INTERACTION OF NUCLEIC ACIDS, I. PHYSICAL BINDING OF THYMINE, ADENINE, STEROIDS, AND AROMATIC HYDROCARBONS TO NUCLEIC ACIDS

By Paul O. P. Ts'o and Ponzy Lu

THE JOHNS HOPKINS UNIVERSITY AND CALIFORNIA INSTITUTE OF TECHNOLOGY

Communicated by James Bonner, November 13, 1963

In a previous communication,[1] it was shown that the helix-coil transition temperature, T_m, of thymus DNA and helical poly A is lowered by pyrimidines, purines, nucleosides, their analogues, and derivatives. The order of their effectiveness indicates that hydrophobic and stacking interactions are important.

To investigate the mechanism by which the T_m is reduced is one principal purpose of this research. It has been proposed that these compounds may interact more strongly with the coil form of nucleic acids than with the helical form, thereby shifting the equilibrium in favor of the coil form.[1] The validity of this explanation has been investigated by equilibrium dialysis to measure the binding of these compounds to nucleic acids. The coil form of nucleic acids was found to have a much higher affinity than the helical form for these compounds as predicted.

The significance of the hydrophobic and stacking interaction of the bases in nucleic acids is now recognized,[1-4] and has been quantitatively studied in our laboratory.[5] This knowledge provides new insight into properties and functions of nucleic acids which were not previously understood or anticipated. Many hydrophobic compounds are biologically active but chemically inert. These include, for example, steroids and polycyclic carcinogens. This new concept suggests that such compounds may interact strongly with nucleic acids.

The second principal purpose of this investigation is to measure the affinity of DNA for these compounds, again by equilibrium dialysis. Estradiol-β-17, testosterone, and diethyl stilbesterol were chosen as representative of the sex hormones, naphthalene and phenanthrene as representative of aromatic hydrocarbons. The results indicate that the affinity of DNA for these compounds is interestingly high, especially the denatured form of DNA. It is noteworthy that the affinity of RNA, poly A, and poly U for these compounds is one or two orders of magnitude lower than that of denatured DNA.

Materials and Methods.—Radiochemicals: Commercially available radiochemicals were used without further purification or carrier dilution. Thymine-methyl-H^3 (6.64 c/mM), adenine-H^3 (2.16 c/mM), estradiol-17β-6, 7-H^3 (150 mc/mM), and testosterone-1, 2-H^3 (784 mc/mM) were obtained from New England Nuclear Corporation, Boston. Caffeine-1-methyl-C^{14} (0.66 c/mM) was obtained from Tracerlab, Waltham, Mass.

Diethyl stilbesterol-H^3 (296 mc/mM) and phenanthrene-H^3 (16.38 mc/mM) were obtained from Volk Radiochemical Company, Skokie, Illinois. Naphthalene-1-H^3 (168 mc/mM) was obtained from International Chemical and Nuclear Corporation, City of Industry, Calif.

Nucleic acids: Native DNA: Calf thymus DNA (highly polymerized) was purchased from Nutritional Biochemicals Corporation, Cleveland, Ohio. A 10 mg/ml solution of this DNA in 0.0025 *M* Na_2HPO_4, 0.005 *M* NaH_2PO_4, and 0.001 *M* ethylene diamine tetraacetic acid, pH 7.1 (HMP) was sheared at 0–5°C in a VirTis "45" high-speed mixer (manufactured by the VirTis Corporation, Gardner, N. Y.) for 20 min at full speed. The solution was then centrifuged to remove a small amount of white residue. The final DNA solution was made up in 0.5 *M* in NaCl. This native DNA solution gave a 30% increase in optical density at 260 mμ when heated above the T_m temperature (70° in HMP; 95° in HMP and 0.5 *M* NaCl).

Denatured DNA: The native DNA solution was sonicated for 20 min at 5°C at maximum power output in a 50-watt 9-kc Magnetostriction Oscillator (Model s-102A, from the Raytheon Manufacturing Company, Waltham, Mass.). The DNA solution was then heated at 100°C for 20 min, cooled, and sheared again for five min in the VirTis.

DNA concentration was measured spectrophotometrically at 260 mμ. All measurements were made on the native DNA in HMP taking E_{max} as 6.6×10^3 per mole of nucleotide.

