Interactions between Polyamines and Nucleotides†

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ABSTRACT: We investigated interactions between naturally occurring polyamines (putrescine, spermidine, and spermine) and nucleotides using an anion-exchange resin method. At pH 6.1, one molecule of any polyamine binds to one molecule of AMP, ADP, or ATP. At pH 7.5, one molecule of putrescine interacts with one molecule of any adenine nucleotide, whereas two molecules of spermidine or spermine can bind one molecule of any nucleotide. Thus, unlike the one-to-one interaction between Mg^{2+} and nucleotide, interactions between polyamines and nucleotides are variable. The multiplicity of the interactions is dependent upon pH and the type of polyamine, indicating that the complex formation between polyamines and nucleotides is affected not only by charge interactions but also by structural features of polyamine molecules. Apparent formation constants for complexes of any polyamine with various nucleoside diphosphates are fairly constant regardless of variations in the base and sugar moieties of the nucleotides. Thus it appears that the phosphate moiety of the nucleotide is the significant determinant for polyamine affinity. When determined at pH 7.5, the apparent formation constant for spermine-AMP (1:1) (K_1 = 360 M^{-1}) was twice as high as that for Mg-AMP; constants for spermine-ADP (1:1) (K_1 = 1250 M^{-1}) and Mg-ADP were close to each other; and the constant for spermine-ATP (1:1) (K_1 = 9500 M^{-1}) was approximately 40% lower than that for Mg-ATP. Thus, it is possible that spermine competes with Mg^{2+} for nucleotides in vivo and, consequently, may have a regulatory role in nucleotide-dependent enzymic reactions by influencing intracellular levels of free and Mg-bound nucleotides.

Since the discovery of polyamines as microbial growth factors (Herbst and Snell, 1948), many studies have shown that the timing and extent of fluctuations in polyamine levels in eukaryotic cells also correlate with changes in the rate of growth (Cohen, 1971; Tabor and Tabor, 1972; Bachrach, 1973; Russell, 1973). Polyamines affect many biological processes such as stimulation of DNA synthesis (Brewer and Rusch, 1966; Schekman et al., 1972), RNA synthesis (Raina and Cohen, 1966; Russell and McVicker, 1973), and protein synthesis (Martin and Ames, 1962; Igarashi et al., 1973). Because the total intracellular concentrations of putrescine, spermidine, and spermine reach millimolar levels (Cohen, 1971; Tabor and Tabor, 1972; Bachrach, 1973; Russell, 1973) and because these polyamines constitute a major portion of multivalent intracellular cations, it is conceivable that interactions between the polyamines and anionic metabolites exist in vivo, that the interactions are widespread, and that they may be responsible for the diverse effects of polyamines.

Nucleotides are involved in many enzymic reactions. It is known that strong interactions exist between Mg^{2+} and nucleotides and that intracellular nucleoside triphosphates exist mostly as Mg complexes, which are presumably the true substrates for enzymes that require these nucleotides. Therefore, it is of particular interest to see whether or not the naturally occurring polyamines—putrescine, spermidine, and spermine—interact with the nucleotides in the way that Mg^{2+} does, and to what extent such interactions might affect the formation of Mg-nucleotide complexes. Results of our present studies indicate that polyamine-nucleotide interactions do exist and that they are strong enough to affect those between Mg^{2+} and the nucleotides.

Experimental Procedures

All nucleotides used in this study were obtained from P-L Biochemicals. Dowex AG1-X2 anion exchange resin (mesh 200–400) was from Bio-Rad Laboratories and was made into the Cl^- form with 1 N HCl by a column method followed by a water wash. The treated resin was kept refrigerated in 100 mM Tris-Cl (pH 7.5) or in 100 mM Pipes[1] (pH 6.1) until used within a 2-week period. Other chemicals, which were of analytical grade or better, were purchased from local commercial sources.

