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On the Interaction of Nucleotides with Poly-L-lysine and Poly-L-arginine. I. The Influence of the Nucleotide Base on the Binding Behavior*

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ABSTRACT: The binding of 5′-ribo- and 5′-deoxyribonucleoside monophosphates by poly-L-lysine and poly-L-arginine has been studied by the equilibrium dialysis technique. The different nucleotides were compared under standard conditions, in order to determine the influence of nucleotide bases on the binding parameters. It was found that the binding behavior is indeed dependent of the nature of the base; whereas the individual pyrimidine nucleotides exhibit little or no differences, the purine nucleotides exhibit large differences among each other and differ substantially from the pyrimidine nucleotides. In every case guanylic acid was found to bind most strongly. Reasons for observed differences in the binding behavior are discussed.

The interaction of nucleic acids with basic proteins is a subject which touches on the question of whether interactions of this kind in nucleoproteins, especially in nucleohistones, can be specific enough to play a role in the regulation of gene expression. Many authors have studied complexes of polynucleotides with basic polypeptides (Chargaff *et al.*, 1953; Spitnik *et al.*, 1955; Lucy and Butler, 1955; Johns and Butler, 1964; Akinrimisi *et al.*, 1965; Tsuboi *et al.*, 1966; Leng and Felsenfeld, 1966; Sober *et al.*, 1966; Ohba, 1966; Olins *et al.*, 1967). Their results provide evidence for a degree of specificity, namely, that the mutual binding of the two macromolecules is influenced not only by charge but also by the nature and relative amounts of the purine and pyrimidine bases in the polynucleotide. In the work presented here we have examined the interaction of various mononucleotides with basic polypeptides. Not only can the thermodynamic parameters of such a system be determined more accurately than those of a polynucleotide–polypeptide system, but the system is also of intrinsic interest, since nucleotides interact with many enzymes as substrates or effectors.

Our data describe the binding behavior of a number of nucleoside monophosphates toward poly-L-lysine and poly-L-arginine. It is quite obvious that at neutral pH this interaction is mainly governed by nonspecific electrostatic forces between the negatively charged phosphate groups of the nucleotides and the positively charged side chains of the polyamino acid. However, our results show that superimposed on this main electrostatic contribution there are more specific nonelectrostatic effects which depend on the nature of the nucleotide base.

Materials and Methods

The nucleotides were purchased from Schwarz BioResearch Inc. and Sigma Chemical Co. Only nucleotide material with an inorganic phosphate content of less than 1% (Bartlett, 1959) was used. The concentration of the nucleotide solutions was determined by measuring the optical density in the ultraviolet region using the molar extinction coefficients given by Pabst Laboratories and Schwarz BioResearch Inc.

Poly-L-lysine–hydrogen bromide was a gift from the Biophysics Department of the Weizmann Institute of Science. The average residue number per chain was 350 according to viscosity measurements of the poly-ε-carbobenzoxy-L-lysine (Daniel and Katchalski, 1962). Polylysine stock solutions were prepared by extensive dialysis against 0.02 M sodium acetate, beginning with a higher concentration to exchange the Br^- counterion for $acetate^-$. The dialysis tubing for this purpose and the membrane used in the equilibrium dialysis experiments were the same and were also treated in the same way. In the beginning about 20% of the ninhydrin-positive material passed relatively quickly through the dialysis tubing; thereafter no ninhydrin-positive material was detectable in the dialysate.

To determine the concentration of the lysine residues the ninhydrin method of Yemm and Cocking (1955) was slightly modified. By enhancing the concentration of methyl Cellosolve (Yanari, 1956), it is possible to compare the ninhydrin assay of a poly-L-lysine sample without hydrolysis with that of a standard poly-L-lysine solution, an aliquot of which had been hydrolyzed and the lysine concentration of which had been determined

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by the usual ninhydrin method (Yemm and Cocking, 1955).

