP and purine (Figure 5 and Table IV), taken in conjunction with earlier spectroscopic, X-ray diffraction, vapor pressure osmometry, and proton magnetic resonance results. It is seen that the present BP–purine and BP–5′-AMP spectral data, as well as earlier spectra of BP dissolved in 50% ethanol saturated with caffeine or with 1,3,7,9-tetramethyluric acid (TMU) (Booth *et al.*, 1954), or BP dissolved in 0.2% aqueous caffeine solution (Reske and Stauff, 1965), exhibit comparable red shifts to those arising from BP interaction with the benzene and pyridine π-electron systems. These data should be considered in conjunction with the following measurements. (i) The detailed analyses by X-ray diffraction of the molecular structure of cocrystals of BP and TMU by Liquori's group (Damiani *et al.*, 1965, 1966). Their results indicate a parallel stacking of one BP plane and two TMU planes alternately such that each BP molecule is "sandwiched" between two TMU molecules at an average perpendicular separation of 3.45 Å. (ii) The extensive investigation by proton magnetic resonance and vapor pressure osmometry on the association of purine, 6-methylpurine (Ts'o *et al.*, 1963b; Ts'o and Chan, 1964; Chan *et al.*, 1964), nucleosides (Schweizer *et al.*, 1965a; Broom *et al.*, 1967), and nucleotides, especially AMP (Schweizer *et al.*, 1965b). These results showed conclusively that all these compounds associate extensively in solution by mode of vertical stacking of the bases. A similar conclusion was also reached independently by Jardetzky (1965). (iii) Our proton magnetic resonance studies of purine in ethanol at varying purine concentrations reveal no characteristic downfield shift of the aromatic proton lines with decreasing concentration, thus indicating the absence of stacking interactions. Further, we find that the red shift in the visible spectrum of BP is absent when it is dissolved in a 0.3 M purine solution in ethanol. It thus appears that the presence of the observed BP red shift is fully dependent upon the presence of strong stacking interactions of the bases, which take place in aqueous solution but not in organic solvents (Chan *et al.*, 1964). This conclusion is in agreement with that of Van Duuren (1964). From a careful fluorescence study, he concluded that there is very little interaction of TMU with BP when dissolved in benzene, even though such an interaction can be observed in aqueous alcohol solution (Booth *et al.*, 1954). On the basis of all this information, it is safe to conclude that the bathochromic shift of the spectra of BP with purine and with 5′-AMP is directly associated with the formation of face to face vertical stacks. (c) The bathocromic shift (7–11 mμ) in the spectrum of BP when complexed with DNA (Table IV) is well known, and here we also report a large shift (11–13 mμ) for the complex of BP–helical poly A. This represents a considerable increase in the magnitude of the BP red shift accompanying a change in environment from stacked monomeric π-electron systems to a relatively rigid polymer system. If the BP were in the polymer system no longer involved in face-to-face interactions, but were now oriented, for example, coplanar with or perpendicular to the DNA bases, one would expect a reduction in the base-induced perturbation of the BP π-electron system due to an extensive reduction in the overlap of BP molecular orbitals with base molecular orbitals. While one cannot accurately predict the accompanying spectral changes, it is highly unlikely that this alteration in BP environment would lead to the large observed increase in the red shift. Rather, this spectral increase is considerably more compatible with a model whereby BP in a polynucleotide environment associates by vertical stacking with the bases in a manner similar to that of the monomer system. As the polynucleotide helix provides a more rigid and regular structure than the stacks of monomers, the BP may be considered to be more tightly locked in its face-to-face arrangement with the bases. This provides a more stable geometry for the action of the intermolecular π-electron dispersion forces, and could thereby lead to the observed increase in the magnitude of the associated red shift. It should be remembered that because of the large base to BP ratio (from 1000 to 3000) there is virtually no direct interaction between bound BP molecules in the

BP–DNA complex, a situation very different from the acridine dye–DNA complex which can exhibit a low base to dye ratio of from 1 to 20.