The binding of polyamines and Mg^{2+} to nucleotides was analyzed by an anion-exchange resin method (Walaas, 1958) with some modifications. The test mixture contained 50 mM Tris-Cl (pH 7.5) or Pipes (pH 6.1), 0.1 mM nucleotide, various concentrations (0.1–10 mM) of polyamine or $MgCl_2$, and 2.5 mg (by dry weight) anion-exchange resin in a volume of 2 mL. With occasional mixing, equilibration was complete in 2 h at 30 °C. After the removal of the resin by centrifugation, the amounts of nucleotide remaining in solution were determined by absorption measurement of the supernatant fraction. Assumptions made are that uncomplexed nucleotides exist both free in solution and bound to the resin and that complexed nucleotides stay in solution but are not bound to the resin. The latter was confirmed by the following experiments. One milliliter of 2 N HCl was added to 1 mL of the supernatant fraction (1st Sup) and to the remaining 1 mL of reaction mixture containing resin. The acidified resin mixture was incubated for 1 h at 30 °C with occasional mixing. A 1-mL supernatant fraction (2nd Sup) was removed after centrifugation. Although higher absorption values were obtained for the 2nd Sup than for the 1st Sup due to release of nucleotide from the resin into solution by acidification, no difference in radioactivity was found between the two supernatant fractions when either [^{3}H]spermidine or [^{3}H]spermine (New England Nuclear) was used as a cation. Similarly, no difference in Mg^{2+} contents in the two supernatant fractions was observed when Mg^{2+} was used as a cation and atomic absorption spectroscopy was used for quantitation of this ion. The same observations were also

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[1] Abbreviations used: Pipes, 1,4-piperazine-*N*,*N*′-bis(2-ethanesulfonic acid).

made when no nucleotide was present in the assay media, confirming that no free cation (polyamine or Mg^{2+}) was bound to resin.

Since variable amounts of a cation were added to reaction mixtures containing a fixed level of a nucleotide, the concentration of cation–nucleotide complex as well as that of uncomplexed nucleotide in the mixtures varied among the reaction tubes in which a fixed amount of anion-exchange resin was present. Therefore, it had to be determined whether or not the amount of nucleotide bound to resin was a linear function of free nucleotide in solution. This was done in a separate experiment where the amount of resin added was fixed and the concentration of nucleotide (AMP, ADP, or ATP) was varied between 0.025 and 0.40 mM in the absence of cation. The results from this experiment proved that the amount of nucleotide bound to resin is simply proportional to the concentration of free nucleotide.

We used the following equations, derived as stated in the Appendix, to quantitatively assess the formation of cation–nucleotide complex at equilibrium when Mg^{2+} or polyamine and nucleotide were mixed together in the presence of anion-exchange resin:

$$\frac{1}{K_d} = \frac{1}{K_d^\circ} + \frac{K_1}{K_d^\circ}[M] + \frac{K_1K_2}{K_d^\circ}[M] + \ldots \quad (A)$$

$$\frac{1}{[M]}\left(\frac{K_d^\circ}{K_d} - 1\right) = K_1 + K_1K_2[M] + \ldots \quad (B)$$

where

$$K_1 = [MN]/[M][N]$$

for

$$M + N \rightleftharpoons MN$$

and where

$$K_2 = [M_2N]/[M][MN]$$

for

$$M + MN \rightleftharpoons M_2N$$

K_d is an operational quantity which is determined by dividing the amount of nucleotide bound to resin by the amount of nucleotide remaining in solution. [M] and [N] represent free cation concentration and free nucleotide concentration, respectively. The assumptions made are presented in detail in the Appendix. Some of the assumptions are self-evident and the rest have been verified in the experiments described in the preceding sections or in the Results section. A plot of $1/K_d$ against [M] will give $1/K_d^\circ$ as the intercept at [M] = 0 (primary plot). Then a plot of $(1/[M])[(K_d^\circ/K_d) - 1]$ against [M] will give K_1 as the intercept at [M] = 0, K_1K_2 as the initial slope, etc. (secondary plot). Thus, we used these plots to obtain K_1, K_2, etc.