Redeproteinization procedure: The DNA was dissolved to 3.3 mg/ml in 0.1 *M* ammonium acetate. This solution was sheared in the VirTis mixer for 8 min at full speed and at 0–5°C. Sodium dodecyl sulfate (S.D.S.) was then added to 2%. After shaking for 10 min at room temperature, the S.D.S. was twice extracted with one volume of an amyl alcohol-chloroform mixture (1:3, V/V). Subsequently, the DNA was precipitated with ethanol, collected, and washed twice with ethanol and ether.

Poly A helix form: Poly A (from Miles Chemical Company, Elkhart, Ind.) was dissolved in 0.05 *M* tris at pH 7.5 to 10 mg/ml. This solution was dialyzed against 0.5 *M* NaCl in 0.1 *M* acetate buffer at pH 4.5 at 5°C. The solution became a solid gel. The gel was sheared for 30 min in the VirTis mixer at full speed, and subsequently sonicated for 45 min at full power. The resulting poly A solution gave a T_m of 70°C in 0.2 *M* acetate, 0.5 *M* NaCl, and 45% increase in O.D. at 260 mμ at temperature above the T_m.

Poly A coil form: Poly A was dissolved in 0.05 *M* Tris, pH 7.5 to 10 mg/ml. This solution was made up to 0.5 *M* in NaCl before used. Poly A concentration was measured at 257 mμ in 0.05 *M* Tris, pH 7.5 taking E_{max} as 10.5×10^3 per mole bases.[6]

Other biopolymers: Poly U was prepared by Dr. Roland F. Beers, Jr. E_{max} was taken as 9.2×10^3 per mole bases in 0.05 *M* Tris pH 7.5 at 260 mμ.[6]

Yeast sRNA was isolated according to the procedure of Holley *et al.*[7] Phosphorous and absorbancy determination gave an E_{max} of 8×10^3 in 0.05 *M* Tris, pH 7.5 at 260 mμ.

Pea ribosomal RNA was obtained as described previously.[6] The E_{max} in 0.1 *M* NaCl, 0.001 *M* $MgCl_2$, and 0.1 *M* NaAc pH 5.3 was found to be 7.3×10^3 at 260 mμ.[6]

Calf thymus histone (from CalBiochem., Los Angeles, B grade) was dissolved in HMP 0.5 *M* NaCl at 5°C to 3 mg/ml. The soluble portion, which is about 50% of the material, was used in the experiments. About 10% of the soluble fraction is dialyzable, and this correction has been applied. Histone concentration was measured by Biuret determination.

Visking dialysis tubing no. 8 was used for all the experiments. The tubing was washed with 1% EDTA, boiling distilled water, hot 5% $NaHCO_3$ solution, and 90% ethanol.

Experimental procedure: Equilibrium dialysis: Dialysis tubings containing one ml of nucleic acid solution were inserted in small tubes which contained one ml solution of the appropriate radioactive compounds. The tubes were then mounted on a rack rotating at 75 rpm in a thermostated incubator at 5 ± 0.5°C.

Leakage of nucleic acids through the bag during dialysis was usually less than 1%. There was no detectable loss of nucleic acid due to absorption on the tubing. On the other hand, losses of the radiochemicals due to absorption on the tubing were considerable: *ca.* 20% for the nitrogen bases and steroids, *ca.* 30% for naphthalene, and as high as 70–80% for phenanthrene. In the calculation of the binding data, only the actual concentration inside and outside the tubing were used.

All dialyses were carried out for seven days. Evidence that the dialysis had reached equilibrium came from three sources. (1) In 4–5 days, equilibrium had been reached in control tubes in which the dialysis tubing contained only buffer solution for all compounds except phenanthrene. (2) For experiments of testosterone with denatured DNA and thymine with helical poly A, the same value of nk was obtained when the radioactive compounds were placed inside the tubing with the nucleic acids. (3) For experiments of estradiol-β-17 and testosterone with denatured DNA, the same value of nk was obtained when a period of dialysis much longer than 7 days was adopted.