Poly-L-arginine–$0.5H_2SO_4$ was purchased from Yeda Research and Development Co., Israel. The average molecular weight was about 28,000 (lot AR 14). To exchange the sulfate counterions for acetate ions, 65 mg of poly-L-arginine–$0.5H_2SO_4$ was suspended in 6 ml of water; while stirring the suspension 6.5 ml of 0.2 M barium acetate was added dropwise. After stirring 4 hr at room temperature the $BaSO_4$ was spun down by centrifugation and the supernatant was dialyzed against 0.3 M sodium acetate and then extensively against water.

After hydrolyzing an aliquot of the stock solution, the concentration of the arginine residues was determined by the ninhydrin method (Yemm and Cocking, 1955). The poly-L-arginine concentration of prepared solutions was compared with that of the stock solutions by the biuret method (Zamenhof, 1957).

The binding studies were carried out by the equilibrium dialysis method. Dialysis cells made from Perspex, the two chambers of which were 30 mm in diameter and 3 mm in depth, also glass cells devised by Karush and Karush (1967), available from Bellco Glass Co., Vineland, N. J., were used. The Perspex cells contained 1.5 ml of solution for each chamber, while the glass cells contained on each side 1.0 ml. The membranes were cut from sausage casing ($^{20}/_{32}$ Visking Co., Chicago, Ill.), allowed to swell in a solution of 1% NaCl and 0.1% EDTA (pH 7) for about 24 hr, then boiled for 30 min in water, and finally placed in 0.02 M sodium acetate. The cells were fixed on an apparatus rotating in a water bath.

By Fick's diffusion law it is possible to determine from diffusion measurements the time which is required to approach conditions within a 1% deviation from the equilibrium. With the Karush cells and the purine nucleotides we determined a time of 30 hr at 0° and of 15 hr at 30° under the above conditions. In our experiments a time of 48 hr at 0° and 24 hr at 30° was taken. For every experiment a control was also done, the cell of which contained on one side the highest nucleotide concentration used and on the other side 0.02 M sodium acetate, to determine whether the approach to the equilibrium conditions was close enough.

The binding behavior was measured at pH 7 in 0.02 M sodium acetate. Although sodium acetate is a very weak buffer at this pH, we preferred it to other available buffers because its very weak affinity both to nucleotides and to the charged side chains of the basic polyamino acids would minimize competition with the binding reaction under study. Another consideration was the buffer concentration. In order to minimize the magnitude of the membrane equilibrium (Donnan, 1911) one should employ a high salt concentration. This, however, would reduce the binding of nucleotides by polylysine or polyarginine, which is mainly electrostatic and thus dependent on the ionic strength. We therefore chose to obtain higher binding values by employing a low buffer concentration, and to calculate the contribution of the Donnan (1911) effect and correct for it.

The calculation of the Donnan effect requires the knowledge of the effective charge per lysine or arginine residue. From the chloride ion activity measurements in the presence of poly-L-lysine at pH 4 H. A. Saroff (personal communication) calculated an effective charge of 0.63/lysine residue; we therefore used a value of 0.6 for our calculation. With poly-L-lysine the corrections due to the Donnan equilibrium were about 5–7% of the free nucleotide concentration, whereas the amount of bound nucleotides was in the magnitude of 40–300% of the free nucleotide concentration. With poly-L-arginine these numbers are still more favorable (3–5 and 70–500%) because of the larger affinities involved and the lower polypeptide concentration. It is obvious that the error of the calculated contribution of the membrane equilibrium, depending mainly on the uncertainty of the effective charge, is relatively small. For similar reasons the polyamino acid concentration was kept constant and the nucleotide concentration was varied. The poly-L-lysine concentration was about 2 mM lysine residues and the poly-L-arginine concentration was 1.3 mM arginine residues.