Results

We tested the anion-exchange resin method for assessing the cation–nucleotide complex formation using Mg^{2+} as cation and AMP, ADP, and ATP as complexing agents at pH 7.5. A plot of data based on eq A (primary plot) gave straight lines with any of the nucleotides over the Mg^{2+} concentration range between 0 and 10 mM. A plot of the same data based on eq B (secondary plot) produced straight lines with any of the nucleotides with a slope of zero, indicating that one magnesium ion binds to one molecule of each nucleotide. The calculated apparent formation constants ($K_f = K_1$) of 160 M^{-1} for Mg-AMP, 1190 M^{-1} for Mg-ADP, and 17 200 M^{-1} for Mg-ATP are close to the previously reported values determined by several different methods (Walaas, 1958; Burton, 1959; O'Sullivan and Perrin, 1964; Martell and Schwarzenbach, 1956; Rudolf and Fromm, 1969). Thus, the anion-exchange resin method is suitable for the study of charge interactions based on complex formation such as exists between Mg^{2+} and nucleotide. Since one of the objectives of this study was to determine whether or not polyamines interact with nucleotides in the way that Mg^{2+} does, we used this anion-exchange resin method for studies with polyamines.

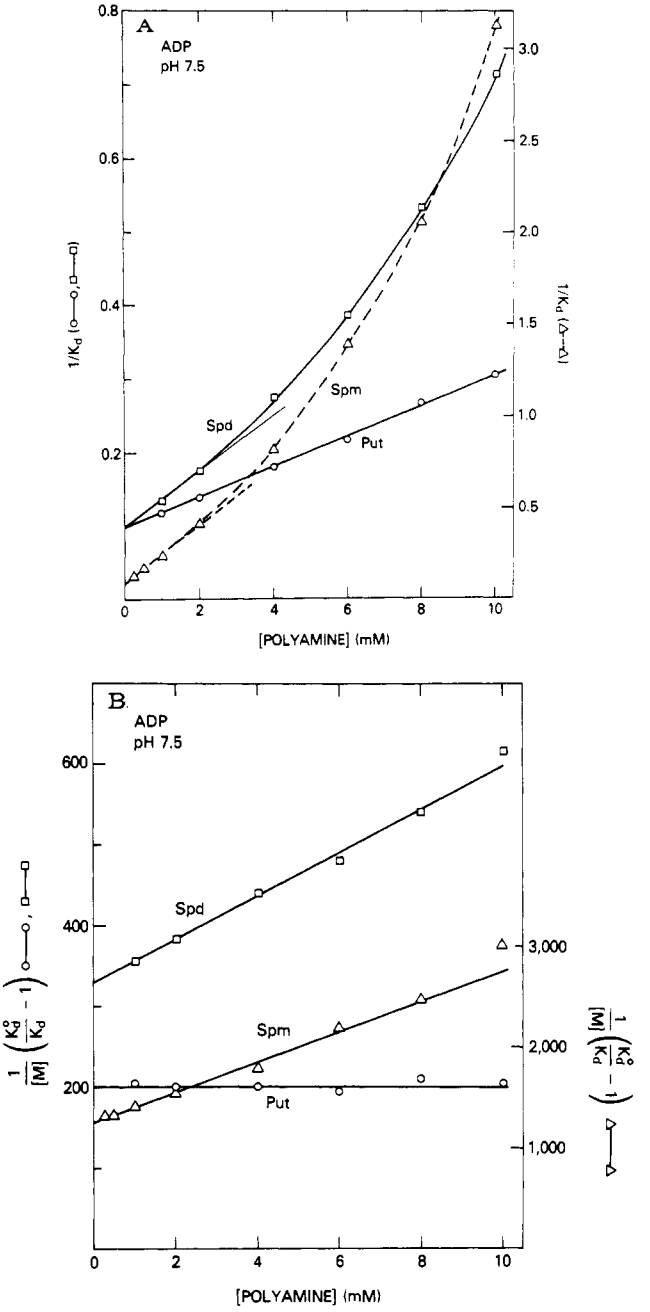

FIGURE 1: Interactions of ADP with naturally occurring polyamines at pH 7.5. Values of $1/K_d$ were determined by anion-exchange resin method as described under Experimental Procedures. (A) Primary plots of data obtained for ADP–polyamine interactions. (B) Secondary plots of the same data used for A. Values of K_d° were estimated from A. [M] represents the concentration of polyamine.

