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Stability Constants of Metal Complexes with Mononucleotides

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The formation constants of mononucleotide complexes with Ca(II), Mg(II) Co(II) and Mn(II) are determined. It has been shown that the formation constants of these metal complexes mainly depend upon the nature of the metal and the length of the phosphate chain in the nucleotide. With a few exceptions the stability of the complexes are characterized by the sequence Ca<Mg<Co<Mn. With increased length of the phosphate chain in the nucleotide the stability increases in the order * AMP(UMP)<ADP (UDP)<ATP(UTP). The influence of the heterocyclic base of nucleotides in metal complex formation is studied with several purines and pyrimidine riboside phosphates and with flavin nucleotides.

Enzymatic transphosphorylation reactions involving mononucleotides as the phosphate donor or acceptor depend upon metal activation. It has therefore been suggested that the metals chelate with ligand groups in the nucleotide and in the enzyme apoprotein. Information of the structure and function of such complexes may be obtained by investigations on model systems. In the present work data on the stability constants of metals with purine-, pyrimidine- and flavinnucleotides will be presented.

In recent years several investigators have determined the stability constants of adenine nucleotides with metals, particularly Mg and Ca [1-5]. The stability constants of Sr-complexes with adenosine-3′-monophosphate and cytidine-3′-monophosphate have been reported by Schubert [6], and an approximate value of the stability constant of manganese with ATP has been given by Cohn and Townsend [8]. Investigations on the weakly associated complexes of sodium and potassium with adeninenucleotides have been carried out by Melchior [7].

* Throughout the paper the following abbreviations will be used: ATP: adenosinetriphosphate, ITP: inosinetriphosphate, GTP: guanosinetriphosphate, CTP: cytidinetriphosphate, UTP: uridinetriphosphate, ADP: adenosinediphosphate, UDP: uridinediphosphate, AMP: adenosine-5′-monophosphate, UMP: uridine-5′-monophosphate, FMN: flavin mononucleotide, FAD: flavin adenine dinucleotide.

In the present work complex formation of metals with nucleotides have been studied by means of the ion exchange resin method according to Schubert [9]. The significance of three main factors for the formation constants has been considered. This involves the nature of the metal ion, and the structure of the nucleotide, particularly the length of the phosphate chain and the configuration of the heterocyclic base. Four metals most commonly required by phosphorylation reactions involving nucleotides have been investigated, which include two alkaline earth metals: Ca(II) and Mg(II), and two metals of the first transition series: Mn(II) and Co(II).

EXPERIMENTAL

Method. The ion exchange method described by Schubert [9,11] has been used for the determination of the formation constants. In the experiments solutions of nucleotides with and without addition of metals were equilibrated with an anion exchange resin. The distribution of nucleotides between the resin and the solution was determined by ultraviolet absorption measurements.

Materials

Resin. The syntetic organic anion exchanger Dowex-1, 200—400 mesh, in the chloride form was used. The resin was put through several alternate Cl^- and OH^- cycles with 5 % solutions of NaCl and NaOH. Subsequently the resin was equilibrated with 0.1 M NaCl. The supernatant solution in contact with the resin was adjusted to pH 8.0—8.2 with dilute NaOH solution. The mixture was stirred for an hour, pH of the solution tested again and readjusted until no change of pH took place after stirring. The resin was filtered, washed rapidly with distilled water and air dried. Analyses showed a moisture content of 11.6 %. All experiments were conducted with the air dried resin.

Reagents. The solutions of sodium chloride and the solutions of the chlorides of Mg, Ca, Co and Mn were prepared from Merck products, analytical grade. All nucleotides used were derived from "Sigma" Chemical Company, Saint Louis, Mo. The purity of these compounds was investigated by the chromatographic procedure of Hurlberth *et al.*[10] on Dowex-1 resin by the gradient elution technique with a formic acid system. If ultraviolet absorbing impurities were detected, pure fractions of the nucleotide to be tested were collected and lyophilized before use.

Procedure

Ten 25 ml flasks were used in each experiment. To eight of these flasks (two were used as blanks) were added a weighed amount of the prepared Dowex-1 resin and subsequently solutions of buffer, nucleotide, metal chloride and 0.1 M NaCl. The 0.01 M tris (hydroxymethyl)-aminomethane buffer used, pH 8.2, was prepared in 0.1 M NaCl. Solutions of nucleotides were prepared in 0.1 M NaCl, adjusted to pH 8.2 and added (freshly made) to the flasks. The final volume and ionic strength in the flasks were 20 ml and 0.1, respectively.