Results

The mathematical treatment of the data from binding studies is a simple one, if all binding sites along the macromolecule exhibit the same binding affinity toward a small molecule that is to be bound. Equation 1 which

$$K_a = \frac{r}{C_f(n - r)} \tag{1}$$

describes this situation is deduced either by statistical treatment of the mass equations that govern the association of small molecules or adsorbates with a large macromolecule (Klotz, 1946) or is deduced from the Langmuir isotherm (Steiner and Beers, 1961). The unit of reference is the macromolecule if one is dealing with a homogeneous solution of macromolecules of the same molecular weight, or the residue unit if one is dealing, as in our case, with macromolecules, the molecular weights of which follow a statistical distribution. Thus, r means the number of adsorbates bound per reference unit, and C_f is the equilibrium concentration of the free nonbound adsorbates. At saturation, if C_f becomes infinite, $r = n$, the number of available binding sites per reference unit. If the residue is the unit of reference it is more convenient to take $1/n$ which means the number of residues available to bind one small molecule. K_a is the intrinsic association constant.

To determine the parameters K_a and $1/n$ one plots either r/C_f against r according to Scatchard's (1949) proposal

$$r/C_f = nK_a - rK_a \tag{2}$$

or $1/r$ against $1/C_f$ according to Klotz (1949).

$$\frac{1}{r} = \frac{1}{n} + \frac{1}{K_a n}\frac{1}{C_f} \tag{3}$$

TABLE I: The Binding Parameters of the 5′-Ribonucleoside Monophosphates and the 5′-Deoxyribonucleoside Monophosphates with Poly-L-lysine in 0.02 M Sodium Acetate (pH 7).

	30°		0°	
	K_a (mM^{-1})	$1/n$	K_a (mM^{-1})	$1/n$
5′-Gp	3.25	2.7	3.25	2.1
5′-Ap	2.25	3.0	2.2	2.4
5′-Cp	2.2	3.6	2.35	3.6
5′-Up	2.25	3.6	2.4	3.75
5′-dGp	4.1	3.5		
5′-dAp	2.45	3.3		
5′-dCp	2.6	4.1		
5′-dUp	2.6	4.1		
5′-dTp	2.15	3.7		

TABLE II: The Binding Parameters of the 5′-Ribonucleoside Monophosphates with Poly-L-arginine at 30° in 0.02 M Sodium Acetate (pH 7).

	K_a (mM^{-1})	$1/n$
5′-Gp	14.5	3.4
5′-Ap	9.4	3.6
5′-Cp	5.3	3.5
5′-Up	4.6	3.4

All the binding data were plotted according to these two equations. Within the accuracy of our data none of the plots revealed evidence of curvature; therefore the best fitting straight line was drawn. (Only the system 5′-Up–polyarginine[1] shows a very small departure from linearity in the direction of an anticooperative effect. As this departure is within the error of estimation we did not account for it.) From each type of plot the values $1/n$ and K_a were determined for the different systems. The numbers in Tables I and II are average values for both plots.

Figures 1 and 2 and Table I show the results of the binding experiments with the 5′-ribonucleoside monophosphates and the 5′-deoxyribonucleoside monophosphates and poly-L-lysine at 30 and 0°, respectively. It is obvious that the binding behavior of the pyrimidine nucleotides is the same within experimental error, whereas the purine nucleotides exhibit marked differences. Figures 1 and 2 represent so-called Klotz plots, where the intercept at the ordinate is equal to the $1/n$ values (lysine residues per bound nucleotide). It is evident that these values are larger with the pyrimidine

