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Bifunctional complexones

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Abstract. In the review, a systematic account is given of the methods of synthesis of bifunctional complexones and conjugates based on them, data on the physicochemical and biological properties of these compounds and their applications are analysed, and the prospects in the search for new ligands are examined. The bibliography includes 160 references.

I. Introduction

In the course of the development of the chemistry of complexones, attempts have been frequently made to construct molecules combining, together with the chelate fragment ensuring complex-forming properties, groups of atoms incapable of participating in the formation of complexes but imparting to the chelating agent new functions which were not previously characteristic of this compound.[1] The first 'hybrids' should legitimately include the analytical indicators synthesised at the beginning of the 1950s; Xylenol Orange **1**, phthalein complexone, alizarin complexone, thymolphthalexone, fluorexon, etc.[1,2] The chelating iminodiacetic acid (IDA or H_2ida) fragment and chromophoric groups are successfully combined in the molecules of these substances (Fig. 1, see over).

Later attempts were made to introduce into the complexone molecule a hydrophobic hydrocarbon group, imparting surface-active properties to the chelating agent,[3–6] paramagnetic spin labels,[7] etc.

However, complexones bound covalently to proteins, which appeared at the end of the 1970s, have aroused and continue to arouse the greatest interest.[8–12] This is associated with the fact that the products of the interaction of the protein of a bifunctional complexone and a cation — conjugates — have found extensive application in biology and medicine (see Fig. 2, p. 941).[1,8–13]

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Despite the fact that all the 'hybrid compounds' mentioned above can perform simultaneously at least two fundamentally different functions, the term 'bifunctional complexones' became firmly established only after the introduction of the last variety of chelating agents.

At the present time, the term 'bifunctional complexones' embraces a range of substances containing, apart from the chelating fragment, a reactive group ensuring the covalent attachment of such a complexone to biological macromolecules (peptides, proteins).[1]

Various modifications of diethylenetriaminepentaacetic acid (DTPA or H_5dtpa), ethylenediaminetetraacetic acid (EDTA or H_4edta), and certain other complexones are usually employed to produce bifunctional complexones. Isothiocyanate, bromoacetamide, carboxymethoxy-, diazonium, or other similar groups are employed as groups with affinity for proteins.

So far as possible, the bifunctional complexones should satisfy the following requirements:

(i) To retain the ability to form thermodynamically highly stable complexes after immobilisation on the protein.

(ii) To become rapidly attached to the protein and to retain the cation in the chelating component for a sufficiently long time compared with the half-life of the radioactive label or the period required for natural elimination from the organism, which presupposes the existence also of kinetic stability (the lowest possible rates of dissociation of the complex in vivo), in addition to the thermodynamic stability.

(iii) Not to exert a significant influence on the biological properties of the antigen.

Furthermore, the conjugate obtained must have a low toxicity and must not accumulate in the organism.

By virtue of the ability of the chelating fragment to bind cations into highly stable complexes, the conjugates acquire properties characteristic of the given metal: radioactivity, paramagnetism, the ability to absorb or emit photons, the ability to catalyse certain chemical reactions, etc.,[14,15] whilst variation of the protein component in the conjugate, for example the combination of the bifunctional complexone with various monoclonal antibodies, significantly increases selectivity in the accumulation of the cation-label or the cation-base of the therapeutic preparation in an in vivo situation.[16,17]

The bifunctional complexone not only ensures the attachment of the cation to the protein but frequently lowers, as a result of complex formation, the toxicity of the metal and increases its 'acceptability' to the organism.[18]

Studies in the field of the synthesis and application of bifunctional complexones have been prosecuted extremely