In preliminary experiments the appropriate amount of resin at the concentration of nucleotide chosen was determined (*i. e.* approximately 75 % of the nucleotide bound to the resin). The affinity of the purine-nucleotides for the resin was greater than that of the pyrimidine-nucleotides. In experiments with adenosine-, guanosine- and inosinetriphosphate, 25 mg of resin at concentrations of 0.6×10^{-4} M of nucleotide was used. In experiments with cytidine- and uridinetriphosphates 50 mg resin was used at a similar range of nucleotide concentration. With ADP and UDP 100 mg and 200 mg, respectively, were necessary to obtain 70 % binding of nucleotide to the resin. With AMP and UMP the absorption of nucleotides was somewhat lower, even when the amount of resin was in-

creased (AMP 500 mg, UMP 1 000 mg). In experiments with flavinnucleotides appropriate absorption was obtained with 200 mg (FAD) or 300 mg (FMN).

The upper limit of the nucleotide concentration to give a constant and reproducible distribution coefficient with 25 mg resin was found experimentally to be 1×10^{-4} M. No deviation in the distribution coefficient (K_d^0) was found for the concentration range employed, ($0.5-0.625 \times 10^{-4}$ M).

Solutions of divalent metals as chlorides, dissolved in 0.1 M NaCl were added to the flasks at different levels of concentration. The concentration range chosen depended upon the affinity of the metal for the nucleotide. Usually three concentrations were employed in each experiment. Because of the high affinity for the nucleosidetriphosphates, low concentrations of metals could be used in these experiments ($0.625-3.75 \times 10^{-4}$ M). With nucleosidediphosphates a range of concentration of $0.125-1.5 \times 10^{-3}$ M was used. A higher concentration was required when the affinity for the nucleotide was low. In the case of AMP, UMP, FMN and FAD $0.75-7.5 \times 10^{-3}$ M had to be used. The same formation constants were found at low and high metal concentrations. Thus, the small variations of the ionic strength in different experiments did not influence the results, since the supporting electrolyte had the much higher ionic strength of 0.1. Theoretically, the effect on K_d of increasing ionic strength in experiments with nucleosidetri- and diphosphates was negligible, when calculated according to Schubert [6]. In experiments with nucleosidemonophosphate a maximal deviation of 6 % of K_d was calculated when 7.5×10^{-3} M metal solution was used.

The general procedure in a typical experiment was as follows: Two flasks contained nucleotide without metal ion added. These flasks were used to determine the nucleotide equilibrium between the resin and the solution. Two other flasks containing nucleotide but no resin were used as blanks. In the remaining 6 flasks metal solutions at different concentrations were added together with nucleotide. The glass-stoppered flasks were placed on a mechanical shaking apparatus and shaken vigorously for 3 h, which was sufficient to obtain equilibrium. The shaking was carried out at 23°C in a constant temperature room. Thereupon the resin was spun down by slight centrifugation, and the nucleotide concentration was measured by ultraviolet absorption at 260 mμ or other appropriate wavelengths in a Beckman Spectrophotometer Model DU in an aliquot of the supernatant. In the experiments with FAD the manganese complex formation was determined by U.V. absorption measurements. The other metal complex formation constants were investigated by determining the fluorescence in a Farrand Spectrofluorometer, using excitation maximum of FAD at 360 mμ and fluorescence maximum at 520 mμ.

The method is based upon the assumption that metal nucleotide complexes exert the same U.V. absorption spectrum as the free nucleotide. This was confirmed as no shift in absorption maximum wavelength nor any change in degree of absorption was observed by addition of metal solutions to nucleotides. This is in accordance with observations made by Nanninga [4] and by Blum [12]. No effect by the metals on the fluorescence of FAD was observed either.

Calculations

The formation constant is defined by

$$K_f = \frac{(MA)}{(M)\ (A)} \tag{1}$$

where M represents the metal and A represents the complexing anion. The formation constant is regarded as an equilibrium constant for the successive reactions between the metal ion and the ligand groups giving the complex [13]. In the present work performed at pH 8.2, the phosphate chain of the nucleotides does not take up protons, and the intermediate and terminal phosphate groups are negatively charged. In a buffered solution the anions are taken up by the resin until equilibrium. The addition of Me^{2+} reduces the concentration of free nucleotide in solution and thus decreases the uptake of negatively charged nucleotide by the anion exchanger.