All experiments were carried out over a 20-fold concentration range of the substrate but at one concentration level of the biopolymer. Except as noted, at least six different concentrations with triplicate samples were used for the determination of nk. When high concentrations of nucleic acid were used because of the low value of nk, the viscosity of the solution became a problem. The viscosity was effectively reduced by shearing of the polymer in the VirTis homogenizer and by sonication.

Binding constant measured by increase in solubility: There are difficulties in doing the dialysis experiment with phenanthrene. The loss of this compound due to absorption on the tubing is large, and the results do not always indicate that equilibrium has been reached. Therefore, the nk value was also measured by change of solubility of phenanthrene in the presence of denatured DNA. Solid phenanthrene-H^3 was added to buffer solution or to buffer solution containing denatured DNA, and the solution rotated at 5°C as in the dialysis experiments. After 5, 11, and 17 days, the solutions were filtered, and the radioactivity of the solutions was determined.

Experiments demonstrating that the binding of the radiochemicals is exclusively onto the nucleic acids: Three types of experiments were done to demonstrate that the binding of these radiochemicals is indeed by DNA only and not by contaminating histones. (1) DNA, redeproteinized with the procedure described above, gave the same nk as the untreated DNA for the binding of estradiol-β-17 and testosterone (see Fig. 2). (2) After incubation with pancreatic DNAase, the denatured DNA samples all became dialyzable, and the dialyzed solutions failed to bind testosterone. This experiment conclusively shows that the substance which binds testosterone is destroyed by DNAase. (3) Finally, the nk of histone for testosterone and phenanthrene binding was determined (see Fig. 3). The low value of nk indicates that histones, if present as slight contaminant, will not significantly affect the results. Nevertheless, the DNA used in the bulk of the experiments was redeproteinized.

Partition coefficients: Partition coefficients of the radiochemicals between heptane and the aqueous solutions of 0.5 M NaCl and HMP, pH 7.1 were measured by equilibrating one ml of heptane with one ml of the buffer-salt solution of the radiochemical. This was done at the same temperature and with the same rotating device used in the dialysis experiments.

Counting procedure: Radioactivity was measured by the Packard TriCarb liquid scintillation spectrophotometers (Models 314 DC and 314 EX) with automatic sample changers. For counting tritium, the counting bottle contained 0.2 ml sample and 15 ml of counting mixture (120 gm naphthalene, 4.0 gm 2,5-diphenyloxazole, PPO, and 0.05 gm 1,4-bis-2-(5-phenyloxazolyl)-benzene, POPOP, per liter of p-dioxane. For counting carbon-14, the bottle contained 0.2 ml sample, 10 ml of ethanol, and 10 ml of counting mixture (7.0 gm PPO and 0.2 gm dimethyl POPOP per liter of toluene).

Results.—The experimental design and the computation of the binding constants was based on the following:

$$\frac{1}{r} = \frac{1}{nk}\frac{1}{[S]_F} + \frac{1}{n}, \qquad (1)$$

where r = moles of bound substrate, $[S]_B$/moles of unit nucleotide in nucleic acids, n = the number of the binding sites per unit nucleotide in nucleic acid, k = the binding constant of the unit nucleotide to the substrate of interest, and $[S]_F$ = the molar concentration of the free substrates. In these experiments, the concentration of the substrate was always very low, and the binding sites were far from saturation because of the large polymer : substrate concentration ratio.

Eight compounds were chosen for binding studies. These are: thymine, adenine, caffeine, estradiol-β-17, testosterone, diethylstilbesterol, naphthalene, and phenanthrene. As for the biopolymers, thymus DNA, yeast soluble RNA, pea seedling ribosomal RNA, poly A, poly U, and thymus histone were chosen.

The graphs, slopes of which yield the values of nk for the binding studies of testosterone to both native and denatured DNA, are presented in Figures 1 and 2 as illustration of actual data. Other data are summarized in Table 1.

