A Gel Chromatographic Procedure That Corrects for Donnan Effects in Studies of Ligand Binding: Its Application to the Interaction of Copper Ions with Bovine Serum Albumin and Deoxyribonucleic Acid†

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ABSTRACT: The binding of copper ion to bovine serum albumin and deoxyribonucleic acid has been studied by gel chromatography on Sephadex G-25, using a procedure that has been developed in order to make allowance for the Donnan redistribution of small ions between the mobile and stationary phases of the column. A study of copper binding to bovine serum albumin in 0.02 *I* acetate (pH 4.0) is used to illustrate the feasibility of making these corrections for Donnan effects. No redistribution of small ions is detected in gel chromatography of DNA–Cu^{2+} mixtures in 5 mM KNO_3. The results obtained for this system compare favorably with data obtained by direct estimation of free ligand with a Cu^{2+}-specific electrode. Both experimental techniques indicate a maximum of 0.5 binding site/nucleotide residue, the strength of binding being enhanced substantially by heat denaturation of the Cu^{2+}–DNA solutions.

The binding of Cu^{2+} ions to DNA produces a marked decrease in the thermal stability of the macromolecule, the main features of the Cu^{2+}–DNA system being well established by the work of Eichhorn (1962), Eichhorn and Clark (1965), Hiai (1965), and Venner and Zimmer (1966). More recent studies have been concerned with determination of the number of interacting sites and of the stability constants of the complexes formed (see Bach and Miller, 1967; Bryan and Frieden, 1967; Zimmer *et al.*, 1971, and references quoted therein); a variety of different experimental techniques have been used.

In the present investigation the binding of Cu^{2+} to DNA in 5 mM KNO_3 is studied by gel chromatography on Sephadex G-25, a technique employed previously by Bryan and Frieden (1967) to study the same interaction. However, a disadvantage of existing gel chromatographic methods for the study of ligand binding (Hummel and Dreyer, 1962; Nichol and Winzor, 1964; Cooper and Wood, 1968) is that any Donnan redistribution of small ions resulting from confinement of the macroion to the mobile phase of the gel column is not taken into account. A modified procedure is described here which corrects for these Donnan effects. The feasibility of so doing is illustrated with a study of copper binding to bovine serum albumin in 0.02 *I* acetate (pH 4.0), a system exhibiting a very pronounced redistribution. Results obtained with the Cu^{2+}–DNA system are then presented and compared with data obtained by Cu^{2+} ion potentiometry.

Materials and Methods

Preparation of DNA Solutions. Bacterial DNA was isolated from *Escherichia coli* K12 by the method of Marmur (1961) and then precipitated twice from phenol solutions in order to remove traces of protein. Stock solutions approximately 1 mM with respect to phosphate (10^{-3} M P) were prepared by addition of the DNA to 5 mM KNO_3 and gentle, continuous agitation of the solution at 4°. After 7 days any undissolved DNA was removed by centrifugation at 18,000*g* for 1 hr. Spectrophotometric grade chloroform was then added (to give *ca.* 0.2%) to the supernatant before storage at 4°.

Immediately prior to use for binding studies, the stock solutions were suitably diluted with 5 mM KNO_3 and the concentration of the resultant solutions determined spectrophotometrically using a molar extinction coefficient ϵ(P) of 6740 at 260 nm (Mahler *et al.*, 1964). Copper ion potentiometry indicated the presence of negligibly small copper concentrations ($<10^{-7}$ M) in these solutions of DNA, the solvent having been prepared from reagent grade KNO_3 and glass-distilled, deionized water.

Preparation of Albumin Solutions. Crystalline bovine serum albumin, obtained from the Commonwealth Serum Laboratories, Melbourne, Australia, was dissolved in 0.02 *I* acetate (pH 4.0) (0.02 M potassium acetate, pH adjusted with acetic acid). Concentrations of the albumin solutions (*ca.* 3%) were then determined spectrophotometrically at 280 nm on suitably diluted aliquots; an extinction coefficient ($E_{1\,cm}^{1\%}$) of 6.6 (Kronman and Foster, 1957) was used.