It is convenient to employ a simple distribution coefficient K_d, as suggested by Schubert [9] for determining the formation constants.

$$K_d = \frac{\% \text{ in exchanger}}{\% \text{ in solution}} \times \frac{\text{volum of solution}}{\text{mass of resin}} \quad (2)$$

The term K_d^0 is taken as the value of distribution coefficient in the absence of a specific complexer, such as the complexing metal cation. Therefore the value of K_f can simply be formulated:

$$K_f = \frac{(K_d^0 / K_d) - 1}{(Me^{2+})^n}$$

where (Me^{2+}) represents the molar concentration of the complexing divalent metal, K_d the measured distribution coefficient for a given concentration of the metal and n the number of ions of Me^{2+} relative to nucleotide in the complex. K_d^0 and K_d are determined by measuring the fraction of nucleotide present in the solution at equilibrium.

Eqn. (3) can be written:

$$1/K_d = 1/K_d^0 + (Me^{2+})^n \cdot K_f/K_d^0 \quad (4)$$

A plot of $1/K_d$ *versus* (Me^{2+}) for proper values of n gives a linear correlation. By extrapolating to $(Me^{2+}) = 0$, the value of $1/K_d^0$ is obtained.

By the present application of the anion exchange resin method an accuracy of $\pm$ 5 % (standard error of mean) was obtained.

RESULTS

The type of complex formation of metals with mononucleotides is illustrated in Fig. 1. By plotting $1/K_d$ (the reciprocal distribution coefficient) against metal concentration (eqn. 4) a linear correlation is obtained. This type of curve was found without exception in all experiments. Thus, in these complexes one mole of the nucleotide combines with one mole of the metal. This is in accordance with experiments performed by Martell and Schwarzenbach [3] who demonstrated monometallic complex formation with inorganic triphosphates as well as with the adenosine phosphates AMP, ADP and ATP. In the

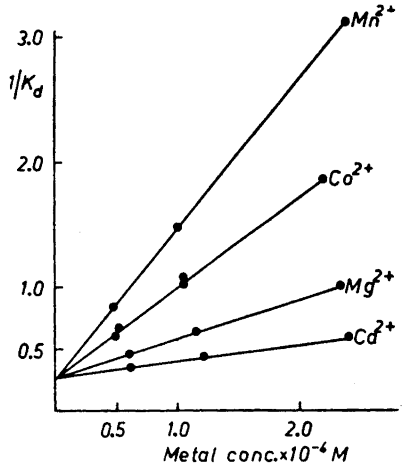

Fig. 1. Complex formation (1:1) of ATP with divalent metals. Determination by anion exchange resin method. See text for experimental conditions.

Table 1. Apparent stability constants (log K_f) of metal complexes at $\mu = 0.1$, pH: 8.2 and 23°C in 0.1 M NaCl.

Metal	ATP	ADP	GTP	ITP	UTP	UDP	CTP
Ca^{2+}	3.77	2.82	3.58	3.76	3.71		3.81
Mg^{2+}	4.04	3.15	4.02	4.04	4.02	3.17	4.01
Mn^{2+}	4.75	3.94	4.73	4.57	4.78		4.78
Co^{2+}	4.62	3.68	4.63	4.74	4.55		4.48

present work this type of metal complex formation has been demonstrated with pyrimidine and purine riboside phosphates as well as with FMN and FAD. Apparently monometallic complexes are characteristic for these group of biologically important substances.

In Table 1 the stability constants of metals with nucleoside triphosphates ATP, GTP, ITP, UTP, CTP and two of the corresponding diphosphates, ADP and UDP, are reported. The formation constants of the different metals with these compounds increase in the order Ca<Mg<Co<Mn. Thus, the lowest formation constants are obtained with Ca(II) and somewhat higher with Mg(II). This is in accordance with earlier observations on adenine nucleotides [3,5,4], and corresponds to the sequence of stability of the alkaline earth metal complexes in general as pointed out by Williams [14].