Interactions of nucleotides with polyamines were investigated at pH 7.5 under the same conditions as with Mg^{2+}. As shown in Figure 1A, a primary plot of data obtained with ADP

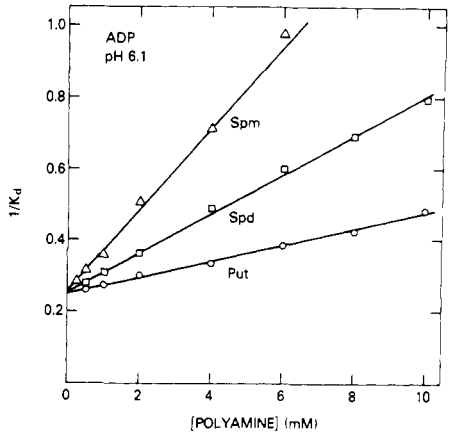

FIGURE 2: Interactions of ADP with naturally occurring polyamines at pH 6.1. Experiments were carried out under the same conditions as those in Figure 1 except that a pH 6.1 buffer was used. Data on the ADP-polyamine interactions are presented as primary plots.

and putrescine gave a straight line over the putrescine concentration range tested (up to 10 mM). The figure also revealed that a primary plot for the interaction of ADP with spermidine or spermine was curvilinear and that the upward curvature increased with increasing polyamine concentrations. A secondary plot of the same data (Figure 1B) yielded a straight line with the slope of zero for the putrescine-ADP interaction, while a secondary plot for the spermidine-ADP or spermine-ADP interaction produced a straight line with a positive slope. The results indicate that M complexed with N successively to give rise to species MN and M_2N, that no further binding of M to M_2N existed, and that a type of complex formation between N and M with species like MN_2 did not occur (see Appendix). The same patterns in these plots were also obtained for the interactions between AMP and polyamines and between ATP and polyamines. Thus, putrescine binds to AMP, ADP, or ATP with a stoichiometry of one to one; and two molecules of spermidine or spermine can combine with one molecule of AMP, ADP, or ATP. It is noted that charges on either polyamine or nucleotide molecules were not completely neutralized in most of these interactions.

Values of K_1 and K_2 were calculated from the secondary plots and summarized in Table I. There is a graded increase in the apparent formation constants (among K_1s or K_2s) for complexes of a given polyamine with AMP, ADP, and ATP. The increment is not directly proportional to the number of negative charges on the nucleotides. Similarly, there is a graded increase in the apparent formation constants (among K_1s or K_2s) for complexes of a given nucleotide with putrescine, spermidine, and spermine. The increment is not directly proportional to the number of positive charges on the polyamines. Among the values of K_1, the K_1 of 82 M^{-1} for putrescine-AMP complex is the lowest and the K_1 of 9500 M^{-1} for spermine-ATP complex is the highest. Interestingly, the values for spermidine-AMP and putrescine-ADP complexes are essentially the same; the values for spermine-AMP, spermidine-ADP, and putrescine-ATP complexes are close to each other; and the values for spermine-ADP and spermidine-ATP fall in the same range. Values of K_2 are 10 to 30% of the values of K_1 for respective complexes, indicating that the affinity of free polyamine for free nucleotide is much higher than that for polyamine-complexed nucleotide.

We further investigated the interactions between polyamines and adenine nucleotides at pH 6.1 in order to see whether differences in charges on the nucleotide molecules affect the

TABLE I: Apparent Formation Constants for Polyamine-Adenine Nucleotide Complexes.[a]

Nucleotide	Constant	Values of constants for nucleotide complexes with polyamines (M^{-1})		
		Putrescine	Spermidine	Spermine
AMP	K_1	82	230	360
	K_2	0	22	38
ADP	K_1	200	330	1300
	K_2	0	82	120
ATP	K_1	290	900	9500
	K_2	0	280	2400

[a] Complex formation between polyamines and adenine nucleotides was assessed at pH 7.5 and values of K_1 and K_2 were calculated from the secondary plots of data as described under Experimental Procedures.

TABLE II: Apparent Formation Constants for Polyamine-Adenine Nucleotide Complexes at pH 6.1.