The binding of testosterone (Fig. 3) and of phenanthrene by calf thymus histone has also been studied. It is important to know whether the small but inevitable histone contamination of DNA is of concern. The binding of steroid hormones and of aromatic hydrocarbons by histones is interesting in itself. At 5°C, the

TABLE 1

BINDING CONSTANTS OF BASES, STEROIDS, AND AROMATIC HYDROCARBONS ONTO NUCLEIC ACIDS[a]
(All in 0.5 *M* NaCl together with buffer AT 5°C)

$$nk\ (M^{-1})^{c} = \frac{[\text{complex}]}{[\text{free nucleotide-unit in nucleic acid}]\ [\text{free substrates}]}$$

	Thymine	Adenine	Caffeine	Diethyl-stilbesterol	Estradiol-β-17	Testos-terone	Naphtha-lene	Phenan-threne
Thymus DNA[f] (heat-denatured)	<1[b]	9.0	7.0[d]	30	64	220	20	2800[e]
Thymus DNA[f] (native)	<1[b]	<1[b]	<1[b]	<1[b]	14	15	<1[b]	300
Poly A coil[g]	<1[b]	5.7[d]	5.8	—	6.2	7.0	—	30[b]
Poly A helix[h]	—	7.1	<1[b]	—	4.0	<1[b]	—	—
Poly U[f]	—	—	—	—	—	<1[b]	—	7[b]
Yeast sRNA[f]	—	—	—	<1[b]	<1[b]	4[i]	—	63
Pea ribosomal RNA[f]	—	—	—	—	—	4[b]	—	65[b]

[a] The range of polymer concentration used is around 0.01–0.025 *M* of nucleotide unit. For the experiment with denatured DNA and steroids and phenanthrene, the concentration is around 0.005 *M*.
[b] Experiments were made only at 1–3 levels of the concentration of the radiochemicals. For value of n*k* less than 1, usually three repeats of the lowest workable concentration were taken.
[c] The standard error of measurement and computation of n*k* for a given sample of nucleic acid is estimated to be ±4% for n*k* value over 100, ±8% for n*k* value below 20, and ±12% for n*k* value below 10. For DNA, the value of n*k* is very sensitive to the helical content and random-coil content of the sample.
[d] Points scattered more than usual, the standard error is around ±20%.
[e] n*k* from the solubility data was 2800 ± 100, and from dialysis experiments varies from 1900 to 2800. Because of the difficulties in dialysis, the value of 2800 was taken.
[f] In 0.5 *M* NaCl, 0.01 *M* phosphate, pH 7.1.
[g] In 0.5 *M* NaCl, 0.05 *M* Tris buffer, pH 7.5.
[h] In 0.5 *M* NaCl, 0.1 *M* Na-acetate, pH 4.5.
[i] n*k* values of 2 and 6 were obtained from two different preparations; an average is reported here.

value of n*k* for testosterone binding is 0.014 $(g/l)^{-1}$ and for phenanthrene binding is 0.3 $(g/l)^{-1}$. When the values are converted to the same unit of $(g/l)^{-1}$, the values for the bindings of testosterone and phenanthrene by the denatured DNA are 25–35-fold higher than those for binding by histone.

The order of the values of n*k* for these eight compounds is more or less the same for all nucleic acids. Thus, a search of physical properties of the compounds was made in the hope of finding the germane one. In Table 2, partition coefficients of the eight compounds between heptane and aqueous buffer at 5°C are given, together with the values of n*k* of these compounds with denatured DNA.

Preliminary investigation on the temperature dependency of n*k* has been made. The n*k* value of testosterone binding to denatured DNA in 0.5 *M* NaCl and HMP was found to be 80 at 27°C, 110 at 15°C, and 170 at 5°C for this particular prepara-

TABLE 2

PARTITION COEFFICIENTS OF BASES, STEROIDS, AND AROMATIC HYDROCARBONS BETWEEN HEPTANE AND AQUEOUS BUFFER AT 5°C*

Compound	Coefficient	n*k*†
Adenine	~0.0004‡	9.0
Thymine	~0.001‡	<1
Caffeine	~~0.01‡	7.0
Estradiol-β-17	0.19	64
Diethyl stilbesterol	0.78	30
Testosterone	1.6	220
Naphthalene	8.2	20
Phenanthrene	800–1200†	2800

* For comparison, the values of n*k* of these compounds to denatured DNA are also listed. Coefficient = conc. in heptane/conc. in 0.01 *M* phosphate, pH 7.1, 0.5 *M* NaCl.
† Data from Table 1.
‡ The coefficient is too large or too small for accurate measurement.