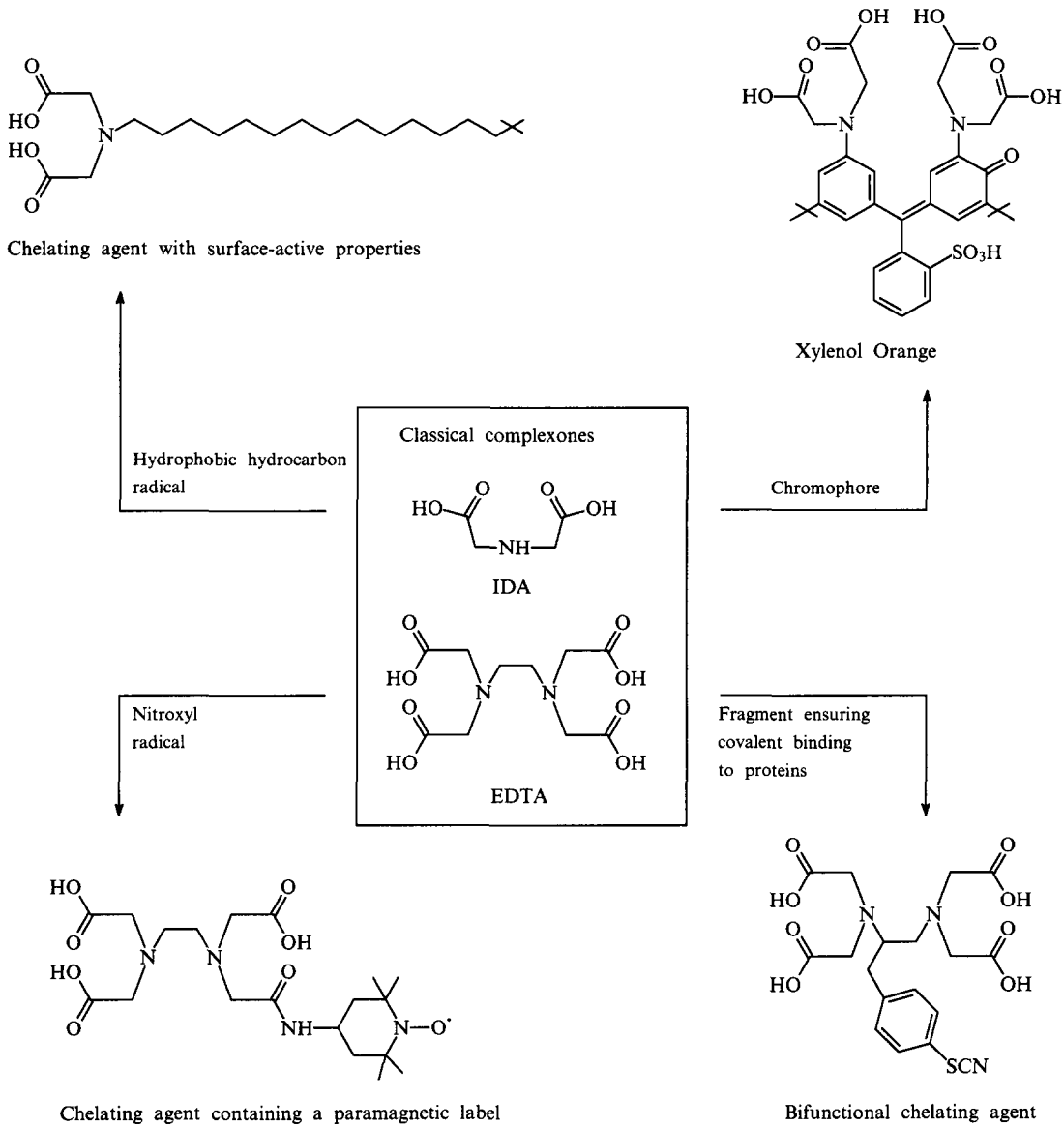


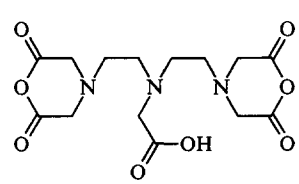
Figure 1. Different versions of the coupling of chelating and other functional fragments. IDA = iminodiacetic acid; EDTA = ethylenediaminetetraacetic acid.

vigorously. The possibility of the application of complexes of radioactive cations in the diagnosis and treatment of different forms of cancer,[19-24] in immunological research,[25-31] in the study of enzymes,[32-34] and in the study of various organs[35-37] has been discovered. Equally extensive prospects have arisen in connection with the complexes of paramagnetic (NMR tomography)[38-40] and fluorescent-active cations.[41-44]

II. Methods of synthesis of bifunctional complexones

Nowadays it is possible to differentiate two approaches to the modification of complexone molecules. The first ensures the formation of a covalent antigen–complexone bond as a result of chemical reactions involving at least one of the acetate groups of the complexone (Fig. 2*a*, compound **1**). The potential denticity of the chelating component of the bifunctional complexone can then be reduced, since the complex-forming capacity of the oxygen atom, arising when the acetamide group is bound to the antigens, is lower than that of the analogous atoms in the ionised carboxy-group.

The starting materials for the preparation of bifunctional complexones are mixed (A-DTPA) and bicyclic (B-DTPA) anhydrides of DTPA, the bicyclic anhydride of EDTA, the *N*-hydroxysuccinimidic ester of DTPA (E-DTPA),[10,45-47] and certain other ligands.[10,46,48,49]



B-DTPA

The synthesis of such complexones is distinguished by its simplicity. Thus B-DTPA is obtained under heterogeneous conditions by heating DTPA in an excess of acetic anhydride and pyridine at 65 °C for 24 h with subsequent filtration of the reaction mass in order to separate the deposit of the target product.[50] Sometimes a mixture of pyridine and dioxane is used as the reaction medium.[45]