The importance of the π-electron dispersion forces ("polarization bonding") in the formation of BP–base complexes has been previously recognized (Booth *et al.*, 1954; Liquori *et al.*, 1962; Van Duuren, 1964). In a recent paper from our laboratory, based on proton magnetic resonance and vapor pressure osmometry studies of purine nucleosides, we have proposed a spatial model for the stacks of adenosine in water and have indicated the importance of London dispersion forces for the formation of these stacks (Broom *et al.*, 1967).

Strictly speaking, the model of face-to-face vertical stacking of BP with the bases in DNA and helical poly A, though it supports the model of intercalation, does not presuppose helicity in the region of the nucleic acids immediately surrounding the BP molecule. For instance, this type of binding of BP to the nucleic acids could take place at an interrupted region or at a local disordered region of the double helix. This concept of face-to-face stacking is in accord with the experience we have had with the photochemistry and radiochemistry of BP–nucleic acid complex (Ts'o and Lu, 1964a; Rapaport and Ts'o, 1965).

There are other findings reported here which are relevant to the mechanism of the binding of BP. The apparent free energy of BP binding to the nucleosides and nucleotides is around 3–3.5 kcal with binding constants of around 2–5 $\times$ 10^2 at 5°. This apparent free energy is increased to about 5.5–6.0 kcal for the binding of BP to DNA and RNA under various conditions with binding constants about 2–6 $\times$ 10^4. Thus, the binding of BP to the polymer has about twice the amount of apparent free energy as the binding of BP to the monomers. This observation qualitatively supports the concept of the mode of the binding of BP to the nucleic acids discussed above, *i.e.*, the insertion of the BP molecules between two bases in the polynucleotide chain, as one would expect a roughly additive contribution to the free energy arising from interaction of a BP molecule with two adjacent but weakly interacting bases.

The effect of electrolytes on the binding of BP to the native and denatured DNA observed here is in general agreement with those reported by Kodama *et al.* (1966), Boyland and Green (1964b), and Boyland *et al.* (1964). Increase in salt concentration reduces the binding constant of BP to native DNA but enhances the binding constant of BP to denatured DNA (Tables III and V). The precise meaning of this phenomenon is not very clear, though an obvious explanation would be that the site of binding of BP to nucleic acids is not the region of the tightly intertwined double helix, and not the region of random coil, but a region in which the stacking and the hydrogen-bonding interactions of the base pairs are less than that of the regular helix but more than that of the random coil (Boyland and Green, 1964b). There is an observation reported here not easily explained by the above notion. The binding of BP to poly A is greatly enhanced going from the single-stranded stacked form in neutral solution (Holcomb and Tinoco, 1965) to that of the double helical form in acidic solution (Rich *et al.*, 1961). The binding constant is increased from 5 to 6.5 kcal. According to the X-ray analyses (Rich *et al.*, 1961), however, the helical poly A has a very compact structure. It is not clear at present from the stereochemical point of view, why the intercalation of BP molecules between the bases in this rather compact helix should be energetically more favorable than that for the native DNA and single-stranded but base-stacked poly A. One alternative could be offered owing to the fact that the poly A helix consists of many interrupted regions (Fresco and Doty, 1957). These interrupted regions may constitute the proper sites for the binding of BP. The frequency of these interruptions corresponds to the average degree of polymerization of the single strands. Some preliminary results in our laboratory indicate that the number of BP bound per number of nucleotides in the acidic poly A does have the expected correlation to the degree of polymerization. Unfortunately, the variation in the binding data is too large, the distributions of the molecular weight of the poly A samples are too broad, and the differences in the average degree of polymerization of poly A samples employed are too small for a very definite conclusion. Thus the answer to this problem must await further investigation with poly A samples with very narrow molecular weight distributions and very large differences in degree of polymerizaton. The outcome of this investigation would be relevant to the binding mechanism of BP to native DNA.