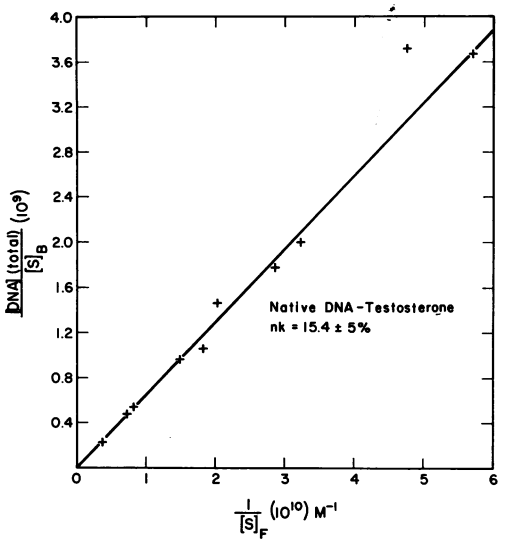

FIG. 1.—Binding curve of testosterone to native thymus DNA.

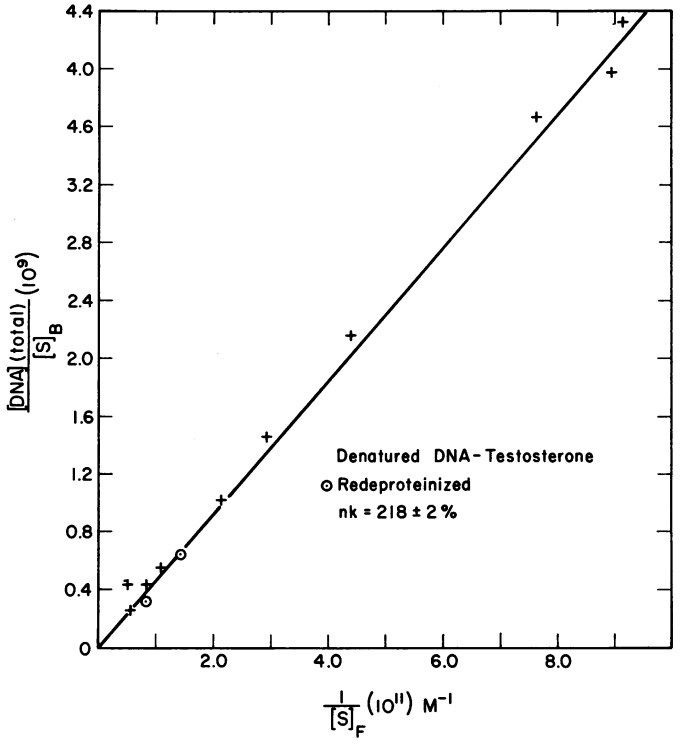

FIG. 2.—Binding curve of testosterone to heat-denatured thymus DNA. -⊙-⊙-, data from redeproteinized DNA.

tion. The optical absorbancy of this denatured DNA sample at 0.5 *M* salts was unchanged at these three temperatures.

Discussion.—The experimental conditions will be reviewed first.

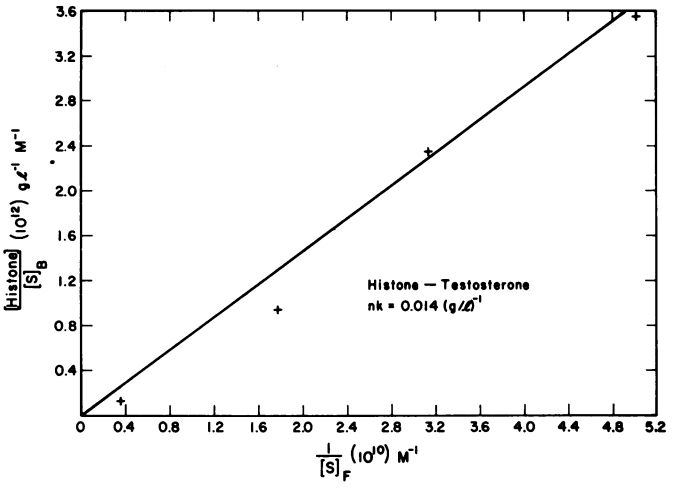

FIG. 3.—Binding curve of testosterone to soluble calf thymus histone.