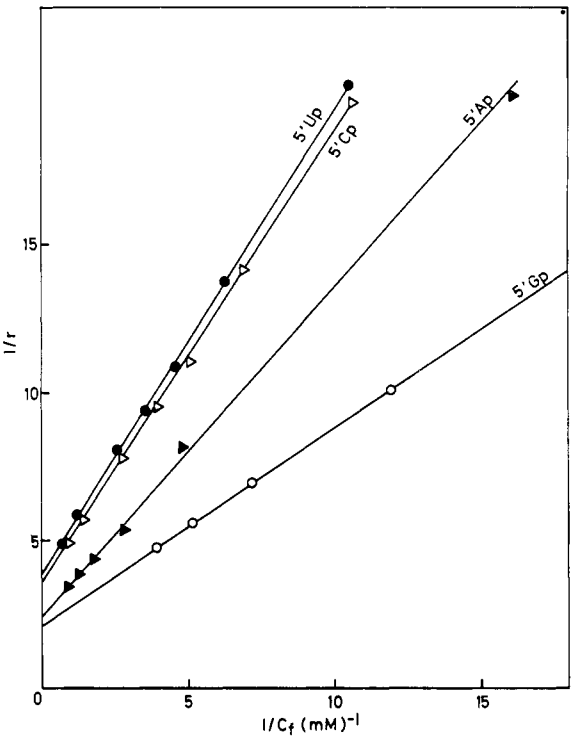

FIGURE 1: Comparison of the binding behavior of the 5′-ribonucleoside monophosphates with poly-L-lysine at 0° in 0.02 M sodium acetate (pH 7) (Klotz plot).

derivatives than with the purine nucleotides; this indicates that the purine nucleotides are more closely packed along the poly-L-lysine molecule. Table I shows that the apparent association constants of the different nucleotides are about the same; only guanosine nucleotides possess considerably larger constants. The different behavior of the adenine nucleotides from the pyrimidine nucleotides demonstrated in the figures can only be seen in the $1/n$ values.

Figure 3 and Table II show the results of the binding experiments with the 5′-ribonucleoside monophosphates and poly-L-arginine at 30°. In this case the $1/n$ values (arginine residues per bound nucleotide) are the same within experimental error. The apparent association constants exhibit a small difference between 5′-Up and 5′-Cp, whereas the purine nucleotides bind much stronger, especially 5′-Gp. In general the association constants are much larger (twice up to four times) than those with poly-L-lysine.

Table I also shows the binding parameters of 5′-thymidine monophosphate with poly-L-lysine at 30°. In order to clear up whether the small difference in the binding behavior between 5′-dTp and the other deoxypyrimidine nucleotides is not an experimental artifact, we did two binding experiments with poly-L-lysine at 0°. In the first experiment we compared 5′-dUp and 5′-dTp, and in the second one 5′-dCp and 5′-dTp. In both cases the plots indicate that 5′-dTp binds less strongly than 5′-dUp and 5′-dCp.

Table III shows the hypochromicity which arose upon the binding of the nucleotides to poly-L-lysine. In 0.02 M sodium acetate at pH 7 we measured the

[1] Abbreviations used are as given in *Biochemistry* 5, 1445 (1966).

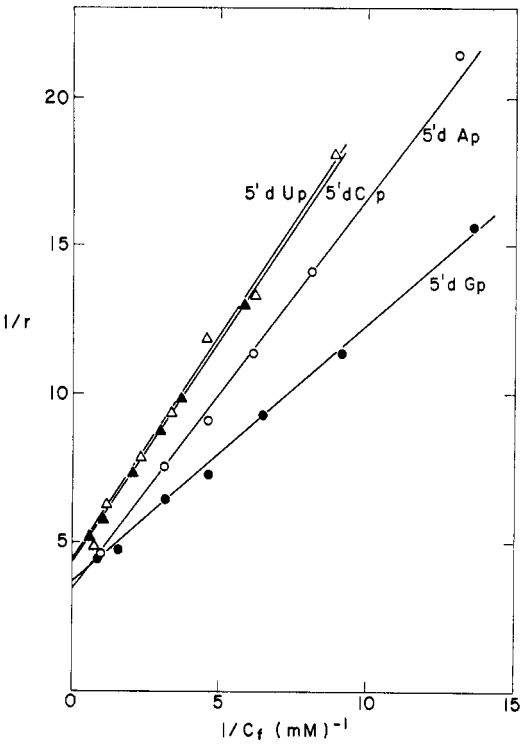

FIGURE 2: Comparison of the binding behavior of the 5′-deoxyribonucleoside monophosphates with poly-L-lysine at 30° (0.02 M sodium acetate pH 7) plotted according to Klotz (1949).