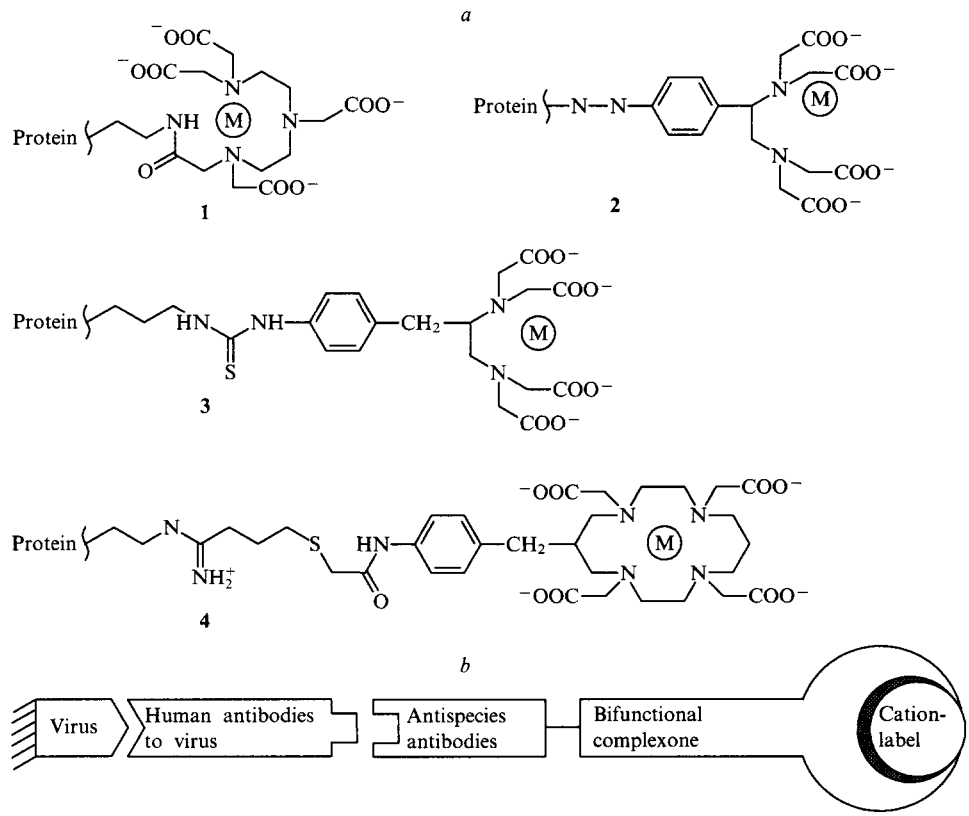
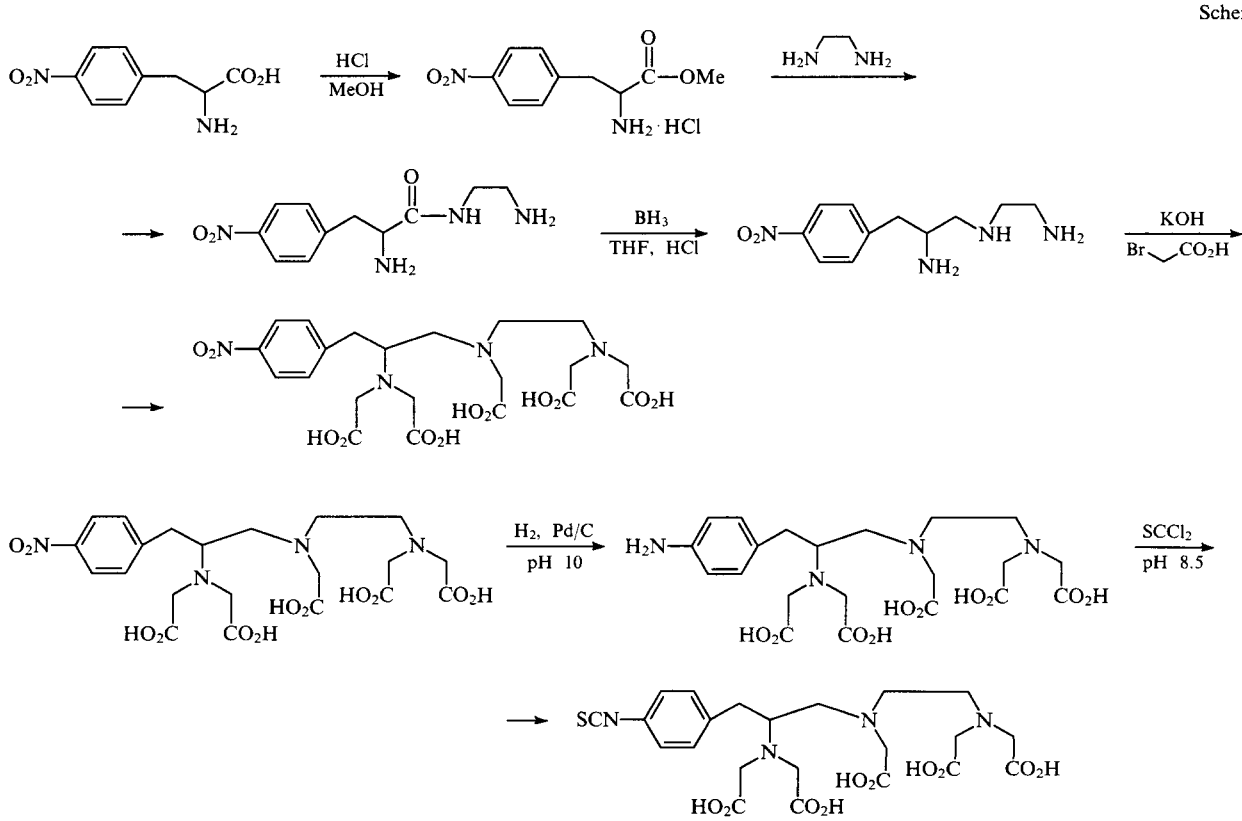
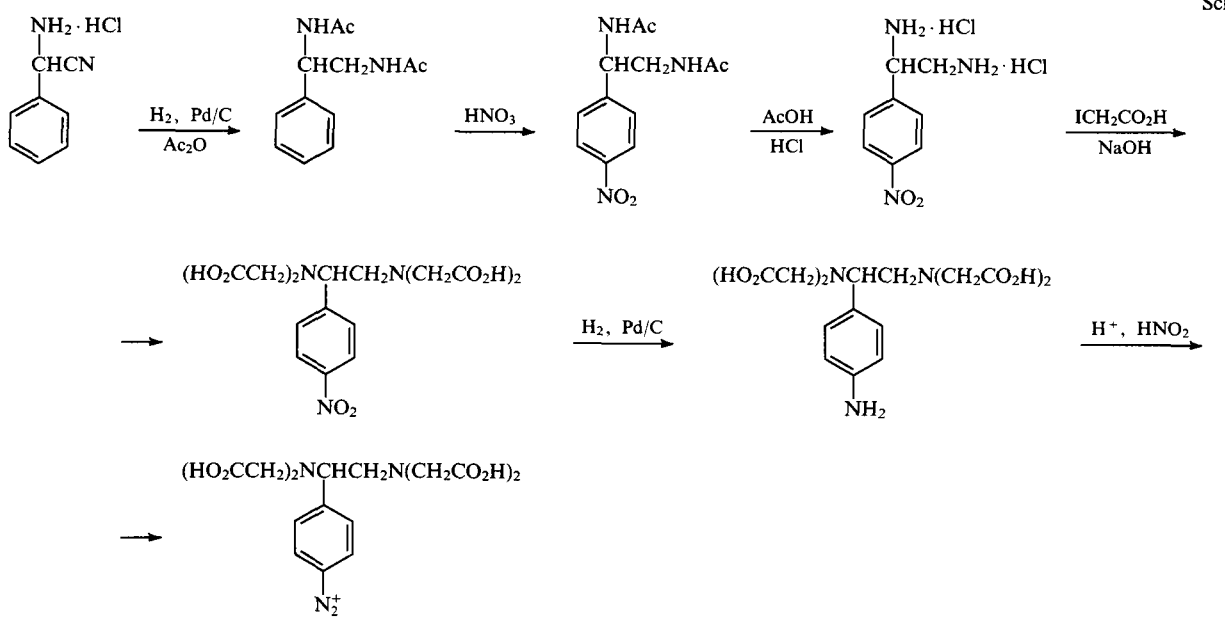


Figure 2. Conjugates formed by bifunctional complexones (*a*) and a general scheme for the diagnosis of viral diseases with the aid of bifunctional complexones (*b*).