Nucleotide	Constant	Values of constants for nucleotide complexes with polyamines (M^{-1})		
		Putrescine	Spermidine	Spermine
AMP	K_1	45	80	170
	K_2	0	0	0
ADP	K_1	93	210	450
	K_2	0	0	0
ATP	K_1	140	700	5100
	K_2	0	0	0

complex formation. While each of the nucleotides has one less negative charge[2] at pH 6.1 than at pH 7.5, the number of positive charges on each of the polyamines at pH 6.1 is not different from that at pH 7.5, that is, 2 for putrescine, 3 for spermidine, and 4 for spermine at both pHs. The primary plots of data obtained for interactions between any of the polyamines and any of the nucleotides produced only straight lines with a positive slope (representative data shown in Figure 2). The secondary plots of the same data gave straight lines with zero slope similar to the case for the putrescine-ADP interaction at pH 7.5. The results indicate that stoichiometry of the polyamine binding to nucleotide at pH 6.1 is one to one. Unlike the complex formation at pH 7.5, the binding of a second molecule of spermidine or spermine to a polyamine-nucleotide complex does not occur at pH 6.1. Thus, lowering the pH of the reaction medium leads to a simple one-to-one interaction between the polyamine and the nucleotide; it also causes the weaker interaction as can be seen in Tables I and II; the apparent formation constant (K_1) for any given polyamine-nucleotide complex at pH 6.1 (Table II) is 50 to 60% lower than that at pH 7.5 (Table I).

Recently we reported that naturally occurring polyamines had profound stimulatory and inhibitory effects on the activity of bovine liver nucleosidediphosphate kinase and that the effects were selective with respect to both polyamine and nu-

[2] The pK_a of primary phosphate of all nucleotides is 0.9–1.0, whereas pK_as of secondary phosphates of AMP, ADP, and ATP are 6.1, 6.3, and 6.5, respectively (Pabst Laboratories circular OR-10, 1956). At pH 7.5, therefore, AMP, ADP, and ATP should have 2, 3, and 4 negative charges on phosphate groups, respectively. At pH 6.1, species of one less negative charge exist as a major population of the nucleotides in solution.

cleoside diphosphate substrate (Nakai and Glinsmann, 1977). We were interested in whether the specificity of the effects could be explained by a selective complex formation between a particular type of polyamine and a specific nucleoside diphosphate; that is, we were interested in whether the complex between a given polyamine and a nucleoside diphosphate is affected by differences in the base moiety or the sugar moiety. Therefore, we investigated interactions at pH 7.5 using three naturally occurring polyamines and ten nucleoside diphosphates. The primary plots and the secondary plots of data obtained with any given polyamine and nucleotide produced the same patterns as those seen in Figure 1 for ADP. Interactions are such that one molecule of putrescine combines with one molecule of nucleotide and that two molecules of spermidine or spermine bind to one molecule of the same nucleotide. Values of K_1 and K_2 were determined from the secondary plots and presented in Table III. Values of K_1 for putrescine-nucleoside diphosphate complexes range from 150 to 220 M^{-1}; values of K_1 for spermidine complexes are between 250 and 340 M^{-1} and those of K_2 vary from 45 to 90 M^{-1}; and the apparent formation constants for spermine complexes are in the range of 1150–1350 M^{-1} (K_1) and of 100–170 M^{-1} (K_2). Although there are small variations (8 to 19%) among the K_1 formation constants for complexes of nucleoside diphosphates with a given polyamine, variations in the constants are not consistent with respect to differences in the base and sugar moieties. Substitutions in the base and sugar moieties of the nucleotides have no significant influence on the interactions that can be assessed by the anion-exchange resin method. The consistent stoichiometry of binding and the close K_1 values among the putrescine complexes, among the spermidine complexes, and among the spermine complexes indicate that charge interactions between amino groups of polyamines and phosphate groups of nucleotides play a major role in determining the characteristics and extent of the overall binding.

TABLE III: Apparent Formation Constants for Polyamine-Nucleoside Diphosphate Complexes at pH 7.5.