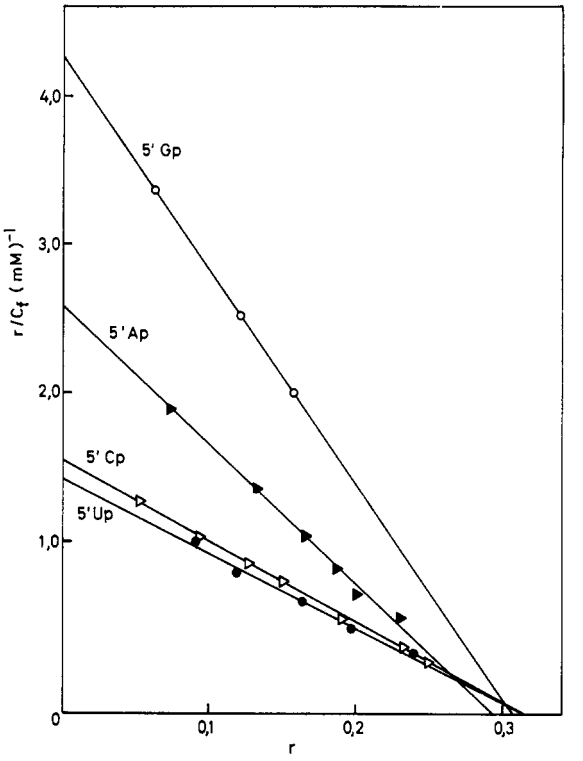

FIGURE 3: The binding behavior of the 5′-ribonucleoside monophosphates with poly-L-arginine at 30° (in 0.02 M sodium acetate, pH 7) plotted according to Scatchard (1949).

difference in absorption between each system and its components at the same concentrations (at the characteristic absorption maximum of each nucleotide). The concentration of poly-L-lysine was 3 mM and that of the nucleotides about 1 mM. Using the binding parameters the amount of bound nucleotides for each system was calculated. The hypochromicity is related to the absorption of the bound nucleotides, assuming that the free nucleotides will not contribute to it. (In order to determine the nucleotide concentration for the equilibrium dialysis experiments, we measured the absorption at diluted concentration, where we did not detect hypochromicity.)

For comparison Table III also contains the hypochromicity of the corresponding dinucleoside monophosphates at pH 7 from a paper of Warshaw and Tinoco (1966). While there is correspondence with ApA, GpG, and CpC, 5′-Up shows in our system almost the same hypochromicity as 5′-Cp or 5′-Gp; on the other side UpU exhibits only a small hypochromicity compared with the other dinucleoside monophosphates.

At higher molar ratios of 5′-Gp to poly-L-lysine a precipitate is formed. We studied this phenomenon with different nucleotides and with both poly-L-lysine and poly-L-arginine (manuscript in preparation). In some cases this precipitate containing most of the polyamino acid turned out to be a liquid phase, very small in volume and more dense than water; in other cases it was a real precipitate. Table IV shows in which system this phenomenon occurs (+) and in which not (−). It was studied under the same conditions as the binding experiments.

TABLE III: The Hypochromicity (H) of the 5′-Ribonucleoside Monophosphates with Poly-L-lysine (Room Temperature, in 0.02 M Sodium Acetate, pH 7) as Per Cent of the Absorption of the Bound Nucleotides (See Text) (Last Column, the Hypochromicity of the Dinucleoside Monophosphates from the Paper of Warshaw and Tinoco (1966)).

	λ_{max} (mμ)	H (%)		H (%)[a]
5′-Gp	252	6.1	GpG	6.9
5′-Ap	259	11.4	ApA	9.4
5′-Cp	271	6.1	CpC	7.2
5′-Up	262	5.8	UpU	1.7

[a] Measured at the characteristic absorption maxima of the dinucleoside monophosphates which are identical or almost identical with the characteristic absorption maxima of the respective mononucleotides (Warshaw and Tinoco, 1966).