Scheme 1



Scheme 2



E-DTPA is also synthesised under mild conditions by the reaction of DTPA with *N*-hydroxysuccinimide in the presence of triethylamine and dicyclohexylcarbodiimide.[27,51]

The possibility of obtaining carboxyalkylated derivatives of ethylenediamine, diethylenetriamine, and their analogues containing secondary amino-groups by the interaction of the above amines with monochloroacetic acid in the presence of calcium oxide is extremely promising.[52-54] The presence of the 'free' amino-group in the structures of such ligands gives rise to hope for their employment in the synthesis of bifunctional complexones with a specified structure.

Another approach in the creation of bifunctional complexones is based on the incorporation in the hydrocarbon skeleton of the complexone of a centre reactive in relation to proteins (Fig. 2*a*, compounds **2**–**4**). In this case, the chelating capacity of the complexone is retained after its immobilisation on proteins, but the synthesis itself is made significantly more complex. As a rule, either a phenyl or an alkylphenyl group with an isothiocyanate fragment in the *para*-position relative to the chelating component is introduced into the hydrocarbon skeleton of the complexone (EDTA, DTPA).[55,56] On subsequent conjugation, the isothiocyanate (diazonium, azide, haloacetamide) group participates in the formation of a covalent bond with the protein.

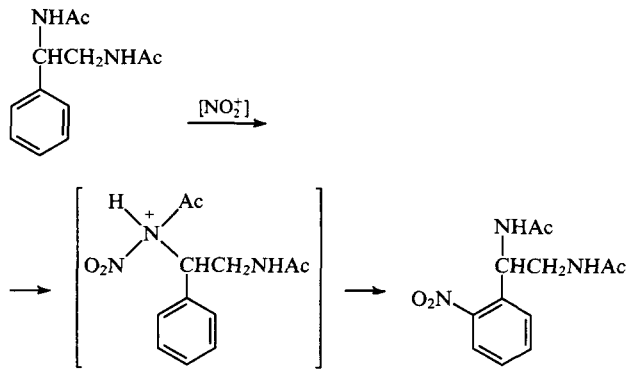
The synthesis of 1-benzyl-substituted derivatives of DTPA and EDTA is a two-stage process. The nitrobenzyl derivatives of the complexones are obtained initially, after which the functional group of the benzyl substituent is converted into the fragment employed for coupling to proteins, for example via Scheme 1 (see previous page).[34]

Scheme 2 represents the process involving the formation of a modified EDTA containing a benzenediazonium group in the ethylene bridge.[34]

It is noteworthy that the nitration of the amine, obtained by the catalytic hydrogenation of the hydrochloride of 1-phenylglycine nitrile in acetic anhydride, leads, according to NMR spectroscopic data, to the formation of a mixture of *ortho*- and *para*-isomers in molar proportions of 2:3. The formation of the *ortho*-isomer is probably preceded by the formation of an intermediate in the nitration of the diamine—an unstable *N*-nitroamide (Scheme 3).

A disadvantage of the method described above is also the low yield (< 30%) of diaminotetraacetic acid in the stage involving alkylation of the corresponding diamine dihydrochloride with an

Scheme 3



excess of iodoacetate or bromoacetate.[57] The use of methyl 2-amino-4-phenylbutyrate as the initial compound[10] instead of the hydrochloride of 1-phenylglycine nitrile makes it possible to reduce to a considerable extent the amount of the *ortho*-isomer formed on nitration and to obtain the analogous bifunctional complexone in a high yield. The small amount of the *ortho*-isomer (~10%) is easily removed by crystallisation.

The reason for the appreciable decrease in the yield of the *ortho*-isomer in this case is the separation from the aromatic ring of the chelating group produced by a distance equivalent to two hydrocarbon units and the consequent decrease in the risk of β-elimination.

New possibilities in the synthesis of bifunctional complexones have arisen by virtue of the use of α-aminoacids (α-AA) as the initial compounds.[34]

α-AA → α-AA ester → α-AA amide → α-AA diamine

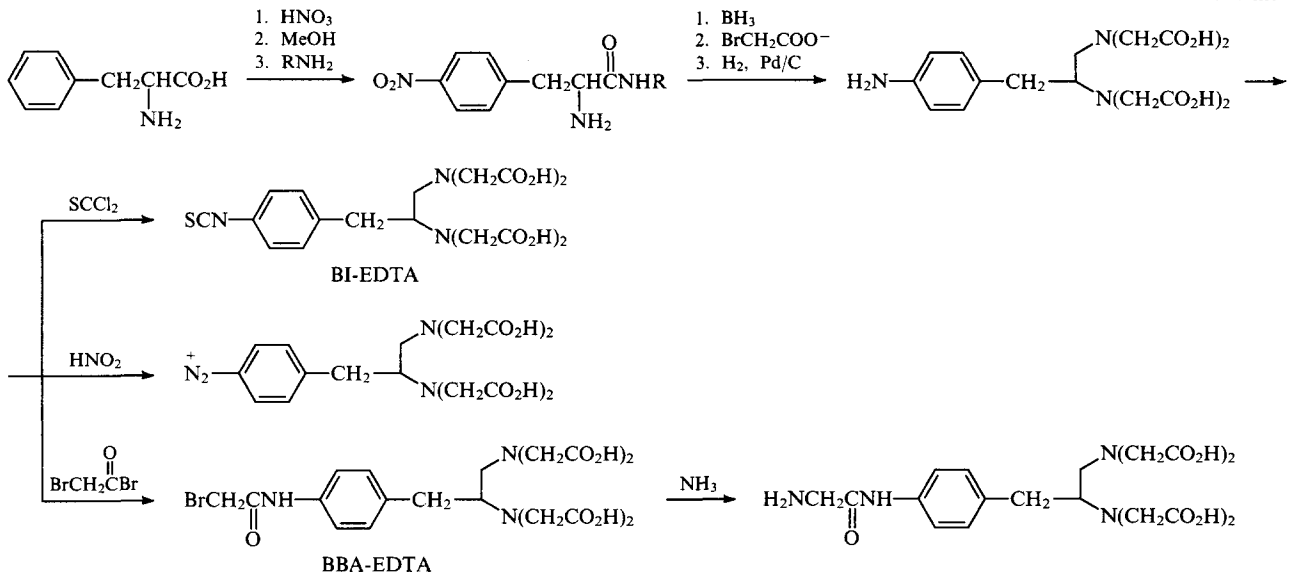
→ bifunctional complexone

By employing amines with different structures for the preparation of the amide, it is possible to vary widely the chelating properties of the target product.