Gel Chromatography. Basis of the Modified Procedure for Studying Ligand Binding. The binding of copper ions to a macromolecular acceptor has been studied by a gel chromatographic procedure based on that of Hummel and Dreyer (1962), which entails preequilibration of a gel column with a specified concentration of ligand. The gel type was selected such that acceptor and complex(es) are excluded from the stationary phase in order to ensure the identity of their rates of migration. Addition of a small volume of acceptor and subsequent elution with more of the preequilibrating ligand solution yields the profile shown schematically in Figure 1a. The increase in total ligand concentration coinciding with the acceptor peak at V_A reflects the binding of ligand, whereupon the amount bound may be calculated by trapezoidal integration to find the area of this peak, or, alternatively, of the corresponding negative peak that occurs at V_L, the elution volume

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of ligand (not shown). We have obviated the need for integration by increasing the volume of the applied zone of acceptor sufficiently for the elution profile to contain a plateau region instead of a peak (see Figure 1b). The concentration of bound ligand is then obtained simply as the difference between this plateau value, $(\bar{m}_L^{\alpha})_m$, and the preequilibrating concentration, $(m_L^{a})_m$; these concentrations are denoted as mobile phase concentrations (m subscript) since an elution profile records the composition of the mobile phase at the exit plane of the column (Nichol *et al.*, 1967).

A disadvantage of both of these methods, or, indeed, of the frontal gel chromatographic procedure (Nichol and Winzor, 1964; Cooper and Wood, 1968; Nichol *et al.*, 1971) is the assumed identity of the free ligand concentrations in the mobile and stationary phases of a column, an assumption inherent in the use of the preequilibrating ligand concentration $(m_L^{a})_m$ for the concentration of free ligand in the α-plateau region. The problem, therefore, is to devise a procedure that avoids this assumption, since it implies the absence of any significant Donnan redistribution of small ions across the semipermeable gel membrane.

Consider the second type of experiment (Figure 1b), involving the migration of an extended zone of charged macromolecular acceptor on a gel column preequilibrated with a known concentration $(m_L^{a})_m$ of ionized ligand L. Initially, the free ligand concentration in the vicinity of the acceptor zone changes from its original value because of complex formation and also because of Donnan redistribution. However, provided that the complex acceptor zone migrates down a sufficiently long column, the situation is reached eventually where the change in free ligand concentration resulting from complex–acceptor migration is due *solely* to Donnan effects: under these circumstances the total amount of *free* ligand in a given segment of column remains unchanged. On the basis that the gel chromatographic distribution coefficient, K_D (Gelotte, 1960), reflects the fraction of stationary phase that is accessible to solute, we may write an expression for the amount of free ligand contained in a narrow segment of column in which the total volume accessible to ligand is ΔV.

Prior to the arrival of the α plateau at some fixed point in the column, the amount of ligand contained in the segment is $\Delta V(m_L^{a})_m$, since in the absence of acceptor the concentrations of ligand in the mobile and stationary phases are identical $[= (m_L^{a})_m]$. This situation exists again after the α plateau has proceeded beyond the point under consideration; hence the concentration of ligand reverts to $(m_L^{a})_m$ after elution of the complex–acceptor plateau and maintains this value until the volume V_L is reached, at which stage the ligand concentration decreases. When the point under consideration is contained within the α plateau the total amount of free ligand is expressed as the sum of the amounts in the mobile and stationary phases. Since the ratios V_A/V_L and $(V_L - V_A)/V_L$ represent the fractions of the volume increment that are the mobile and the stationary phases, respectively, it follows that

$$(m_L^{a})_m = (m_L^{\alpha})_m(V_A/V_L) + (m_L^{\alpha})_s(V_L - V_A)/V_L \quad (1)$$

Subscripts m and s denote mobile and stationary phases, respectively.

Equation 1 still contains two quantities of undetermined magnitude, *viz.*, $(m_L^{\alpha})_m$ and $(m_L^{\alpha})_s$, but by subjecting the α-plateau mixture to frontal gel chromatography under the same conditions, the concentration of ligand in the stationary phase, $(m_L^{\alpha})_s$, is obtained directly from the size of the more slowly migrating boundary on the trailing side (Nichol *et al.*, 1969). The concentration of free ligand in the mobile phase, $(m_L^{\alpha})_m$, may then be obtained from eq 1, and hence the concentration of bound ligand determined by difference between this quantity and $(\bar{m}_L^{\alpha})_m$, the total concentration of ligand (free and complexed) observed in the α-plateau region of the elution profile.

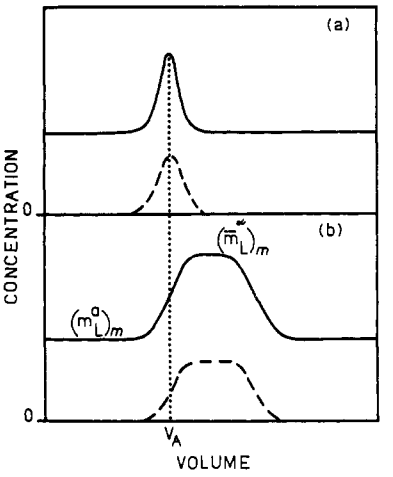

FIGURE 1: Schematic representation of two gel chromatographic methods of investigating ligand-acceptor interactions: (a) the Hummel and Dreyer (1962) procedure; (b) a proposed method. In each case the solid line refers to the ligand profile and the broken line to acceptor concentration.

Gel Chromatography of Albumin–Copper Mixtures. 3% bovine serum albumin (35 ml) in 0.02 *I* acetate (pH 4.0) was applied to a 2.1 × 54 cm column of Sephadex G-25, preequilibrated with a specified concentration of copper $[(m_L^{a})_m]$ dissolved in the acetate buffer; this copper solution was also used to elute the protein from the column, the flow rate of which was maintained at 40 ml/hr. The eluate was divided into 0.8-ml fractions, which were analyzed for protein and copper contents; spectrophotometric measurements at 280 nm were used for the former, while copper concentrations were determined by the cuprethol method (Woelfel, 1948), which had been shown to yield the *total* copper content of albumin–copper mixtures. By combining the remnants of fractions that definitely represented the α-plateau region (Figure 1b), 9 ml of protein–copper mixture was obtained from such an experiment.

Albumin–copper mixture (18 ml) obtained from duplicate runs of the above type was subjected to frontal gel chromatography on a 1.2 × 14.7 cm column of Sephadex G-25, preequilibrated with 0.02 *I* acetate buffer containing the same specified copper concentration $(m_L^{a})_m$ as before; in this instance copper was omitted from the acetate buffer used to develop the descending (trailing) side of the elution profile. A flow rate of 12 ml/hr was used with this column, and the eluate was monitored by the cuprethol procedure.

Gel Chromatography of DNA–Cu^{2+} Mixtures. In experiments designed to check for the existence of any significant Donnan effect with the DNA–Cu^{2+} system, 20 ml of DNA (1.8×10^{-4} M P) in 5 mM KNO_3 was preequilibrated with 0.15 mM Cu^{2+} and applied to a 1.4 × 70 cm column of Sephadex G-25 that had been preequilibrated with 5 mM KNO_3 containing 0.1 mM Cu^{2+}; the higher concentration of ligand in the applied mixture was chosen so that the concentration of free copper in the mixture was in the vicinity of $(m_L^{a})_m$. Column flow rate throughout the application of the sample and also the subsequent elution with 5 mM KNO_3–0.1 mM Cu^{2+} solution was maintained at 20 ml/hr. The column effluent was monitored continuously at 254 nm in order to obtain the DNA

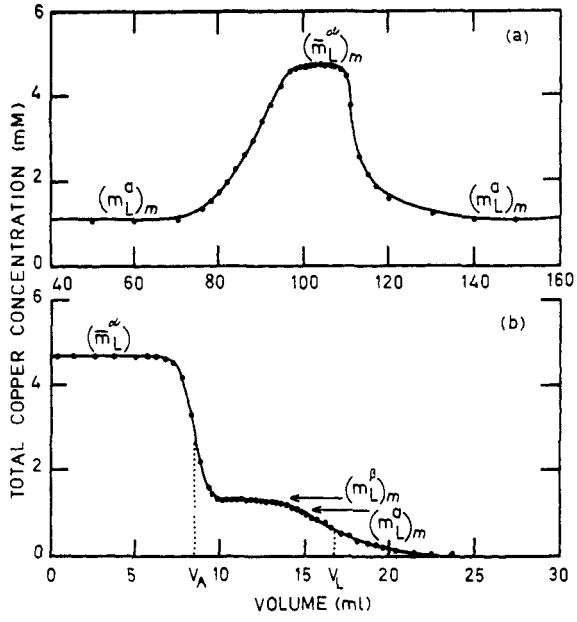

FIGURE 2: Elution profiles obtained in a gel chromatographic study of the binding of copper to bovine serum albumin in 0.02 *I* acetate (pH 4.0): (a) the extended zone experiment in which 3% albumin (35 ml) was applied to a 2.1 × 54 cm column of Sephadex G-25 preequilibrated with 0.02 *I* acetate (pH 4.0) containing 1.12 mM copper; (b) the trailing side of the elution profile in a frontal experiment in which the α-plateau mixture from (a) was applied to a 1.2 × 14.7 cm column of Sephadex G-25 preequilibrated with the acetate–1.12 mM copper solution (see the text).

elution profile, and then collected in 1-ml fractions, which were analyzed for copper by the cuprizon method (Somers and Garraway, 1957): total copper in DNA–Cu^{2+} mixtures is obtained by this procedure. Fractions corresponding to the α-plateau region were pooled and added to the corresponding plateau material from a duplicate run. The resulting DNA–Cu^{2+} mixture (20 ml) was then applied to a 1.2 × 14.7 cm column of Sephadex G-25, preequilibrated with 5 mM KNO_3–0.1 mM Cu^{2+}, and eluted with the same solution. A flow rate of 12 ml/hr was used with this column, and the eluate again was monitored for copper by the cuprizon procedure.

Experiments were also performed in which the frontal run was omitted. Known amounts of Cu^{2+} were added to DNA solutions (1×10^{-4} MP) in 5 mM KNO_3, some of these mixtures being denatured by heating at 75° for 10 min before being subjected to gel chromatography. A smaller (1.2 × 45 cm) column of Sephadex G-25 was then used, and the volume of the applied zone decreased proportionately to 10 ml.

Cu^{2+} Ion Potentiometry. Measurements of the free Cu^{2+} ion concentration in solutions containing both Cu^{2+} ions and DNA (either native or heat denatured in the presence of Cu^{2+} ions) were made using an Orion specific cupric ion activity electrode Model 94-29 in conjunction with a Radiometer K100 flow-through calomel reference electrode. Full details of the method have been described elsewhere (Holman and Jordan, 1972).

TABLE I: Gel Chromatographic Evaluation of the Extent of Binding of Copper to Bovine Serum Albumin in 0.02 *I* Acetate (pH 4.0).

Protein Concn[a] (M × 10^{-4})	Ligand Concn (mM)					
	$(m_L^a)_m$	$(\bar{m}_L^\alpha)_m$	$(m_L^\alpha)_s$	$(m_L^\alpha)_m$	r	r_{app}
4.48	1.12	4.70	1.37	0.78	8.8	8.0
4.48	0.60	3.30	0.75	0.40	6.5	6.0

[a] Molecular weight of bovine serum albumin taken as 67,000 (Squire *et al.*, 1968).

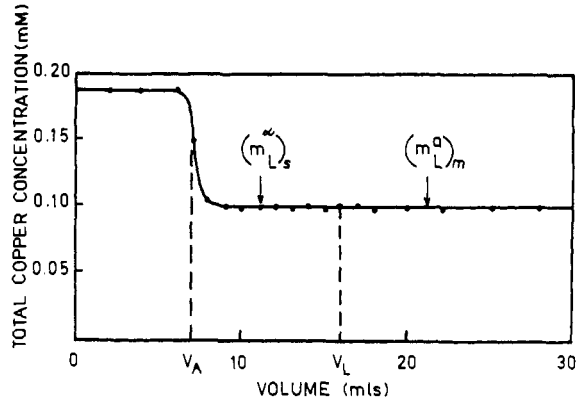

FIGURE 3: Trailing side of the elution profile obtained in frontal gel chromatography of the α-plateau mixture from an extended zone run on 1.8×10^{-4} MP DNA in the presence of 5 mM KNO_3–0.1 mM Cu^{2+}, which was also used to elute the DNA–Cu^{2+} mixture from the column (see the text).

Results

Albumin–Copper Interaction. Elution profiles obtained in gel chromatography of 3% bovine serum albumin on Sephadex G-25 columns preequilibrated with 1.12 mM copper solution are shown in Figure 2. The upper half (Figure 2a) illustrates the creation of the α plateau, in which the *total* copper concentration has increased approximately fourfold over the preequilibrating value; the finding that the copper concentration reverts to $(m_L^a)_m$ after elution of the albumin–copper zone establishes the fact that the α plateau is, indeed, in chemical and Donnan equilibrium with the preequilibrating concentration of copper. Figure 2b shows the trailing side of the elution profile obtained in the frontal gel chromatographic experiment on the α-plateau mixture; the concentration of ligand separating as the β plateau reflects its level in the stationary phase of the α region (Nichol *et al.*, 1969), *i.e.*, $(m_L^\beta)_m = (m_L^\alpha)_s$. The fact that this value differs markedly from $(m_L^a)_m$ signifies the existence of a pronounced Donnan effect.

Results of this experiment, together with a second employing a lower copper concentration, are summarized in Table I to permit quantitative assessment of the magnitude of the Donnan redistribution and of its effect on the binding function r (Klotz, 1946). The second and third columns list the preequilibrating and α-plateau concentrations, respectively, while the values given in column 4 refer to concentrations in the β plateaux of the frontal experiments. The fifth column tabulates the values of $(m_L^\alpha)_m$ calculated from eq 1 with values of 78 and 180 ml for V_A and V_L, respectively. Magnitudes of the binding function r calculated from $(\bar{m}_L^\alpha)_m$ and $(m_L^a)_m$ are given in the second last column, while the final column lists the apparent values obtained by assuming the identity of $(m_L^\alpha)_m$ and $(m_L^a)_m$.

DNA–Cu^{2+} Interaction. Figure 3 presents the trailing side of the elution profile obtained in the frontal gel chromatographic experiment on an α-plateau mixture of DNA and Cu^{2+} ions. In contrast with the result shown in Figure 2b there is no discernible difference between $(m_L^\beta)_m$ and $(m_L^a)_m$, and hence

the extent of the Donnan redistribution of Cu^{2+} ions is sufficiently small to have escaped experimental detection under these conditions (DNA–Cu^{2+} in 5 mM KNO_3). Accordingly, the difference between $(\bar{m}_L{}^{\alpha})_m$ and $(m_L{}^{s})_m$ was taken as the concentration of complexed Cu^{2+} in subsequent experiments.

The results of gel chromatographic binding studies on the DNA–Cu^{2+} system in 5 mM KNO_3 are denoted by open symbols in Figure 4, which presents the data in Scatchard (1949) form: circles refer to the interaction between Cu^{2+} and native DNA, and the triangles to DNA–Cu^{2+} mixtures that had been heat denatured at 75° for 10 min. Also shown in Figure 4 are the results of Cu^{2+} ion potentiometric studies on DNA–Cu^{2+} mixtures, the closed circles and closed triangles relating to experiments with the native and heat-denatured DNA samples, respectively.

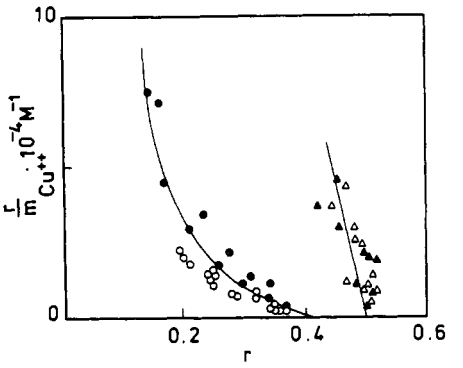

FIGURE 4: Scatchard plots of binding results obtained by gel chromatography and Cu^{2+} ion potentiometry with the DNA–Cu^{2+} system in 5 mM KNO_3: (○) native DNA (gel chromatography); (●) native DNA (potentiometry); (△) denatured DNA–Cu^{2+} mixtures (gel chromatography); (▲) denatured DNA–Cu^{2+} mixtures (potentiometry).

Discussion

Figure 2 and Table I serve to illustrate a gel chromatographic procedure whereby Donnan effects may be taken into account in the quantitative characterization of the binding of charged ligands to macroionic acceptors. Previously it has been necessary to neglect this correction, and thus studies of relatively weak ion binding to highly charged polyelectrolytes at low ionic strength have generally been avoided. In the cases of the albumin–copper and DNA–Cu^{2+} interactions under investigation, the ligand is contributing negligibly to the ionic strength of either system, which is provided by uni-univalent salt (potassium acetate or potassium nitrate). For such cases the Donnan redistribution may be expressed (Svensson, 1946) as

$$\left[\frac{(m_L{}^{\alpha})_m}{(m_L{}^{\alpha})_s}\right]^{1/z_L} = \frac{(m_K{}^{\alpha})_m}{(m_K{}^{\alpha})_s} = \left\{1 - \frac{z_A(\bar{m}_A{}^{\alpha})_m}{2(I^{\alpha})_m}\right\} \qquad (2)$$

where z_L and z_A are the valences of the charged ligand and macroion, respectively, and $(\bar{m}_A{}^{\alpha})_m$ refers to the total molar concentration of acceptor; $(I^{\alpha})_m$ is the ionic strength of the mobile phase in the α-plateau region and thus equals the concentration of potassium ion $(m_K{}^{\alpha})_m$ for the present systems. From eq 2 it is evident that the acceptor valence, its concentration, and also the ionic strength largely determine the magnitude of the Donnan effect. These factors dictated the choice of conditions for the present study of the albumin–copper system, which involved high concentrations of protein at low ionic strength and a pH at which uncomplexed bovine serum albumin bears a net charge of approximately +28 according to the titration curves of Tanford *et al.* (1955). Of the nine copper atoms that on average are bound per molecule of albumin in the first experiment listed in Table I, approximately six would be present as $[CuOAc]^+$ and three as Cu^{2+}, assuming equal binding affinities for the two copper species (Reynolds *et al.*, 1973) and a value of 150 M^{-1} for the association constant describing the interaction between Cu^{2+} and acetate (Bale *et al.*, 1957); thus an averaged valence in the vicinity of +40 is predicted for the acceptor on the basis of conservation of charge. The existence of free ligand in two charged forms clearly precludes exact prediction of the expected difference between $(m_L{}^{\alpha})_m$ and $(m_L{}^{\alpha})_s$, but approximate calculations indicate the experimental results to be eminently reasonable. A point of interest is the observation (Table I) that an error of about 50% in the free ligand concentration has led to an error of only 10% in r; however, this is not to be regarded as a general observation, since it only reflects the relative magnitudes of $\{(\bar{m}_L{}^{\alpha})_m - (m_L{}^{\alpha})_m\}$ and $\{(\bar{m}_L{}^{\alpha})_m - (m_L{}^{s})_m\}$. Furthermore, since evaluation of the number of binding sites per acceptor molecule and the equilibrium constant also requires a value of the free ligand concentration, the 50% error in the latter quantity would be the major source of error.

Failure to detect any significant Donnan redistribution of ligand with the Cu^{2+}–DNA system (Figure 3) is, in fact, also to be expected on the basis of eq 2. Even in the extreme case of there being no counterion-screening effect on the DNA phosphate charges or on the bound metal ion charges (Daune *et al.*, 1966; Miller and Bach, 1968), the concentrations of Cu^{2+} in the mobile and stationary phases should differ by considerably less than 1%; since screening reduces the effective charge per phosphate residue in native DNA to approximately −0.2 under the present conditions (Mathieson and Matty, 1957), the magnitude of this difference would be decreased still further. Clearly, such concentration changes cannot be detected experimentally, and hence Donnan effects may be neglected with the DNA–Cu^{2+} system in 5 mM KNO_3.

Further confirmation of the above prediction of the magnitude of Donnan effects in gel chromatography of DNA–Cu^{2+} mixtures under these conditions is the agreement between results obtained by the column method and those involving the direct estimation of free Cu^{2+} potentiometrically (Figure 4). Both techniques indicate a maximum of 0.5 for r with either native or heat-denatured DNA, with the strength of ligand binding much greater in the latter case. Thus we conclude that Donnan effects cannot be detected experimentally in gel chromatographic studies of the DNA–Cu^{2+} system under these conditions (I = 0.005), and should not, therefore, have been regarded as a potential source of error in quantitative studies of Cu^{2+} binding by this method (Bryan and Frieden, 1967).

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Cupric Ion–Adenosine Triphosphate System. Proton Magnetic Resonance Line-Broadening Studies†

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ABSTRACT: An investigation of Cu^{2+}-induced broadening of the H_8 and H_2 signals in the proton magnetic resonance (pmr) spectra of the Cu(II)–ATP and Cu(II)–AMP systems, containing very low Cu^{2+} concentrations ($\leqq 10^{-4}$ M) and very low Cu:nucleotide ratios ($2–4 \times 10^{-4}$) with D_2O as solvent, has been carried out. The pD dependences and concentration dependences of these line broadenings were correlated with the changing distribution of the various species in hydrolytic equilibrium in the ranges pD 4.4–10.4 and 0.02–0.25 M. The results indicate that 1:1 complexation, with backbonding, predominates in the Cu(II)–ATP system at a pD $\leqq$ 5.4, where only unhydrolyzed complexes exist, when the ATP concentration is 0.02 M, but that at 0.25 M ATP a considerable fraction of the backbound unhydrolyzed complexes have 1:2 stoichiometry because of ring stacking of the two adenine groups. Only 1:1 stoichiometry seems to prevail for the hydrolyzed species, *i.e.*, $CuATP(OD)^{3-}$ and $CuATP(OD)_2^{4-}$, even at the higher nucleotide concentration. Although backbonding is evident for the monohydroxy complex, it is relatively insignificant for the dihydroxy monomer. Since there should be even less tendency for backbonding in the diol-dimer, $[CuATP(OH)]_2^{6-}$, than in $CuATP(OH)_2^{4-}$, because of the tendency for adenine-ring stacking in the dimer, we believe that these results satisfy one of the requirements of the recent theory proposed by Feldman (*Jerusalem Symp. Quan. Chem. Biochem. IV*, 528 (1972)) for the mechanism of metal ion catalysis of ATP dephosphorylation, namely, that Cu^{2+} is *not* bound to the adenine group of the active species.

Because of the very great catalytic effect of the cupric ion on the nonenzymatic dephosphorylation of adenosine 5′-triphosphate (ATP) (Tetas and Lowenstein, 1963), the Cu(II)–ATP system has been investigated in many laboratories. Spiro *et al.* (1968) concluded that the ground-state active species for this reaction is the diolated dimer, $ATPCu(OH)_2$-CuATP, which had previously been shown by Taqui Khan and Martell (1962a) to be coexistent in the pH 5–9 region with several monomeric complex species, $CuATP^{2-}$, $CuATP(OH)^{3-}$, and $CuATP(OH)_2^{4-}$. Claims that Cu^{2+} is attached to the adenine moiety in the active species (Schneider and Brintzinger, 1964; Miller and Westheimer, 1966) have been disputed by Feldman (1972). Previous demonstrations of the simple fact that Cu^{2+} changes the infrared (ir) spectrum (Schneider and Brintzinger, 1964) and the proton magnetic resonance (pmr) spectra (Eichhorn *et al.*, 1966) of adenine nucleotides do not relate to this particular question, because of the multispecies composition of the system.

We have carried out a study of the pH dependence of the Cu^{2+}-induced broadening of the pmr signals of the adenine moiety of ATP, since complexation of the paramagnetic Cu^{2+} ion by the adenine moiety should be detectable by such line broadening (Swift and Connick, 1962) and since this

† From the Department of Radiation Biology and Biophysics, University of Rochester School of Medicine and Dentistry, Rochester, New York 14642. *Received September 21, 1973.* This paper is based on work supported in part by the U. S. Public Health Service Training Grant No. DE-00175 and in part by the U. S. Atomic Energy Commission Contract AT-11-1-3490 and has been assigned Report No. UR-3490-380. Presented in part at the 162nd National Meeting of the American Chemical Society, Washington, D. C., Sept 1971. This work is part of the Ph.D. Thesis of V. W., University of Rochester, 1973.

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