Discussion

The pK_a for the dissociation of the second phosphate proton of nucleoside monophosphates, at an ionic strength of 0.02, is about 6.5 (Phillips *et al.*, 1965).

TABLE IV: Qualitative Comparison under Which Conditions and in Which Systems Precipitation or Phase Separation Occurs (+) or Not (−).

	Poly-L-lysine		Poly-L-arginine	
	30°	0°	30°	0°
5′-Gp	+	+	+	+
5′-Ap	−	+	+	+
5′-Cp	−	−	−	+
5′-Up	−	−	−	−

TABLE V: Reciprocal Michaelis Constants of the 3′-Nucleotidase of Mung Bean Sprouts (Loring *et al.*, 1966) (Last Column, the Apparent Association Constants of the 5′-Ribonucleoside Monophosphates with Poly-L-arginine (from Table II)).

	$1/K_m$ $M^{-1} \times 10^{-2}$		K_a (mM^{-1})
3′-Gp	15	5′-Gp	14.5
3′-Ap	9.0	5′-Ap	9.4
3′-Cp	0.7	5′-Cp	5.3
3′-Up	1.3	5′-Up	4.6

Therefore, at pH 7 the ratio of concentrations of monovalent to divalent anions is about 1:3. Assuming that the divalent anion binds more strongly than the monovalent anion, one should expect that at pH 7 the binding behavior of a nucleotide toward basic polyamino acids is pH dependent. In addition, if the above assumption is true, the apparent binding behavior of different nucleoside monophosphates should depend on the respective pK_a values. Fortunately Phillips *et al.* (1965) showed that the pK_a values of the second phosphate proton dissociation are independent of the nature of the base and, as they showed in the case of adenosine monophosphate, they are independent of the presence or absence of the 2′-OH group.

The association constants measured in our system are apparent constants depending on pH and ionic strength. However, as all measurements are done under standard conditions, it is possible to compare the binding behavior of the different nucleotides.

That the interaction of a nucleoside monophosphate with poly-L-lysine or poly-L-arginine depends on the nature of its base is also demonstrated by the phenomenon of precipitation or phase separation (Table IV). Comparing Table IV with Tables I and II, it is evident that there is correspondence between the binding constants and the occurrence of this phenomenon. As a contribution of the bases to the binding affinities one can assume (a) neighbor–neighbor interaction of the bound nucleotides as it is known with polynucleotides and described as stacking effects and (b) base-dependent nonelectrostatic affinities between a nucleotide and its binding site.

That neighbor–neighbor interactions of the bound nucleotides may play a role is indicated by the hypochromicity effects (Table III) arising upon binding of nucleotides to poly-L-lysine. The order of magnitude is comparable to the hypochromicity of dinucleoside monophosphates (Warshaw and Tinoco, 1966), except for UpU. Furthermore the binding strength parallels the tendency of stacking of the different nucleotide bases (Ts'o *et al.*, 1963). Therefore nucleotide stacking may contribute largely to the observed differences in the binding behavior.

But if neighbor–neighbor interactions alone were responsible for the differences of the binding affinities, one should expect cooperative behavior. Yet the binding data do not reveal any cooperative effect, even in the case of 5′-Gp and poly-L-arginine with the largest association constant (Figure 3 and Table II). In this latter case a strong cooperative effect is demonstrated at higher 5′-Gp concentrations, but in this concentration region precipitation occurs and the binding site parameter changes from 3.5 arginine residues to 2 residues per bound 5′-Gp, indicating that this cooperative effect is connected with a complete change of the binding behavior (manuscript in preparation). Nevertheless, it is possible that moderate cooperativity will not be detected within the accuracy of our data. Furthermore a cooperative effect due to stacking may be compensated by an anticooperative effect due to charge repulsion. Base–base interaction of the bound nucleotides should, however, lead to a temperature dependency of the binding constants. Table I does not reveal such an effect, but this could be masked by other processes, like a process which leads to a denser packing of the bound nucleotides at 0°, as is indicated by the $1/n$ values of Table I. On the other hand, if there are any nonelectrostatic interactions between a nucleotide and its binding site, it is reasonable to assume that these affinities go along with the tendency of stacking. Especially in the case of polyarginine one could discuss π–π electron interactions between the nucleotide bases and the guanido groups.