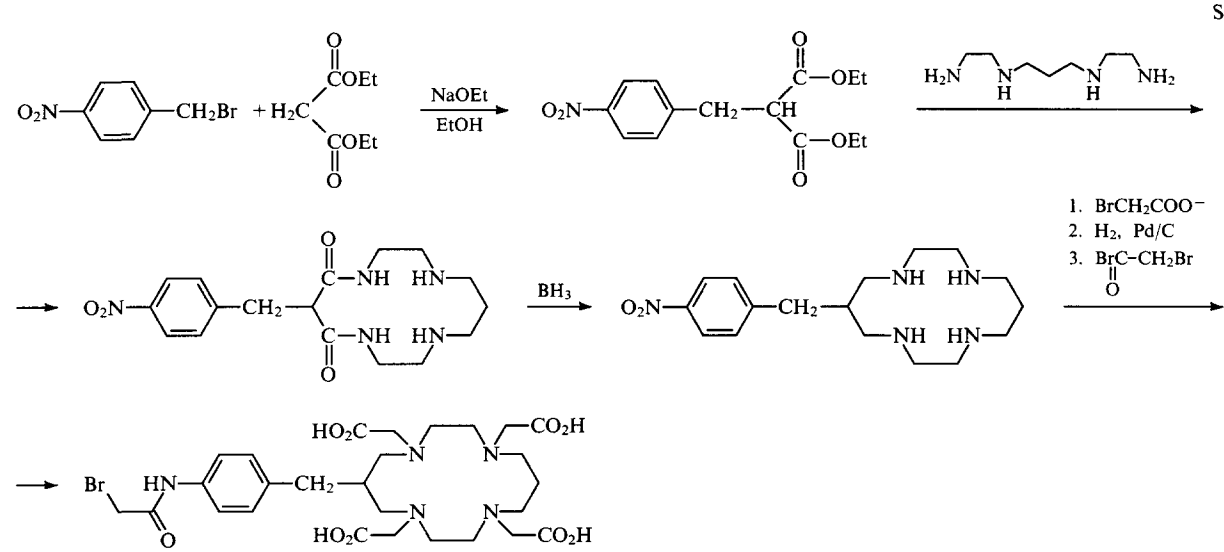
Scheme 4 illustrates the synthesis of bifunctional reagents in which phenylalanine is employed as the initial compound.[27]

The only serious disadvantage of the above synthesis is the need to use the toxic thiophosgene in the stage where the amino-group is substituted by the isothiocyanate group.

Scheme 4



Scheme 5



The multistage nature of the process is to a large extent compensated by the virtually quantitative yield of (*S*)-*N*-4-{2,3-bis[bis(carboxymethyl)amino]propyl}phenylbromoacetamide (BBA-EDTA) and its phenyl isothiocyanate analogue (BI-EDTA).[55]

Derivatives of tetraazacyclotetradecanetetraacetic acid (TATA of H_4tata) (Scheme 5) have also been obtained in a high yield using a readily available starting material.[58]

III. Synthesis of conjugates based on bifunctional complexones

In the preparation of conjugates, the sequence of reactions in the synthesis of the final products can be various. For BBA-EDTA, BI-EDTA, and the anhydrides of DTPA and E-DTPA, it is recommended that the chelating agent is initially coupled to the protein and then the cation-label is introduced into the system. For TATA, the synthesis is usually carried out in the opposite sequence,[10] but the first version is also permissible.

The coupling of the anhydrides of TATA and DTPA to proteins proceeds as a rule readily and under mild conditions. For example, in order to obtain the conjugates based on B-DTPA, the dried and purified anhydride of DTPA is treated with a bicarbonate buffer solution of the protein (10–15 mg ml^{-1} in 0.05 M solution of sodium bicarbonate at pH 8.5). The free DTPA is removed from the bound protein with Sephadex-650 molecular sieve or by centrifugation.[59]

The combination of B-DTPA with proteins is statistical in character: some of the protein molecules always remain free, whilst others combine with several B-DTPA fragments at once. Calculation shows that, for the average ratio [protein]:[B-DTPA] = 1:3, only a small proportion of the macromolecules remain unbound to the complexone, whereas for the 2:1 ratio more than 60% of the protein reacts with B-DTPA.[59]

It is quite evident that the proteins can lose activity as a result of immobilisation, the degree of degradation increasing with increase in the number of attached complexone molecules. It has been noted that the probability of side reaction involving so called 'cross' binding to the antibodies i.e. the 'cross linking' of the protein molecules with one another, is relatively high for A-DTPA. The chelating capacity of the complexone then diminishes sharply and it becomes necessary to carry out an additional purification of the reaction mixture in order to remove such 'blank' conjugates with a double molecular mass.[33]

Comparison of the relative effectiveness of the conjugation of B- and A-DTPA showed that the latter reagent is unstable, hydrolysing at an elevated temperature, and it is therefore not

possible to isolate and analyse it. Accordingly, the determination of the protein : anhydride ratio involves significant difficulties. On the other hand, B-DTPA is stable and the synthesis involving it is to a large extent susceptible to monitoring. A satisfactory reproducibility of the coupling reaction is attained as a result.[59]

It has been established that the effectiveness of the incorporation of the radioactive indium-111 into systems obtained by treating various proteins with B-DTPA ranges from 30% to 60% of the indium-111 isotope introduced, depending on the nature of the antibodies.[32]