All experiments were carried out at 5°C. At this temperature, the value of nk was higher than those obtained at 15°C and 27°C. Increase in temperature usually leads to increase in the content of coil form of nucleic acids. The optical absorbancy measurement of the denatured DNA in 0.5 M salt was found to be unchanged in the range of 5–27°C, an indication that the change in conformation is slight, if any. Since the affinity of these compounds is so much greater for the coil form than for the helical form of nucleic acid, the elevation of temperature is expected to increase the value of nk. The lowering of nk at higher temperature therefore clearly indicates that the binding process has a negative value for both enthalpy change and entropy change.

All experiments were carried out in a solution containing 0.5 M NaCl together with 0.01–0.1 M buffer. The presence of this amount of electrolytes is sufficient to suppress the Donnan effect exerted by the nucleic acids and also places the nucleic acids in a dielectric environment such that its conformation is not sensitive to minor changes of electrolyte concentration.

All the substrates chosen for study are neutral at the pH employed, with the exception of adenine at pH 4.5, the medium required for the formation of helical poly A.[1] At this pH, the adenine is partially protonated. For the other compounds, as well as for adenine at pH 7.0, there is no electrostatic interaction expected between nucleic acid and the uncharged substrate.

The value of r in these experiments is very low, so that the binding sites are far from saturation. Thus, only the values of nk can be deduced from the data where k is the number average association constant for all the binding sites.[8] Also, no information can be deduced about the heterogeneity of the sites. The reciprocal form of the binding equation is used for plotting data because of the following consideration: by choosing the nucleotide (or mole of phosphorus) of the nucleic acid as the unit for the calculation of the binding data, the value of 1/n (most likely less than 10) is much smaller than the value of $1/r$ (10^9 to 10^{10}). Thus, it is justifiable to include the origin as a datum point. This helps in assigning the slope to the line.

The use of nucleotide unit as the basis for calculation is a reasonable approach, but it deserves some reflection. This approach, on one hand, acknowledges our ignorance about the nature of the binding sites and, on the other hand, assumes a uniformity for the primary or secondary structures owing either to regularity or to statistical randomness. It is difficult to imagine a value of n much larger than one or two per nucleotide. Conversely, if a few nucleotides are needed in a cooperative manner to bind the substrates, it is difficult to see how n can be less than $^1/_4$. The value of k, then, represents an average tendency of the association of the nucleotide unit with the substrates.

The first conclusion of this work is that the affinity of these compounds for the coil form of nucleic acids is much higher than that for the helical or native form (Table 1). Therefore, most likely this is the mechanism by which T_m was reduced by these compounds.[1] As a corollary to this conclusion, the value of nk may not be an absolute number unless the secondary structure has been more rigorously defined. Indeed, during the investigation, the value of nk was found to vary with both native and denatured DNA, depending on the past history of the sample. We feel that to a large extent the value of nk obtained for native DNA reflects the extent of the denatured regions or the denatured materials in the samples.

There is one notable exception to this conclusion. The nk of adenine binding to the helical form of poly A appears to be greater than that to the coil form. As mentioned above, adenine is partially protonated at pH 4.5 and therefore interacts with the phosphate groups of poly A. This is an unavoidable situation, since the poly A must also be protonated before it will assume the helical form.[1] This exception is thus an interesting and logical one.

The second conclusion (Table 1) is that the denatured DNA has a two orders of magnitude higher affinity for these substrates than does RNA, natural or synthetic. One is tempted to interpret this data on the grounds that the RNA, especially the yeast sRNA,[9] may have more helical secondary structure than the denatured thymus DNA. However, this is unlikely because the random coil form of both poly A and poly U also has a much lower value of nk than denatured DNA. Proper understanding of this interesting difference between DNA and RNA must await further investigation.

What is the nature of the binding forces between these substrates and the nucleic acids? It cannot be electrostatic interaction and it is unlikely to be totally dependent upon hydrogen bonding, especially in the case of the aromatic compounds. The affinity of various substrates to nucleic acids and the effectiveness of various compounds in reducing the T_m[1] appears to follow similar principles.