Direct interaction between a nucleotide and its binding site is involved in substrate recognition of enzymes dealing with nucleotides. Table V shows the comparison of the reciprocal K_m values of 3′-ribonucleoside monophosphates of the 3′-nucleotidase from mung bean sprouts (Loring *et al.*, 1966) with the apparent association constants of the 5′-ribonucleoside monophosphates with poly-L-arginine. The enzyme recognizes 3′-nucleotides regardless which base is attached; the contribution of the base to the Michaelis constant may therefore represent relatively unspecific affinities which are comparable to those factors which in our case differentiate the binding behavior of the different nucleotides. There is some correspondence.

Whereas with poly-L-arginine the $1/n$ values, the number of amino acid residues per bound nucleotide, are practically equal, these values vary considerably in the case of poly-L-lysine (Tables I and II). The numbers indicate that the purine nucleotides tend to be more

densely packed along the poly-L-lysine molecule than the pyrimidine nucleotides. Ts'o *et al.* (1963) showed that purine derivatives associate more strongly in aqueous solution than pyrimidine derivatives. This could be the reason for the smaller $1/n$ values of the purine nucleotides.

The conformation of nucleosides and nucleotides in solution is still under discussion; Klee and Mudd (1967) assume from optical rotatory dispersion measurements that the conformation of purine nucleosides is different from that of pyrimidine nucleosides. Purine derivatives are thought to be in a *syn* conformation with respect to the 5′ position of the ribose and the 2 carbon atom of the purine ring system. On the other side pyrimidine derivatives which are sterically hindered in the *syn* conformation are thought to have the *anti* conformation (Emerson *et al.*, 1967). This, too, could be a reason for the different binding behavior of purine and pyrimidine nucleotides. Assuming that upon binding a nucleotide approaches the poly-L-lysine molecule with the phosphate group, the *anti* conformation would imply that in the case of thymidylic acid the 5-methyl group would be in the neighborhood of or on the same side as the phosphate group, which could explain the influence of this group on binding.

It is difficult to see how one could compare our results with experiments of several authors studying the interaction of basic polypeptides with polynucleotides. Most experiments done with DNA showed that polylysine preferentially binds to A–T-rich samples (Spitnik *et al.*, 1955; Leng and Felsenfeld, 1966; Ohba, 1966). Poly-L-arginine was shown in one case (Leng and Felsenfeld, 1966) to prefer G–C-rich DNA. In the case of RNA the results of Sober *et al.* (1966) and Latt and Sober (1967a) agree with our data for one could deduce from our results that not only poly-L-arginine but also poly-L-lysine should exhibit some higher affinity to G–C-rich polynucleotides.

Latt and Sober (1967b) showed that the specificity of the interaction of oligolysines with polynucleotides, in the presence of different cations, is markedly influenced by the nature of the cation. These authors deduced from their results that cations may compete with the charged side groups of the basic polypeptides for the phosphate groups of the polynucleotides and may therefore influence or change the binding specificity of the polynucleotides. Some authors carried out experiments in relatively high salt concentrations, or studied the specificity of interaction between polypeptides and polynucleotides in precipitated complexes; these latter experiments may yield binding specificities different from those experiments carried out in solution (Latt and Sober, 1967b). This may be the reason for the different results.