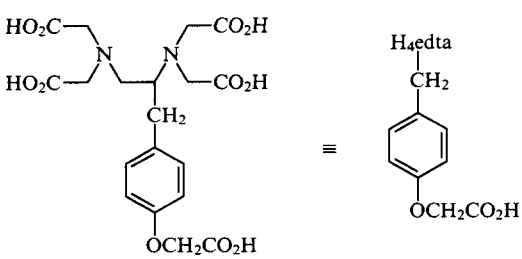
A minimal probability of cross-link formation has been noted for E-DTPA. For example, other conditions being equal, E-DTPA retains its antigen binding activity to a greater extent than B-DTPA — 100% and 70% respectively. Furthermore, the conditions needed to attain the maximum effectiveness of the coupling of E-DTPA to proteins are closer to the natural physiological conditions under which proteins exist than in the case of B-DTPA. In particular, maximum coupling efficiency has been noted for B-DTPA at pH 8.3, whereas in the case of E-DTPA it occurs in the region of pH 7.[60]

The number of variants of the coupling to proteins is much smaller for B-DTPA than for E-DTPA.[60] The bicyclic anhydride is capable of interacting mainly only with the accessible lysine residues, whereas E-DTPA adds also to the histidine and tyrosine fragments. Among the disadvantages of E-DTPA, one should include also its ability to form with proteins conjugates with a higher molecular mass and lower stability in vivo than the conjugates formed by B-DTPA. The practical consequences of this may be a higher degree of accumulation of the E–DTPA-labelled proteins in the liver and a considerable dose of the accumulated activity in the kidneys and in the bladder.[30]

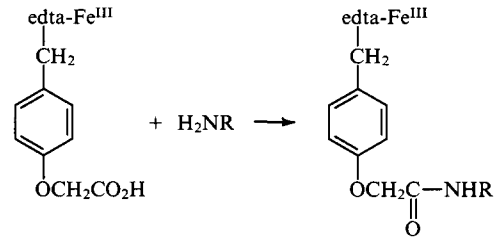
The coupling to proteins of the bifunctional complexones formed as a result of the modification of the hydrocarbon 'skeleton' is usually carried out by simple mixing of the components at pH 8–9 whilst cooling the mixture. The complexone is as a rule taken in excess. At the end of the coupling reaction, lasting on average several hours, the conjugate is separated from the reaction mixture by dialysis. It has been noted that in certain cases, when the average number of chelating molecules bound to the protein is greater than 3, a loss of the immune activity of the antibodies is observed.[10,20,35,37]

It has been established that the haloacetamide groups are distinguished by a low selectivity in reactions with natural macromolecules. These groups interact effectively with a wide range of nucleophilic centres of proteins, including the sulfhydryl and amino-groups. The resistance of these groups to hydrolysis under the conjugation conditions (37 °C, pH 9.5) makes it possible to obtain the necessary number of protein-bound chelating groups ($c > 10^{-5}$ M), which ensures, in its turn, the rapid and quantitative binding of the metal cations in the next stage of the synthesis of the conjugate.[23] The immune activity of the complexone–protein conjugates is retained for the group of compounds under considerations at a level of 77%–84% relative to control antibody specimens.[61]

1-(*p*-Carboxymethoxybenzyl)-EDTA occupies a special place in the overall series of benzyl derivatives of EDTA.



A necessary condition for the formation of the imide bond between this compound and the protein molecule is the preliminary formation of a complex with a metal, which protects the chelating fragment from undergoing the analogous reaction. A cation-label, for example the radioactive indium(III) or iron(III) isotopes, is usually selected as the complex-forming agent. The iron complexonate is obtained by the interaction of the complexone with iron(II) at pH 2. The complexonate produced is rapidly oxidised by atmospheric oxygen to the corresponding iron(III) derivative [subsequently the iron(III) complexonate must be protected against photodegradation]. The iron(III) chelate, taken in excess, readily reacts with human serum albumin at 20 °C and pH 5.[62]



R = protein

If necessary, the iron(III) may be removed from the conjugate by reduction during dialysis, by employing a buffer solution containing ascorbate and citrate ions and EDTA at pH 5. In the absence of ascorbate ions, the iron(III) is not removed from the conjugate.[62]

Cations can be easily introduced into the system by simple mixing of the components (with some excess of the metal) at pH 6, established with the aid of an acetate buffer solution.[10,63] Depending on the nature of the cation and of the chelating component of the immobilised protein, the reaction lasts from several minutes (indium-111)[10] to several hours (yttrium-90).[64] The conjugate is separated by dialysis from the excess unreacted components. It has been shown[30,65] that the addition of EDTA is extremely effective in the stage involving the elimination of the free radioisotope from the conjugate.

IV. Physicochemical properties and structures of bifunctional complexones and the conjugates based on them

There are virtually no literature data on the most important physical, chemical, and biological properties of the bifunctional complexones. This can be accounted for to a large extent by the difficulty of isolating such complexones and of eliminating the impurities from the latter (this is needed for accurate potentiometric and spectroscopic studies) and also by the low yield of the final products. Therefore, in constructing the bifunctional complexone molecule, designed to chelate a specific cation, and also in the initial quantitative and qualitative assessment of its complex-forming properties, one has to be guided by the information accumulated for the usual complexones and complexonates, introducing appropriate corrections into the latter.

1. Thermodynamics of the complex formation reaction

Bifunctional complexones, which constitute a special case of classical complexones, are capable of chelating the vast majority of the cations derived from the metals in the Periodic System.[1,10] For biological and medical purposes, the cations of radioactive isotopes (^{111}In, ^{99}Tc, ^{90}Y, ^{67}Cu, ^{46}Sc, ^{67,68}Ga, ^{153}Gd, ^{109}Pd),[10,66–71] paramagnetic cations (Gd^{3+}, Mn^{2+}, Fe^{3+}),[72–76] and fluorescent cations (Eu^{3+}, Tb^{3+})[77,78] are of greatest interest. The thermodynamics of the complex formation reaction is then considered taking into account the presence of the background biometals, for example calcium, which are present in vivo in an excess relative to the cation-label and behave as a competitor capable of displacing the label from the conjugate, reducing thereby the specificity of the action of the preparation introduced.[1,79–81]

For the estimation of the ability of the bifunctional complexone H_nL to form complexes with the cation of the metal M in a preliminary stage, use is made of the concentration stability constant K_{ML} measured at a fixed ionic strength I and a fixed temperature, i.e.

$$K_{ML} = \frac{[ML]}{[M][L]},$$

and also of the complex formation selectivity index — $K_{ML} : K_{CaL}$ (or $\Delta \lg K = \lg K_{ML} - \lg K_{CaL}$).