With metals of the first transition series the formation constants are considerably higher, perhaps due to the higher ability of these metals to form covalent bonds. It is of interest to note that the stability of Mn(II)-complexes with nucleoside triphosphates and nucleoside diphosphates as ligands are greater than with Co(II). Thus, the nucleotide molecule gives preference for the atomic structure of manganese with half filled 3*d*-shell. In this respect these complexes differ from the usual sequence of stability of metals with many other ligands reported by Irving and Williams [13]. This preference for manganese must be due to the molecular geometry of these nucleotides and is consistent with the high affinity of manganese for ligands of the oxygen type [15]. Thus the importance of bonding to oxygen atoms in the polyphosphate chain of the nucleotides is indicated. Table 1 shows a striking conformity of the formation constants, these being dependent upon the metal ion and the number of phosphate groups and independent of the rest of the nucleotide molecule. There is one exception to this rule, ITP forms a more stable complex with Co than with Mn, an observation which at present cannot be explained in terms of the structure of the ligand. ITP thus follows the ordinary sequence of stability: Mn<Co (Irving and Williams [13]).

Complex formation of the metals with nucleotide ligands containing one phosphate group (AMP, UMP, FMN) or with two "intermediate" phosphate groups (FAD) is of a somewhat different character (Table 2). Complexes of the 1:1 type are formed, but they are weakly associated and the order of

Table 2. Apparent stability constants (log K_f) of metal complexes at $\mu = 0.1$, pH: 8.2 and 23°C in 0.1 M NaCl.

Metal	AMP	UMP	FMN	FAD
Ca^{2+}	1.76		2.06	2.02
Mg^{2+}	1.95	2.25	2.03	2.02
Mn^{2+}	2.31		2.17	2.39
Co^{2+}	2.58		2.41	2.36

stability with different metals is not as characteristic as with the nucleoside triphosphate- and diphosphate groups. This is clearly seen from Table 3 where the relative stabilities of metal complexes with the different ligands are calculated. Particularly it should be pointed out that the great stability of manganese-complexes with nucleosidetriphosphates is not observed with nucleosidemonophosphates or with FAD which contains two phosphate groups in "intermediate" position. The stabilities of Mn(II) and Co(II) complexes with the last mentioned ligands are but slighthly higher than the stability of the Ca(II) and Mg(II) complexes, and the order of stability is not as typical as that obtained with nucleosidetri- and diphosphate compounds.

The significance of the length of the terminal phosphate chain on the formation constant is illustrated by experiments with ATP, ADP and AMP.

Table 3. Relative stability of metal nucleotide complexes (calculated ratio of K_f).

Complexing agent	Ca	Mg	Mn	Co
ATP	1	1.8	9.6	7.1
ITP	1	1.9	8.2	11.3
GTP	1	2.9	14.1	11.1
CTP	1	1.6	9.3	4.6
UTP	1	2.1	11.8	6.9
ADP	1	2.1	13.3	7.3
AMP	1	1.5	3.5	6.5
FMN	1	0.9	1.3	2.2
FAD	1	0.5	2.3	2.2

Table 4. Relative stability of metal complexes with nucleoside mono-, di- and triphosphates (calculated ratio of K_f).

Metal	AMP	ADP	ATP	UMP	UDP	UTP
Ca^{2+}	1	11.4	110			
Mg^{2+}	1	15.7	121	1	8.4	61
Mn^{2+}	1	43.2	268			
Co^{2+}	1	12.8	112			

The relative stabilities of the metal complexes with adenosinemono-, di- and triphosphates are shown in Table 4. At pH 8.2 used in the experiments these nucleotides contain, respectively, 2, 3 and 4 negatively charged oxygen atoms in the phosphate chain. In complexes with Ca(II), Mg(II) and Co(II) addition of one intermediate phosphate group in the molecule (ADP) increases the formation constant by a factor of approximately 10. Two intermediate phosphate groups (ATP) increase the stability of the complex with a factor of approximately 100. Similar results have been reported by Martell and Schwarzenbach [3] in experiments with Ca(II) and Mg(II) complexes of adenosine phosphates. This definitely, indicates the formation of chelating structures of metals with poly-phosphates in purine nucleotides. Similar observations have been made with metal complexes of condensed polyphosphates [16,3]. Experiments performed with Mg-complexes of uridine phosphates (Table 4) show that this is valid also for pyrimidine nucleotides.

The unique properties of manganese to form chelate structures with adenosine polyphosphates is indicated from Table 4. Thus, the stability constants of manganese with AMP, ADP and ATP increase by a ratio of 1:43:268. However, such a preference for manganese is not present in complexes with FAD where the two phosphate groups are in intermediate position.