Nucleotide	Constant	Values of constants for nucleotide complexes with polyamines (M^{-1})		
		Putrescine	Spermidine	Spermine
ADP	K_1	200	330	1250
	K_2	0	82	120
GDP	K_1	170	310	1150
	K_2	0	85	170
CDP	K_1	220	330	1300
	K_2	0	86	140
TDP	K_1	190	310	1350
	K_2	0	82	140
UDP	K_1	200	310	1320
	K_2	0	90	170
dADP	K_1	150	260	1330
	K_2	0	45	110
dGDP	K_1	160	250	1250
	K_2	0	45	100
dCDP	K_1	170	310	1350
	K_2	0	76	130
dTDP	K_1	170	320	1340
	K_2	0	73	150
dUDP	K_1	200	340	1350
	K_2	0	82	160

Discussion

Major objectives of our present study were to assess modes of interactions between polyamines and nucleotides and to determine formation constants for the polyamine-nucleotide complexes. We used an anion-exchange resin method in this study because we were also interested in comparing polyamine-nucleotide interactions with Mg-nucleotide interactions which had been analyzed previously by the same method.

The binding of Mg^{2+} to a nucleotide is strictly one to one and the complex formed is probably nonflexible because Mg^{2+} has a fixed valence conformation. In contrast, interactions between polyamines and nucleotides are variable and multiple, presumably due to the flexible molecular structure of polyamines. At pH 6.1, the stoichiometry of complex formation between putrescine, spermidine, or spermine and AMP, ADP, or ATP is one to one. At pH 7.5, one molecule of putrescine binds to one molecule of nucleotide, whereas two molecules of spermidine or spermine can complex with one molecule of nucleotide. The increase of one negative charge on nucleotide molecules obtained by raising the pH causes spermidine and spermine but not putrescine to bind multiply to nucleotide. Thus, the stoichiometry of the interactions between nucleotides and polyamines is dependent upon pH and the type of polyamine, indicating that it may be determined not only by the number of negative charges on nucleotides relative to that of positive charges on polyamines but also by the distribution pattern of positive charges on polyamine molecules. It is to be noted that pH influences not only the overall stoichiometry of the polyamine-nucleotide interactions but also the affinity of nucleotides for polyamines and that the polyamine-nucleotide interactions do not necessarily neutralize the charges of both cations and anions completely.

It is well accepted that most intracellular nucleotides exist as Mg complexes. It is possible, however, that significant portions of intracellular nucleotides also may be present in sequestered forms with macromolecules such as proteins and with other cations. Complexes of the latter case may in part be with polyamines, because the apparent formation constants for polyamine-nucleotide complexes are high and the concentration of these cations can be in the millimolar range (Cohen, 1971; Tabor and Tabor, 1972; Bachrach, 1973; Russell, 1973). For example, at pH 7.5, the values of K_1 for spermidine-AMP and spermine-AMP are higher than the constant for Mg-AMP; K_1 for spermine-ADP is slightly greater than the constant for Mg-ADP; and K_1 for spermine-ATP is close to the formation constant for Mg-ATP. Thus, these polyamines can compete with Mg^{2+} for complex formation with AMP, ADP, ATP, and other nucleotides. Intracellular polyamine concentrations fluctuate significantly in response to both endogenous and exogenous stimuli (Cohen, 1971; Tabor and Tabor, 1972; Bachrach, 1973; Russell, 1973). The fluctuation likely alters the in vivo levels of polyamine-nucleotide complexes and hence Mg-nucleotide complexes. Since Mg-nucleotide complexes rather than free nucleotides are the true substrates for many enzymic reactions where the nucleotides are involved, naturally occurring polyamines possess great potential for regulating these reactions by complexing with nucleotides. In view of this notion, polyamines were tested for effects on the activity of nucleosidediphosphate kinase and were shown to have significant effects (Nakai and Glinsmann, 1977).

In most of the cases where effects of polyamines were investigated, the polyamines did not serve as a simple replacement for Mg^{2+} or other divalent cations. Unlike the binding of Mg^{2+} to nucleotides, the binding of polyamines to nucleo-

tides appears to be affected not only by charge interactions but also by structural features of polyamine molecules. It is possible that polyamines bind to nucleotides at a site different from that which Mg^{2+} binds to. If so, Mg^{2+} may still bind to some of the polyamine-nucleotide complexes, thereby giving rise to a series of nucleotide species which exhibit different physicochemical as well as biochemical properties from those of polyamine-free Mg-nucleotide complexes. Thus, potentially wide diversity exists for the polyamine-nucleotide interactions which is not present for Mg-nucleotide interactions. Experimental data which support this possibility are that polyamines have profound effects on nucleosidediphosphate kinase reactions in the presence of high concentration of Mg^{2+} and that these effects are selective with respect to the type of polyamines (Nakai and Glinsmann, 1977).