Our analysis of the literature values of the stability constants (see the stability constants handbooks[79-85] and the relevant reviews[86-88]) and inspection of the contents of the computer data bank produced for commercial use by the IUPAC Commission on Chemical Equilibria in 1993[89] revealed that the amount of thermodynamic data, permitting an indirect characterisation of the complex-forming capacities of the bifunctional complexones, is also extremely small. Direct thermodynamic studies on bifunctional complexones have been scarcely carried out. The first reports on this topic appeared comparatively recently.[90]

Table 1 presents certain data for EDTA and DTPA, which permit the qualitative estimation of the trends in the variation of the stabilities of the complexes on passing from classical complexones to their modifications modelling to some extent the bifunctional complexone.

The constants presented in Table 1 show that the introduction of aliphatic and aromatic groups into the ethylenediamine fragment of the complexone not only does not reduce the stability of the complexes but can actually tend to increase it. This effect is apparently associated with the stabilisation by the given group in solution of the ligand conformation most favourable for complex formation.

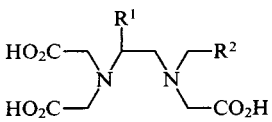
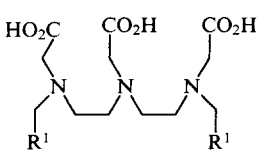
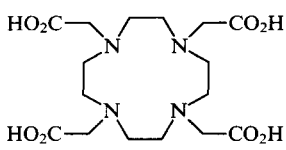
The replacement of one of the carboxy-groups by a hydrocarbon substituent leads unambiguously to a decrease in the overall stability of the complexes. However, there is then a possibility of an extremely significant increase in selectivity in complex formation by the typical cation-labels Cu^{2+} and Gd^{3+} relative to the endogenic background cations Zn^{2+} and Ca^{2+}.

It is remarkable that the replacement of the carboxylate fragment by the acetamide fragment also induces a sharp decrease in the stability constant. This suggests that in the case of gadolinium the decrease in the stability constant is associated with the decrease in the effective denticity of the ligand: the noninvolvement of the acetamide fragments in the formation of coordinate bonds with the lanthanide ion.

Ligands with 'built in' macrocycles are of considerable interest. Apart from the polychelating effect, the macrocyclic effect also makes an additional contribution in this case.[1,94-97] For example, the use of 1,4,7,10-tetraazacyclododecane-*N,N',N'',N'''*-tetraacetic acid (DOTA or H_4dota) instead of EDTA makes it possible to increase the stability of the europium(III) complexonate by ten orders of magnitude. The selectivity in relation to calcium ion increases simultaneously.[1] At the same time, the differences between the stabilities of the copper, calcium, and zinc complexes are abolished to a significant extent.

The next stage in the approach to a real system involves the consideration of the effective (and not concentration) stability constants K_{ef}, in which account is taken of the hydrolysis of the complexonate. The need for such allowance is due to the tendency of polyaminopolycarboxylic acids to be protonated at the physiological pH (7.2). For example, EDTA is present in the equilibrium mixture under such conditions both in the form of the complex and in the form of the anions $Hedta^{3-}$ and H_2edta^{2-}. This reduces significantly the fraction of the complex proper. Furthermore, the aquometal ions are also partly hydrolysed at the above pH and are present in solution in the form of monomeric and polymeric hydroxy-complexes. The constants K_{ef} make it possible to estimate the pH values most favourable for the complex formation reaction between the given ligand and the given cation.[1,98,99]

Table 1. Stability constants of the complexes formed by EDTA, DTPA, DOTA, and their derivatives (I = 0.1; 20–25 °C).

Ligand (H_nL)	R^1	R^2	$\lg K_{ML}$				Ref.
			Ca^{2+}	Zn^{2+}	Cu^{2+}	Gd^{3+}	
	H (EDTA)	COOH	10.79	16.68	18.86	17.27	88
	Et	COOH	11.75	18.23	20.6	18.6	85
	Ph	COOH	11.25	17.05	19.4	17.4	85
	H	Pr	16.1	–	12.7	13.07[a]	85, 91
	H	Ph	6.7	–	16.8	–	85
	COOH (DTPA)		10.89	18.55	21.53	22.56	88
	C(O)NHMe		7.17	12.04	13.03	16.85	18
	Py		7.97	14.02	17.50	16.83	18
 DOTA			15.85	18.90	19.06	28.21	92, 93

[a] Data for europium(III) (I = 1) are presented.

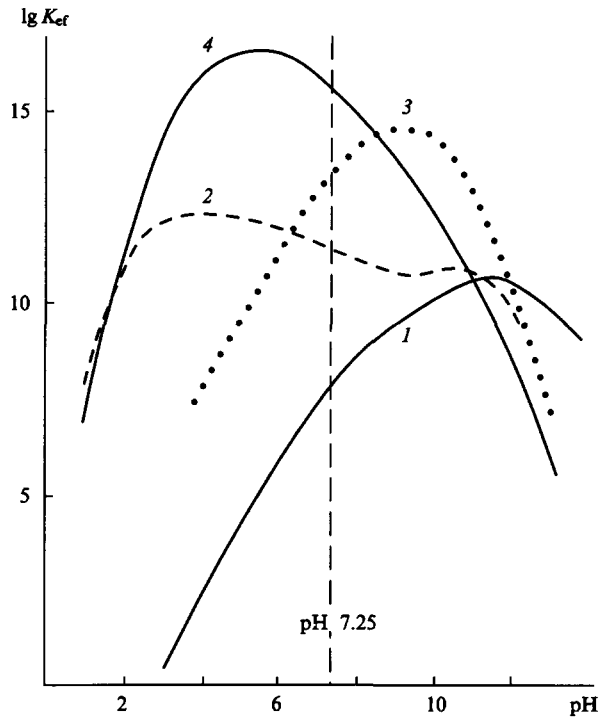


Figure 3. pH dependence of the effective stability constants of the complexes of EDTA with different ions: (*1*) Ca^{2+}; (*2*) Fe^{3+}; (*3*) Sm^{3+}; (*4*) In^{3+}.[100]