It is conceivable to assume that any process which leads to the regulation of DNA transcription on the DNA level should imply recognition of bases along the double-stranded DNA molecule. Especially the two grooves of the DNA could provide exposed parts of the bases for such a process. With the results of different authors, including ours, it is reasonable to assume that the exposed parts of guanine and adenine on the DNA surface are different enough to be recognized. Cerami *et al.* (1967) showed that actinomycin requires the amino group at the 2 position of guanine to be bound. Some alkylating reagents react specifically with the N-7 position of guanine which is exposed in the big groove (Brookes and Lawley, 1961). It should be more difficult, however, to discriminate between uracil and cytosine. This could be one of the reasons why, in DNA*t*uracil changed to thymine, the methyl group in the 5 position lies in the middle of the big groove.

In general, our results show that there is some specificity in the interaction between a nucleotide and a basic polypeptide. This specificity may be due to nucleotide stacking and/or to nonelectrostatic affinities between a nucleotide and its binding site superimposed on the major electrostatic interaction.

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The Nonenzymatic Conversion of Tyrosine into Mono- and Dihydroxyindoles*

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ABSTRACT: The reaction of methyl or ethyl tyrosinate, tyrosineamide, and *N*-methylamide in dilute aqueous acetic acid with *N*-bromosuccinimide (NBS) or bromine results first in rapid *o,o'*-bromination and a commensurate increase of the absorption of the phenolic chromophore at 275–280 mμ. A third mole of NBS produces the labile tribromodienone (*e.g.*, XVIII, λ_{max} 270 mμ) which can either revert to the dibromotyrosine level or rearrange to a 5,7-dibromo-6-hydroxyindole (X) resulting from intramolecular Michael addition of the amino group of the tyrosine side chain to the dienone system. At pH 6 this rearrangement proceeds with loss of two bromines to a 5,6-dihydroxy-7-bromoindole (XXV).

Compound XXV was obtained also by the action of NBS on the ethyl ester (XXX) of 3,4-dihydroxyphenylalanine (dopa) with ethyl 5,6-dihydroxy-7-bromo-2,3-dihydroindole-2-carboxylate (XXXI, isolated as the 5,6-diacetate XXXII) as an intermediate or by-product which is spontaneously dehydrogenated to the corresponding indole XXXIII on standing in alkaline solution. Oxidation of dopa ethyl ester XXX by NBS in addition gave a bromine-free product which was isolated as the *O,O,N*-triacetate XXXIV. Mechanisms for these transformations which make possible for the first time the nonenzymatic conversion of tyrosine derivatives into 6-hydroxy- and 5,6-dihydroxyindoles are discussed.

The action of *N*-bromosuccinimide (NBS)[1] on tyrosine-containing peptides and proteins normally results in the scission of the carboxyl amide bond of tyrosine and formation of a spirodienonelactone (V) (Scheme I) with the concomitant release of a new NH_2-terminal peptide (Schmir *et al.*, 1959; Wilson and Cohen, 1963a). This oxidative as well as electrolytic peptide cleavage (Iwasaki *et al.*, 1963; Farber and Cohen, 1966) has been useful in determining or "auditing" primary sequences of proteins independent of enzymatic procedures (Witkop, 1961; Ramachandran and Witkop, 1967). The reaction pathway with NBS has been shown to involve two discrete steps: (i) rapid consumption of 2 moles of NBS and *ortho* bromination of the phenolic moiety to give the 3,5-dibromotyrosyl residue (I); (ii) slower consumption of a third mole of NBS to afford the comparatively stable spirodienonelactone (V) which may arise by either stepwise or concerted nucleophilic attack of the amide carbonyl group of tyrosine upon a labile tribromo intermediate, such as II or III, to yield the unstable iminolactone IV (*cf.* Schmir and Cunningham, 1965; Cunningham and Schmir, 1966), which undergoes spontaneous hydrolysis to the final products.

As judged by ultraviolet spectroscopy the formation

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[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: NBS, *N*-bromosuccinimide; dopa, 3,4-dihydroxyphenylalanine.