Various studies have described interactions between polyamines and macromolecules, such as nucleic acids and ribosomes (Cohen et al., 1969; Echandi and Algranati, 1975), and the results indicate the presence of definite stoichiometry in certain polyamine-macromolecule complexes (Cohen, 1971; Bachrach, 1973). To our knowledge, however, the present study is the first demonstration that polyamines interact and form complexes with low molecular weight metabolites, e.g., nucleotides. It is conceivable that other metabolites, such as nucleotide sugars, phosphorylated sugars, etc., may interact strongly with these naturally occurring polyamines in vivo and, in turn, may influence the role of these metabolites in various biological processes.

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Appendix

We assume that nucleotide N in solution can bind to resin and that one or more polyamine molecules M can complex with N in solution. We assume further that no M is bound to resin, that the amount of nucleotide bound to resin is simply proportional to the free nucleotide concentration (as in the linear portion of a Langmuir isotherm), and that activity coefficients of all species in solution can be neglected, to a good approximation.

The species in solution have concentrations designated by [M], [N], [MN], [M_2N], [M_3N], etc. Then

$$[N]_t = [N]_r + [N] + [MN] + [M_2N] + \ldots$$
$$[M]_t = [M] + [MN] + 2[M_2N] + \ldots \quad (1)$$

where t = total, r = resin, and the concentrations so labeled are equivalent solution concentrations.

We have the further relation

$$[N]_r = K_d^\circ[N] \quad (2)$$

where K_d° is a constant (which is proportional to the amount of resin present) in view of our assumption above about linear binding.

We define the operational quantity K_d by

$$K_d = [N]_r/([N]_t - [N]_r)$$
$$K_d \text{ approaches } K_d^\circ \text{ when } [M]_t \to 0 \quad (3)$$

The complexing of M to N, in successive steps, is represented by

$$K_1 = [MN]/[M][N]$$
$$K_2 = [M_2N]/[M][MN] \quad (4)$$
$$K_3 = [M_3N]/[M][M_2N]$$

etc.

If we now substitute eq 1, 2, and 4 into eq 3, we find

$$1/K_d = (1 + K_1[M] + K_1K_2[M]^2 + K_1K_2K_3[M]^3 + \ldots)/K_d^\circ \quad (5)$$

A plot of $1/K_d$ against [M] would start linearly, with positive slope, but then curve upward as [M] increases (i.e., the second derivative is positive).

We digress to mention that, if N were to complex to M, with species M, N, NM, N_2M, etc., then a similar analysis shows that a plot of $1/K_d$ against [M] would start linearly but then curve downward.

For practical purposes, it is convenient to rearrange eq 5 as follows:

$$\frac{1}{[M]}\left(\frac{K_d^\circ}{K_d} - 1\right) = K_1 + K_1K_2[M] + K_1K_2K_3[M]^2 + \ldots \quad (6)$$

A plot of the left-hand side of this equation against [M] will give K_1 as the intercept at [M] = 0, K_1K_2 as the initial slope, etc. Thus, this plot can be used to obtain K_1, K_2, K_3, etc. This is analogous to a plot of Π/c against c in osmotic pressure work.

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Functional Rhodopsin Complex Consisting of Three Noncovalently Linked Fragments†

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ABSTRACT: A simple method for the routine preparation of both bovine rod outer segments and rhodopsin of good purity in exceptionally high yields is described. Incubation of rod outer segment disc membranes with the proteolytic enzyme, papain, resulted in the production of a complex in which three fragments of apparent molecular weights 23 000, 15 500, and 6000, referred to as H, M, and L (heavy, medium, and light) fragments, were identified. The H fragment was reduced by 2-mercaptoethanol to give a mixture of polypeptides of molecular weight 9000–4000 showing that it contained the disulfide bond(s) of rhodopsin. To investigate the relationship of the three fragments to the native rhodopsin molecule, several strategic sites on the rhodopsin molecule were specifically labeled. The active site was labeled with 11-*cis*-[15-^{3}H]retinal and SH groups with *N*-ethyl[2,3-^{14}C]maleimide or iodo[^{3}H]-acetamidosalicylate. The L fragment contained an SH group which was modified by either *N*-ethyl[2,3-^{14}C]maleimide or iodo[^{3}H]acetamidosalicylate while the H fragment contained an SH group which was modified only by *N*-ethyl[2,3-^{14}C]-maleimide. The M fragment contained the retinal binding site. The three fragments arose without any significant loss in amino acid residues from rhodopsin and they were inseparable when the digested rod outer segments were solubilized in the detergent lauryl dimethylamine *N*-oxide and then chromatographed on calcium phosphate. The characteristic spectral, bleaching, and regeneration properties of native rhodopsin were fully maintained in cleaved rhodopsin. Assuming that papain can only digest those parts of the rhodopsin molecule that are normally exposed to the aqueous phase, then the mode of cleavage of rhodopsin yielding, after thiolysis, at least five large polypeptides favors a model in which the rhodopsin polypeptide chain alternately threads in and out of the membrane.