The third objective was to investigate the binding of BP to nucleohistones and histones. The results show that the binding of BP to nucleohistones is about 50–60% of that to the DNA (Table V) and the rate of dissociation of the complex as shown qualitatively in gradient ultracentrifugation is also higher than that of the BP–DNA complex (Figures 2 and 6). Three lines of evidence indicate that the binding of BP to the isolated histones is not significant. (a) The maximum amount of BP solubilized by histones per weight is only one-sixth (possibly only one-tenth as shown in the sucrose gradient electrophoresis) of that by the DNA. The rate of the dissociation of the BP–histone complex is also much faster than that of the BP–DNA complex (Figure 8). (b) When nucleohistone is dissociated into DNA and histone components in 1.5 M NaCl, the amount of BP solubilized is the same as that of the DNA alone in this concentration of salt, indicating that the amount of BP solubilized by the dissociated histones is negligible (Table V). (c) When acid was added to extract the histones into solution from the BP–nucleohistone complex, practically all of the BP went down with the DNA precipitate. Thus, these results indicate that the binding of the BP to the nucleohistone take places mostly with the DNA–residual protein moiety. This does not necessarily mean, however, that the bound BP molecules in the nucleohistones do not have any contact with histone molecules. Preliminary experiments in our laboratory showed that photochemically excited BP molecules in the BP–nucleohistone complex do

form chemical linkages with the histone components. The precise reason for the reduction of the binding of BP to the DNA in nucleohistones as compared to isolated DNA in dilute salt solution is not very clear. The two obvious explanations are that of the reduction of the binding sites due to stereochemical arrangement of the histones on the double helix, and that of the general tightening of the double helix of DNA due to the binding of positively charged histones. This second explanation is analogous to the reduced binding of BP to DNA in concentrated salt solution. Most recently, spermidine was found to reduce the binding of BP to DNA (Liquori *et al.*, 1967). Therefore, in regard to this question, more information about the detailed structure of the nucleohistones is needed.

The last objective was to survey the binding of various related polycyclic hydrocarbons to native DNA for comparison. The first conclusion is that the binding of 1,2- or 3,4-benzpyrene is stronger than that of 1,2,3,4- or 1,2,5,6-dibenzanthracene. This observation may be related to the fact that the ring system of the benzpyrenes is more compact than the rather more extended ring system of the dibenzanthracenes. The more compact ring system makes available a greater portion of the molecule for stacking interaction with the DNA bases, and the pyrene nucleus in particular presents a highly concentrated aromatic system. Furthermore, simple steric considerations might lead to a greater efficiency of intercalation for the relatively more compact benzpyrene molecules.

The second conclusion is that the extent (molar ratios) and the affinity (binding constants) of the binding of the carcinogenic compounds to native DNA are the same as those of their respective noncarcinogenic counterparts. This is not unexpected, since at the level of physical binding the relatively nonspecific London dispersion forces would contribute most significantly to the stacking interaction (Pullman *et al.*, 1965). However, in order to have this conclusion fully meaningful in terms of its biological implications, one has to ascertain whether the carcinogenic compounds and the noncarcinogenic compounds have the same specific binding sites on the DNA. In other words, if there are specific sites on the DNA, which are identical in affinity to both carcinogenic and noncarcinogenic compounds, then the physical binding of carcinogen to DNA is unlikely in itself to be the determining step in carcinogenesis. We may conclude from the exchange data that this is *not* the case, *i.e.*, that DNA does not have specific sites, all of which are common to both carcinogens and noncarcinogens. For if both were competing for identical sites on the DNA, the nonradioactive compound added in great excess should completely replace the bound ^3H compound. The fact that a high percentage (60–70%) of the ^3H compound remains bound to DNA after shaking for 2 weeks in the presence of excess nonradioactive compound, indicates that few of the binding sites on the DNA are common to both carcinogens and noncarcinogens. Unfortunately the data do not permit us to distinguish between the two other alternatives. One of these is that the DNA does have specific sites for binding of these two hydrocarbons, but these sites are not common to both. The other alternative is that for the binding of these compounds to DNA, there are many sites which are not specific. Under the condition of apparent saturation of binding (hydrocarbon:base ratio $\approx$ 1:1000), there are still many empty sites available. Thus, the level of the binding is not limited by the number of the binding sites but by the low solubility, *i.e.*, the low chemical potential of the hydrocarbons in aqueous solution. In this situation, the nonradioactive compounds do not need to compete with the ^3H compounds for binding sites, and therefore, no exchange was detected. It should be remembered that whether or not the physical binding process is found to be in itself the determining carcinogenic step, the physical carcinogen–DNA complex may still participate in the actual determining process.

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