Partition coefficients of these substrates between heptane and water were determined (Table 2), in order to provide further insight into this question. Listed together in Table 2 are the values of nk to the denatured DNA. There is a certain amount of correlation between the partition coefficients and the nk. For instance, the hydrocarbon and the steroids all have higher coefficients and higher nk than do purines and pyrimidines. Among the steroids, testosterone has both the highest partition coefficient and the highest nk. Similarly, phenanthrene has the highest values of both nk and partition coefficient among all compounds. There are, however, discrepancies. For instance, naphthalene has a higher coefficient and lower nk than the steroids, adenine has a lower coefficient but higher nk than thymine,

The best conclusion seems to be that the hydrophobic forces, such as interaction of water molecules and close-range van der Waals interaction, do play a very important role. Other factors, such as additional hydrogen bonding sites, stereochemistry, and π-electron attractions, may also be involved. Munck, Scott, and Engel have reported the bindings of steroid hormones on the bases, ribonucleosides, and ribonucleotides.[10-11] They also came to the conclusion that the forces involved are predominantly due to hydrophobic interaction.

Summary.—The affinity constants (nk), where n is the number of the binding sites and k is the average association constant of each site, between certain compounds and nucleic acids have been measured by equilibrium dialysis at 5°C in buffered solutions containing 0.5 M salt. The order of the increasing affinity constants of these compounds to the nucleic acids was found to be: thymine $<<$ adenine, caffeine $<$ naphthalene $<$ diethylstilbesterol $<$ estradiol-β-17 $<$ testosterone $<$ phenanthrene. The affinity constants of the nucleic acids in coil form was found to be much higher than those of nucleic acids in helical form. Desoxyribonucleic acid has a two order of magnitude higher affinity constant than ribonucleic acid. Nucleic acids tested were native and heat-denatured thymus DNA, helical poly A and coil poly A, poly U, ribosomal RNA, as well as yeast sRNA.

We wish to thank Prof. J. Bonner for his active support of this work, and the capable assistance of Mrs. I. Melvin and Miss Elizabeth Nilsen is also gratefully acknowledged. This research was supported in part by grant GM-10316-1, U.S. Public Health Service, and by grant GB-767, National Science Foundation.

[1] Ts'o, P. O. P., G. K. Holmkamp, and C. Sander, these PROCEEDINGS, **48**, 686 (1962).
[2] Herskovits, T. T., S. J. Singer, and E. P. Geiduschek, *Arch. Biochem. Biophys.*, **94**, 99 (1961).
[3] Herskovits, T. T., *Arch. Biochem. Biophys.*, **97**, 433 (1962).
[4] DeVoe, H., and I. Tinoco, Jr., *J. Mol. Biol.*, **4**, 500 (1962).
[5] Ts'o, P. O. P., I. S. Melvin, and A. C. Olson, *J. Am. Chem. Soc.*, **85**, 1289 (1963).
[6] Ts'o, P. O. P., G. K. Helmkamp, and C. Sander, *Biochim. Biophys. Acta*, **55**, 584 (1962).
[7] Holley, R. N., J. Apgar, B. P. Doctor, J. Farrow, M. A. Marin, and S. H. Merrill, *J. Biol. Chem.*, **236**, 200 (1961).
[8] Schellman, J. A., R. Lumry, and L. T. Samuels, *J. Am. Chem. Soc.*, **76**, 2808 (1954).
[9] Spencer, M., W. Fuller, M. H. F. Wilkins, and G. L. Brown, *Nature*, **194**, 1014 (1962).
[10] Scott, J. F., and L. L. Engel, *Biochim. Biophys. Acta*, **23**, 665 (1957).
[11] Munck, A., T. F. Scott, and L. L. Engel, *Biochim. Biophys. Acta*, **26**, 397 (1957).

SPLINE INTERPOLATION AND THE HIGHER DERIVATIVES

BY I. J. SCHOENBERG

UNIVERSITY OF PENNSYLVANIA AND INSTITUTE FOR ADVANCED STUDY

Communicated by Hassler Whitney, November 12, 1963

I. *Introduction.*—Let $[a, b]$ be a finite interval and let us consider the function class

$$F_m[a, b] = \{f(x) | f \in C^{m-1}[a, b], f^{(m-1)} \text{ absolutely continuous}, f^{(m)} \in L^2(a, b)\}.$$

In 1910 F. Riesz[1] gave a characterization of the class $F_1[a, b]$. A formulation different from his but accessible to a similar method is as follows: