In the case of bovine serum albumin, this possibility is lively indeed. Sharp single bands were, however, obtained with myosin, H-meromyosin and lysozyme. Multiple bands were observed with all of the other proteins studied, but in each of these latter cases, excepting only that of BSA, there was reason to doubt the homogeneity of the protein sample. The question as to how commonly a single protein species will give more than one band of precipitate requires further study, but the method deserves closer examination as a criterion of the homogeneity of protein samples.

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# The Preferential Hydration of Proteins in Concentrated Salt Solutions. III. Theoretical[1]

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Equations are developed showing the relation between the binding of ions by macromolecules and preferential hydration. It is suggested that the hydration of the salt ions in solution is the primary factor responsible for the preferential hydration of the macromolecule.

Equations governing the interaction between ions and macromolecules have been developed by a number of investigators.[2] These binding equations are based upon the mass law and treat the macromolecule as possessing a number of specific ion binding sites having definite association constants for interaction with the small ions. Equations have been developed by other workers who have studied the phenomenon of preferential hydration of macromolecules.[3] The macromolecule is considered by these workers to interact with a number of water molecules and a smaller number of ions. If the proportion of water molecules to salt ions in the vicinity of the macromolecule is greater than the proportion of water to salt in the solvent, the preferential hydration is positive. If salt is in excess, the preferential hydration is negative.

Interestingly, investigators studying ion binding often find that proteins bind sizable numbers of salt ions in solution. Other investigators studying preferential hydration find that proteins bind large quantities of water to the exclusion of salt.

In this communication we have joined together the ion binding equations and the preferential hydration equations. As might be expected, the resulting expression clearly shows that at low concentrations of salt ions, the macromolecules preferentially bind salt while at high concentrations of salt, the macromolecules preferentially bind water. Indeed, it becomes clear that ion binding and preferential hydration are, experimentally, difficult to separate.

One result of this treatment is to suggest that the interpretation of ion binding results, such as determined in the e.m.f. cell or by equilibrium dialysis, may contain small but definite errors if a correction for preferential hydration is not made.

In addition, we present in this communication a reasonable physical mechanism for preferential hydration of macromolecules in salt solutions. In this mechanism, attention is directed to the ionized salt which interacts strongly with adjacent water molecules through ion-dipole bonds. As the salt ion is brought to the surface of the macromolecule, electrostatic allegiances shift, new bonds may arise and water molecules may be freed. Should these events be energetically unfavorable, the ion concentration will drop sharply in the surface layer. Effectively, the macromolecule will be preferentially hydrated even though there is little or no positive interaction between it and the water.

## Theory

In this section it is shown that preferential hydration may be expressed mathematically in terms of ion binding by considering all the binding energies, positive and negative, over the entire surface of the macromolecule. The surface layers surrounding the macromolecule will be defined as the monolayer of solvent in contact with atoms of the macromolecule together with several adjacent layers of solvent. For ease of mathematical formulation the sizes of the water molecules and the unhydrated salt ions are considered to be equal so that the surface layers will contain $\lambda$ water molecules and salt ions regardless of the composition of the layers. In general the surface of the large molecule will be inhomogeneous in binding affinities for the salt ions, and it can be divided into groups of sites i, each group containing $\lambda_i$ members possessing an intrinsic affinity constant $k_{ij}$ for a particular ion, j.

For interaction between small molecules and a macromolecule, the mass law may be conveniently written[2]

$$r_{ij} = \frac{\lambda_i k_{ij} m_j}{1 + k_{ij} m_j} \qquad (1)$$

where $r_{ij}$ is equal to the number of moles of salt ions of species j bound at sites of type i, $m_j$ is the molality of the free (unbound) ion, and $k_{ij}$ is the intrinsic association constant for this reaction. Now, if the composition of solvent bound at the $\lambda_i$ sites is to be the same as the composition of the free solvent, then there would have to be $r_{ij}(55.51/m_j)$ water molecules bound at the sites, $\lambda_i$. The difference between this number and the number of water molecules actually bound, $(\lambda_i - r_{ij})$ will be termed the preferential hydration, $\alpha_{ij}$

$$\alpha_{ij} = (\lambda_i - r_{ij}) - r_{ij}\frac{55.51}{m_j} \qquad (2)$$

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(2) Reviewed by I. M. Klotz, in "Proteins," Vol. I, Part B, H. Neurath and K. Bailey, Editors, Academic Press, Inc., New York, N. Y., 1953, p. 763.

(3) S. Katz and H. K. Schachman, *Biochem. et. Biophys. Acta*, **81**, 28 (1955).

The preferential hydration, $\alpha_{ij}$, is equal to the number of water molecules at the sites i in excess or dearth of the number needed to "balance" the bound ions of species j. It may be either positive or negative, and if it is negative the macromolecule is considered to be binding ions preferentially at the sites i.

The $r_{ij}$ may be eliminated between equations 1 and 2 giving[4]

$$\alpha_{ij} = \lambda_i \frac{(1 - k_{ij}\,55.51)}{(1 + k_{ij}\,m_j)} \qquad (3)$$

The $\alpha_{ij}$ can finally be summed over the groups of sites i to give

$$\alpha_j = \sum_i \alpha_{ij} \qquad (4)$$

This quantity, $\alpha_j$, is the preferential hydration of the entire macromolecule with respect to ion species j.

A similar equation can be written for ions of the opposite charge and will in general, give a different value for $\alpha_j$.

**Ion Binding Studies.**—It will now be shown that in some types of ion binding studies the quantity actually measured is the preferential hydration, $\alpha_j$, with respect to a particular ion species as defined by equation 4.

In these studies the molality of a free ion, $m_j$, is measured in a solution containing $g$ kilograms of water, $n_j^0$ moles of ion j, and $n_p$ moles of protein. The usual procedure is then to calculate a number, $r_{obsd.}$, by the type of equation[5]

$$n_p r_{obsd} = n_j^0 - m_j g \qquad (5)$$

On the other hand, the experimental results may be interpreted more properly in terms of the preferential hydration, $\alpha_j$. Upon addition of $n_p$ moles of macromolecule, let $r_j n_p$ be the number of moles of salt ions that are actually bound. Then $(\lambda - r_j)n_p$ moles of water which do not contain salt ions of species i are present at the remainder of the sites. The complete expression for $m_j$ is therefore given by

$$\frac{n_j^0 - n_p r_j}{g - n_p \dfrac{(\lambda - r_j)}{55.51}} = m_j \qquad (6)$$

which can be rearranged to give

$$n_j^0 - m_j g = \frac{-m_j}{55.51}\left[\lambda - r\left(1 + \frac{55.51}{m_j}\right)\right] n_p \qquad (7)$$

But according to equation 5, the left hand side of equation 7 is equal to $n_p r_{obsd}$. Moreover, the expression in brackets on the right hand side of equation 7 is equal to $\alpha_j$. This is directly seen by summing equation 2 over the sites i. Thus, equation 7 becomes

$$r_{obsd} = \frac{-m_j}{55.51}\,\alpha_j \qquad (8)$$

Therefore, the $r_{obsd}$ is simply related to the preferential hydration $\alpha_j$. This is an important result, for equations 3 and 4 show that $\alpha_j$ may be composed of several groups of sites *including those which exclude ions strongly*. Let us consider a simple case of a macromolecule possessing $\lambda_e$ sites at which ions are completely excluded, and $\lambda_b$ sites at which they are bound firmly with an intrinsic association constant $k_0$. Then equations 3 and 4 give

$$\alpha_j = \lambda_e + \lambda_b \frac{(1 - k^0\,55.51)}{(1 + k^0 m_j)} \qquad (9)$$

Should $\lambda_e$ be large and $\lambda_b$ small, appreciable errors can result if $r_{obsd}$ and $\alpha_j$ are interpreted in terms of $\lambda_b$ only. For example, if $\lambda_e = 1000$, $\lambda_b = 10$, and $k^0 = 50$, the calculated binding will be approximately 5% too low when the salt concentration is 0.01 $M$, and approximately 20% too low at a concentration of 0.1 $M$. Even at infinite dilution appreciable errors may occur as may be seen in equation 9 by letting $m_j$ approach zero. A better value can be obtained if $\lambda_e$ is first determined from preferential hydration studies made at very high salt concentrations where the second term on the right in equation 7 becomes small in comparison with $\lambda_e$. In Fig. 1 is shown a plot of equation 9 for a hypothetical protein with $\lambda_e = 1000$, $\lambda_L = 10$ and $k^0 = 50$.

**Equilibrium Dialysis Studies.**—Katz has pointed out that the quantity actually measured in equilibrium dialysis experiments is the specific adsorption coefficient which is defined thermodynamically and may be interpreted in terms of the preferential hydration in simple three component systems such as water, sucrose and protein.[6] A more complicated treatment is necessary for the development of equations which consider preferential hydration in equilibrium dialysis studies of ion binding to macromolecules. The development will not be presented here, but the final expression for a solution containing isoionic protein and a uni-univalent electrolyte is

$$\left[g - \frac{n_p \alpha_+}{55.51}\right]\left[g - \frac{n_p \alpha_-}{55.51}\right] = \left(\frac{n}{m'}\right)^2 \qquad (10)$$

where $\alpha_+$ and $\alpha_-$ are the preferential hydration values for the cation and anion, $g$ is the number of kilograms of water inside the dialysis sac, $n_p$ is the number of moles of the macromolecule, $n$ is the total number of moles of salt (or of either ion) inside the sac and $m'$ is the molality of salt *outside* the sac.

While this equation is readily derived in a rigorous fashion, its validity can be seen upon close inspection. The terms in brackets on the left represent the effective quantities of water in which all the cations or anions, including those bound to the macromolecule, would have to be dissolved to give the molalities $m_+$ and $m_-$, which are the actual molalities of the free (unbound) ions inside the sac. The $(m')^2 = m_+ m_-$, as the condition of equilibrium. When each of these molalities, $m_+$ and $m_-$, is multiplied by the effective quantity of water, the result is equal to the total number of moles of anion or cation inside the sac, $n$.

**Sedimentation Studies.**—As can be seen from an inspection of equation 4, the value of $\alpha_j$ becomes more positive as $m_j$ increases. Therefore by measuring $\alpha_j$ over a wide range of concentrations it may become possible to estimate both the number of sites of exclusion and of binding. Ultracentrifugation may offer an approach to the problem of measuring $\alpha$ in high salt concentrations. If ultracentrifugation is to be used, however, two related problems immediately arise. Equation 4 can be written for both the anion and for the cation, and in general $\alpha_+$ and $\alpha_-$ will be different. But the ultracentrifugation studies give but one value for $\alpha$, and it is not obvious to us how this $\alpha$ is related to $\alpha_+$ and $\alpha_-$. Secondly, if $\alpha_+$ and $\alpha_-$ are different, the number of bound anions and cations are different, and the sedimenting unit carries a net charge. In this case, charge effects, especially the secondary charge effect, could affect the sedimentation properties of the hydrodynamic unit.

For bovine serum albumin, a voracious ion binder, these difficulties may explain the wide variety of results obtained for $\alpha$ by ultracentrifugation in different solvents and at different $p$H values.

For ribonuclease, on the other hand, the experimental value for $\alpha$ remained unchanged in a number of salt solutions and over a wide range of concentrations. It also was independent of $p$H. It appears reasonable to assume that for this protein, $\alpha_+$ and $\alpha_-$ are similar or identical, and that the net charge carried by this protein is small. In this case the centrifugation studies should give $\alpha_j$.

Therefore, if values for $\alpha_j$ are to be measured by centrifugation studies, we feel that measurements should be made over a range of salt concentrations and in a variety of solvents. If under these conditions the calculated $\alpha$ remains constant, it appears reasonable to assume that it is a valid measure of preferential hydration.

## Discussion

Sizable amounts of water are associated with ribonuclease and bovine serum albumin in concentrated salt solutions as shown in our previous studies.[7]

(4) In concentrated salt solutions individual ion activity coefficients should be used in equation 3. In lieu of having these, but less correctly, the mean ion activity coefficients may be used. In equation 3 the activity coefficient for the salt ion will appear as a coefficient of $k_{ij}^0$ in equations 1 and 3 as suggested by C. Tanford and J. G. Kirkwood, *J. Am. Chem. Soc.*, **79**, 5333 (1957), or as suggested in ref. 5.

(5) G. Scatchard, I. H. Scheinberg and S. H. Armstrong, Jr., *J. Am. Chem. Soc.*, **72**, 535 (1950).

(6) S. Katz, *ibid.*, **78**, 300 (1956).

(7) D. J. Cox and V. N. Schumaker, *ibid.*, **83**, 2433, 2439 (1961).

It may be asked if a monolayer of water over the surface of these proteins would account for the large hydration values obtained. The minimum surface areas for these proteins which may be calculated from spheres of equal volume are 3000 Å.$^2$ for RNase and 9000 Å.$^2$ for BSA. From the molecular weight and density of water we may assign a 3.1 Å. cube to each water molecule. Therefore, a monolayer of water around the equivalent sphere would correspond to a preferential hydration value of 0.4 g. $H_2O$/g. protein for RNase. For BSA the corresponding value would be 0.25 g. $H_2O$/g. protein. These values are certainly the same magnitude as the observed results; surface irregularities and deviation from spherical shape will tend to make the approximate calculations too small.

In order to apply this theory to proteins, it is necessary to consider the interaction between salt ions and the several groups of sites which exist at the surfaces of these macromolecules. From equations 3 and 4 it can be seen that for the preferential hydration to be positive at all, at least one of the terms $k_{i,j}^0$ (55.51) must be less than unity. This means that the standard free energy of ion binding must be positive: $\Delta F^0 = -RT \ln (1/55.51) = +2339$ cal. at 20°. In solution the salt ions are symmetrically hydrated with water molecules held in place by strong ion-dipole forces. In order for the salt ion to participate in the monolayer adjacent to the macromolecule, this hydration shell must be distorted. It is difficult to estimate the energies that would be involved in this distortion, but it is reasonable to expect that they are positive and quite large. We suggest that this is the primary factor involved in the preferential hydration of proteins in concentrated salt solutions. For the hydrophobic side chains (valine, phenylalanine, etc.,) the values of $k^0$ (55.51) are probably very small indeed. A shell of water might be expected to form within such hydrophobic regions. This view is, perhaps, essentially different from the suggestion of Klotz[8,9] that the hydrophobic side chains cause an ordering of neighboring water molecules, but the end result might be much the same. Such a shell would tend to exclude the hydrated hydronium ion, thereby changing $pK$ values of acidic and basic groups buried in such regions. For those sites at the surface of the protein formed by peptide bonds and uncharged nitrogen and oxygen atoms, two additional factors must be considered. The free energy would be expected to be somewhat lower than that found for the hydrophobic regions because of the stronger induced dipole that would be created in such regions upon the approach of the ion. On the other hand, hydrogen bonds are thought to exist between water and many of the nitrogen and oxygen atoms on the surface of the protein. The presence of such bonds might contribute +1 or +2 kcal. to $\Delta F^0$, particularly at sites adjacent to peptide bonds. Considering then these two additional factors the preferential hydration probably still is positive, but it may not be complete. Next, it is necessary to consider the sites of charged acidic and basic groups. Ions

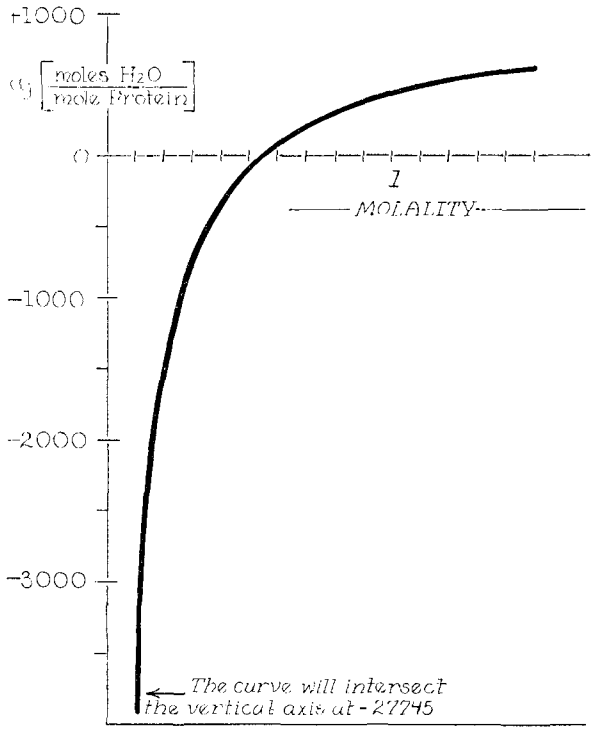


Fig. 1.—Preferential hydration values, $\alpha_j$, for a hypothetical protein possessing 1000 sites from which ions of species j are completely excluded, and 10 ion binding sites for which the intrinsic association constant, $k^0$, is 50. The curve is determined by equation 9.

of the same charge would be strongly repelled, and with respect to these ions the hydration would be large at such sites. For ions of opposite charge the binding affinities at charged sites are probably greatest and the values of $\alpha_{ij}$ small or even negative. The actual values will depend upon the type of ion, type of site, steric factors and the neighboring charge distribution. However, large quantities of $Na^+$, $K^+$ and $Cl^-$, usually do not bind copiously to isoelectric proteins[2] which contain many such charged acidic and basic groups. Therefore, a number of factors must combine to make a specific ion binding site.[10]

In conclusion, it may be pointed out that it is possible to explain hydration without invoking any forces except those of steric exclusion. Hydration would occur if the macromolecules were selectively permeable to water through channels which could admit water molecules but because of their small size would exclude larger solutes. The small hole through the center of the hemoglobin molecule discovered by Perutz and co-workers[11] may be such a channel. Kauzmann (cited by Schachman and Lauffer[12]) has suggested another explanation which does not involve attractive forces as the primary factor. If the density increasing com-

(8) I. M. Klotz, *Science*, **128**, 815 (1958).

(9) I. M. Klotz, in "Symposium on Salt and Water Metabolism," *Circulation 21*, 828 (1960).

(10) Uncharged imidazole residues have been implicated in the binding of the zinc ion to serum albumin. Therefore, charged groups may not be essential for the binding of ions at certain specific sites where they might form coördinate linkages.

(11) M. F. Perutz, M. G. Rossmann, A. F. Cullis, H. Muirhead, G. Will and A. C. T. North, *Nature*, **185**, 416 (1960).

(12) H. K. Schachman and M. A. Lauffer, *J. Am. Chem. Soc.*, **71**, 536 (1949).

ponent (urea, sucrose, etc.,) is larger than the water molecules and considered to be composed of "rigid, uncharged spheres of radius, $a$, it is clear that the center of any of these molecules will not be able to come closer than a distance, $a$, to the 'surface' of the sedimenting particle. This results in a region of thickness, $2a$, surrounding the macromolecules in which the density varies from that of water to that of the solution. According to Kauzmann, this region of varying density can be approximated by assigning a water layer of thickness, $a$, to the macromolecules and assuming that the liquid more distant than $a$, from the surface of the macromolecule has the density of the bulk liquid.[13]" In such cases as these, it is still possible to use the equations developed in the theoretical section of this communication. The $\alpha_j$ will refer to the number of water molecules in the region from which salt is excluded, and the binding affinities, $k^0$, for such regions will be equal to zero.

(13) H. K. Schachman, "Ultracentrifugation in Biochemistry," Academic Press, Inc., New York, N. Y., 1959, p. 233.

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# A Raman Study of the Bromide Solutions of Zinc and Cadmium[1a]

BY WILBUR YELLIN[1b] AND ROBERT A. PLANE

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Raman intensity measurements were made of zinc bromide solutions containing various ratios of total zinc to total bromide. These results led to the following as the most probable assignments for the three polarized lines: $\nu_{172}$ cm.$^{-1}$ ($\rho$ = 0.06), $ZnBr_4^=$; $\nu_{186}$ cm.$^{-1}$ ($\rho$ = 0.09), $ZnBr_2$; $\nu_{205}$ cm.$^{-1}$ ($\rho$ = 0.13), $ZnBr^+$. Comparison with $ZnBr_2$ in non-aqueous solvents indicated that the aqueous $ZnBr_2$ molecule is different from that in other solvents and may be tetrahedral $Zn(H_2O)_2Br_2$. Stepwise formation constants for the three complexes were found in concentrated solution to be: $(ZnBr^+)/(Zn^{++})(Br^-)$ = 0.3, $(ZnBr_2)/(ZnBr^+)(Br^-)$ = 1, $(ZnBr_4^=)/(ZnBr_2)(Br^-)^2$ = 0.2. If $ZnBr_3^-$ were present, its principal Raman band was coincident with that of one of the other species. In cadmium bromide solution of various ratios of total $Br^-$ to total $Cd^{++}$, only one polarized line was found. Coincident with that line, at 166 cm.$^{-1}$ ($\rho$ = 0.08) which was due to $CdBr_4^=$, was the principal line of one (or more) lower species. The lower species appeared to be of less importance than in the zinc system, while the tetrabromo species was of greater importance: $(CdBr_4^=)/(Cd^{++})(Br^-)^4 > 1$.

Of the methods used for studying complex ions in aqueous solution, none is more direct than the measurement of Raman spectra of solutions. This method is direct in that each different complex species present should exhibit its characteristic vibrational spectrum.[2] Thus, the Raman method differs from most other methods which determine some property characteristics of one species (often the activity of the completely dissociated species) and from variations of this property infer the presence of additional species. Furthermore, the very existence of a Raman spectrum shows that the complex species has kinetic identity (*i.e.*, exists for times long enough to allow vibrations) and is bound together by something other than hard-sphere coulombic attractions (*i.e.*, the mean molecular polarizability must change during vibration[3]). Thus, in principle, observation of Raman spectra is a general method for both defining and detecting complex ions in aqueous solutions.

The present work was undertaken in order to study as quantitatively as possibly Raman spectra of concentrated aqueous solutions containing relatively simple complex ions. For the investigation, the bromides of zinc and cadmium were chosen. Both had previously been studied qualitatively.[4,5] Aqueous solutions of $Zn^{++}$ and $Br^-$ had been found to show Raman lines at 172, 184 and 208 cm.$^{-1}$, while non-aqueous solutions of $ZnBr_2$ showed a line at (or near) 208 cm.$^{-1}$ and in some solvents a second line at 172 cm.$^{-1}$. On this basis, the line at 208 cm.$^{-1}$ was assigned to the linear $ZnBr_2$ molecule, that at 172 cm.$^{-1}$ to the tetrahedral $ZnBr_4^=$ (whose other frequencies were found to be 61, 82 and 210 cm.$^{-1}$) and the intermediate line (184 cm.$^{-1}$) found only in water was assigned to $ZnBr_3^-$. No Raman evidence was found for any other species such as $ZnBr^+$, for which non-Raman evidence exists.[6,7] Previous studies of aqueous cadmium bromide solutions detected the existence of only the tetrahedral $CdBr_4^=$.[5] Thus, a primary aim of the present investigation was to determine which lower complexes are formed in aqueous solutions of the bromides of zinc and cadmium. Quantitative intensity measurements were made both for this purpose and to determine the concentrations of the various species as a function of solution composition.

## Experimental

Measurements were made with a Cary Model 81 Raman spectrophotometer. This instrument was altered only in that the filter solution was replaced by pure isopropyl alcohol and sample tubes were used which were specially

(1) (a) This work was supported by the United States Air Force through the Air Force Office of Scientific Research of the Air Research and Development Command, under Contract No. AF 49(638)-279. Reproduction in whole or in part is permitted for any purpose of the United States Government. Some of the results were reported at the 136th National Meeting of the American Chemical Society, Atlantic City, New Jersey, September, 1959. (b) Based on work performed for partial fulfillment of the degree of Doctor of Philosophy.

(2) G. W. Chantry and R. A. Plane, *J. Chem. Phys.*, **33**, 736 (1960).

(3) *Cf.* D. A. Long, *Proc. Roy. Soc. (London)*, **A217**, 203 (1953). L. A. Woodward and D. A. Long, *Trans. Faraday Soc.*, **45**, 1131 (1949).

(4) M. L. Delwaulle, *Compt. rend.*, **240**, 2132 (1955); *Bull. Soc. Chim., France*, **1955**, 1294 (1955).

(5) J. A. Rolfe, D. E. Sheppard and L. A. Woodward, *Trans. Faraday Soc.*, **50**, 1275 (1954).

(6) E. Ferrell, J. M. Ridgion and H. L. Riley, *J. Chem. Soc.*, 1121 (1936).

(7) (a) L. G. Sillen and B. Liljequist, *Svensk kem. Tidskr.*, **56**, 85 (1944); (b) S. A. Shchukarev, L. S. Lilich and V. A. Latysheva, *Zhur. neorg. Khim.*, **1**, 225 (1956).