Rhodopsin, the photoreceptor protein of bovine retinae, consists of a glycoprotein opsin covalently bonded to 11-*cis*-retinal by a Schiff base linkage (Akhtar et al., 1965, 1967, 1968; Bownds, 1967). Light activation photoisomerizes the retinal chromophore from the 11-cis to the all-trans form (Wald, 1968) and this initiates spectrally defined changes in the visual pigment and concomitant protein conformational changes that may alter the membrane conductance leading to visual excitation. In order to describe these processes in molecular terms a knowledge of the structure of rhodopsin and its vectorial organization in the membrane is required.

A number of physicochemical approaches are currently being used to determine the molecular organization of rhodopsin in photoreceptor membranes. One such technique which is specifically aimed at pin-pointing regions of rhodopsin that protrude from the membrane into the aqueous environment involves the limited proteolysis of rod outer segments (ROS)[1] (Bonting et al., 1974; Daemen et al., 1974; Trayhurn et al., 1974a,b; Saari, 1974; Pober and Stryer, 1975; van Breugel et al., 1975; Klip et al., 1976; Towner et al., 1977). We have now subjected ROS, radioactively labeled at various strategic sites on the rhodopsin molecule, to papain treatment and have produced a complex consisting of at least three noncovalently linked fragments which retains many of rhodopsin's characteristic properties. The cleavage mode sheds light on selected aspects of the structure of rhodopsin as it exists in the disc membrane.

Experimental Section

Materials. Fresh cattle eyes were obtained from a local slaughter house; the retinae were immediately removed and stored at −18 °C in the dark. Tween 80 and papain (2× crystallized) were purchased from Sigma Chemical Co., Kingston-upon-Thames, Surrey KT2 7BH, U.K. Ammonyx-LO (a 30% solution of mostly lauryl dimethylamine *N*-oxide) was obtained from Venture Chemicals, Reading, Berks, U.K. Butyl-PBD [5-(4-biphenyl)-2-(4-*tert*-butylphenyl)-1-oxa-3,4-diazole] and NE260 were purchased from Koch-Light Laboratories, Colnbrook, Bucks, U.K., and Nuclear Enterprises, Sighthill, Edinburgh, Scotland, U.K., respectively. NCS tissue solubilizer was obtained from Hopkins and Williams, Chadwell Heath, Essex, U.K. NaB^3H_4, iodo[^{3}H]acetic acid, and *N*-ethyl[2,3-^{14}C]maleimide were purchased from the Radiochemical Centre, Amersham, Bucks, U.K. All other chemicals were reagent grade. Iodo[^{3}H]acetamidosalicylate was synthesized according to the procedure of Holbrook et al. (1973).

Preparation of Rod Outer Segments (ROS). ROS were isolated by modification of the procedure of McConnell (1965). All appropriate operations were carried out under dim red light in a cold room at 4 °C. The buffer solution was 0.067 M potassium phosphate, pH 7. All sucrose solutions contained the buffer.

Retinae (100–200) were thawed, homogenized by two passes

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[1] Abbreviations used: ROS, rod outer segments; [^{3}H]retinal-labeled ROS, rod outer segments labeled with 11-*cis*-[15-^{3}H]retinal; butyl-PBD, 5-(4-biphenyl)-2-(4-*tert*-butylphenyl)-1-oxa-3,4-diazole.