DISCUSSION

In recent years considerable attention has been given to the molecular structure of metal complexes of adeninenucleotides. Thus, it has been suggested by Calvin [17] that a metal chelate structure between the metal and the phosphate residue of the ADP or ATP molecule is formed. On the other hand Szent-Györgyi [18] has proposed a structure where the 6-NH_2-group and the N_7 in the adenine as well as the phosphate residue contribute ligand groups to the metal.

In several investigations the problem has been studied by determination of stability constants of metals with adeninenucleotides. Investigations based upon titration measurements have been reported [2,3,5]. Further the problem has been studied by cation exchange method [1,6], by anion exchange method [4] and paramagnetic resonance absorption [7]. As far as the results are concerned

great discrepancies exist depending upon the method used and the experimental conditions, as discussed by Smith and Alberty [5].

The anion exchange method adopted in the present work has earlier been used by Nanninga [4]. However, the formation constants of Mg and Ca complexes with adeninenucleotides reported by him differ considerable from the results obtained in the present work. On the other hand our results completely agree with the stability of Mg and Ca adeninenucleotide complexes determined by Martell and Schwarzenbach [3] by very accurate titration measurements.

It may therefore be of some interest to point out the limitations and accuracy of the ion exchange method. Several important factors as discussed thoroughly by Schubert [11], must be taken into consideration. By the present application of the anion exchange resin method the equilibrium of nucleotide between the resin and the solution, *i. e.* K_d must be independent of the amount of nucleotides bound by the complexing metal. As mentioned in the experimental part this point was investigated by measuring K_d^0 in a system usually containing 25 mg resin at several concentrations of ATP. Above 10^{-4} M ATP, deviation in K_d^0 occurred in accordance with theoretical calculations [11]. Therefore in all experiments the nucleotide concentration was kept below the critical level. The lower formation constants reported by Nanninga can possibly be explained by the different experimental conditions with regard to the concentration of nucleotide and the amount of resin. Further it has been observed that deviation of K_d^0 occurred when more than 85 % was bound to the resin. Therefore, in each experiment, an amount of resin was chosen which permitted the determination of K_d^0 where 60—70 % of the nucleotide was absorbed. The formation constants were calculated on the assumption that the buffer ions did not form complexes with the reactants. The fact that constant values of formation constants were obtained at different metal concentrations indicated that a single complex was formed. Experimental support for this assumption was further obtained as Tris buffer at different concentrations in the range 0.05—0.005 M had no effect on the formation constant. A weak complexing action of the Na ions in the solution must be expected [8]. Finally it should be mentioned that the conformity of the extrapolated and experimental determinal K_d^0 excludes some of the disturbing factors described by Feldman *et al.*[19]

From the present work the conclusion can be drawn that two main factors determine the formation constants of metalnucleotide complexes, namely the nature of the metal and the length of the phosphate chain. The high stability of metals with di- and triphosphate ligand groups, depends upon terminal localization of the phosphate residue, such as in purine- and pyrimidine nucleotides. On the other hand the stability of metal complexes of FAD containing two intermediate phosphates groups is not increased compared with FMN or AMP.

At the pH employed no particular indication has been obtained of any significance of the heterocyclic base. This is in accordance with the findings of Martell and Schwarzenbach [3] who did not find any complex formation of metals with adenine. In the present work the stability constants of the different purine- and pyrimidinenucleosidetriphosphates were of the same order of magnitude. Further the sequences of stability were always the same, Ca<Mg<Co<Mn. The only exception is ITP characterized by the order Mn<Co.

The more stabile complexes with transition metals than with the group II A must be expected due to the more favourable acceptor factors in the former. However, the different acceptor abilities of cobalt and manganese also influence complex formation with nucleotides. The extent of increased stability characterized by the sequence AMP<ADP<ATP is much more pronounced with Mn(II) than with Co(II).

In the literature some information on the significance of metal nucleotide complexes in activation of enzymes involving phosphorylations has been given. Most commonly a requirement for Mg exists, but activation with Mn, Ca and Co has also been observed [20]. Indications that metal complexes are the actual substrates of the enzymes have been presented. Thus, with fructokinase at high ionic strength [21] and with muscle ATPase activity [22], maximal enzyme activity was obtained at a metal/ATP ratio of 1. At present scanty information is available on the structure of metal nucleotide complexes with the apoprotein part of phosphorylating enzymes [23]. This problem will be more fully discussed in a forthcoming paper. A biochemical significance of the highly stable complexes of Mn with ADP and ATP must be considered, as it is known that manganese can restore respiration and phosphorylations in Ca-inactivated mitochondrial preparations [23].

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