Fig. 3 presents the dependence of the logarithms of K_{ef} on pH for the complexes of EDTA with typical cation-labels: Fe^{3+}, Sm^{3+}, and In^{3+} (curves *2–4*). The corresponding values for the background element (calcium — curve *1*) are presented for comparison. The figure shows that in the alkaline region the complex formation reaction between EDTA and calcium becomes preferable to the reaction with indium or samarium.[100]

A clear idea about the ratio of the concentration and effective constants is provided by the following example. Despite the fact that the concentration stability constant of the iron(III) complex of EDTA is 10^6 times greater than that of the copper(II) complex of EDTA, at pH 7 the chelate $[Cuedta]^{2-}$ is 10^2 times more stable than $[Feedta]^-$ according to the calculations of the effective constants.[101]

It is also noteworthy that the calculations of the effective constants performed by different workers using different initial postulates can differ significantly[100,101] and as a rule require a critical analysis.

The problem of predicting the interaction between the complex-forming agent and the ligand in vitro in multicomponent systems, incorporating, apart from the bifunctional complexone, a series of bioligands and a wide range of background cations is much more complex.[79–81,101] Calculations have shown that the introduction of only one additional component into the In^{3+}–EDTA system reduces the K_{ef} at pH 7.4 by six orders of magnitude.[101]

Finally, difficulties which are truly impossible to overcome are faced by the experimenter when an attempt is made to assess quantitatively the thermodynamics of the complex formation reaction in vivo.[101,102] Nevertheless, on the whole knowledge of the thermodynamic stability constants makes it possible to predict fairly successfully the stability of the conjugate.[68,103]

2. Kinetic stability of the conjugates

Apart from the thermodynamic stability of the conjugates, when the latter are modified it is necessary to take into account also their kinetic stability: the rate of loss by the conjugate of the cation as a result of the dissociation of the complex or the substitution reaction between its 'own' cation-label and the competing biometals. Therefore, together with the problem of creating a complex with the bifunctional complexone which is thermodynamically as stable as possible, the problem of retarding the decomposition of the coordination compound appears no less important.

The rates of formation of the complexonates from the aquometal ions and also of the reverse process involving their dissociation are comparable to the rate of exchange of the water molecules in the aquo-ion, i.e. are determined to a first approximation by the properties of the aquo-ions.[1,104]

The introduction of an element of rigidity into the structure of the complexone molecule, in particular the introduction of macrocycles, makes it possible to retard significantly the dissociation of the complex formed by the complexone. For example, while the electrostatic charge on the anion is the same, the rate of dissociation diminishes sharply in the series EDTA–CDTA–DOTA (CDTA or H_4cdta = cyclohexanediaminetetraacetic acid).[1,104] It has been suggested that the kinetic features established in vitro for the classical complexones are also valid for their bifunctional analogues in vivo.

The kinetics of the loss of the metal atom by chelate complexes under the conditions of the living organism have been investigated.[10,34,105] The decomposition of the complex is a consequence of the operation of several factors at once, both kinetic and thermodynamic. In particular, it has been shown that the copper(II) complexes in human blood serum lose the metal in conformity with the prediction based on the analysis of the properties of simpler compounds. Thus the macrocyclic complexonate formed by TATA loses copper at a rate of 1%–3% in 24 h, the chelate based on the DTPA monoamide loses it at a rate of ~50% in 24 h, while the complex based on benzyl-EDTA loses the metal faster still.

The rate of loss of the metal by indium(III) complexonate has been investigated (Table 2).[105] It follows from the data presented that the coupling of the bifunctional complexone to albumin does not influence significantly the rate of biodegradation of the complex. At the same time, it is necessary to note that the ratio of the rate of degradation of the butylamides of EDTA and DTPA is directly opposite to that for EDTA and DTPA. Nevertheless, on the basis of the coordination possibilities of the modified ligands, the tendency in the variation of the rate of degradation of the modified ligands should be of the same type as for the rates of degradation of the corresponding unmodified ligands. The significant scatter of data between different series of experiments indicates the existence of a poorly monitored error.

Table 2. Kinetics of the in vitro loss of indium(III) by complexones, bifunctional complexones, and their conjugates with albumin.[105]

Ligand	Rate of loss of indium by the complexonate ML(%) in 24 h
1-(*p*-Carboxymethoxybenzyl)-EDTA	0.028 (0.041)
1-Methyl-EDTA	0.060 (0.052)
Albumin-phenyl-EDTA	0.060
Albumin-benzyl-EDTA	0.11
EDTA	0.53 (0.67)
EDTA mono-n-butylamide	29.0
DTPA mono-n-butylamide	0.93
Albumin-DTPA	1.6
	2.5[b]
DTPA	2.3 (2.6)

[a] The transfer of indium from the initial chelate complex ML to the complex with transferrin was investigated in human blood serum at pH 7.3 and 37 °C; the data for a repeated series of experiments are presented in brackets.

[b] A 0.01 M glycine solution was additionally introduced into the system.

3. The structure of the complexonates

Together with the thermodynamic, kinetic, and biological properties of the bifunctional complexones, their structure is an extremely important practical characteristic: the real denticity of the ligand and the coordination number of the cation. This is particularly important for the NMR-contrasted substances, since their capacity for paramagnetic interaction with the substrate is higher, other conditions being equal, the greater the number of water molecules retained in the coordination sphere of the paramagnetic cation incorporated in the conjugate.[106,107]

The structures of the metal complexonates have been examined in brilliant reviews.[108-110] Here we shall dwell only on two most important features discovered by analysing the results of more than 300 X-ray diffraction studies:

(i) The coordination number of the cation coordinated by the complexones frequently proves to be higher than those observed for the same ions in complexes with simple monodentate ligands. For example, manganese(II), cobalt(II), magnesium(II), and iron(III) in their EDTA and DTPA complexes exhibit a coordination number of 7, which is unusual for these metals. Because of this factor, the hexadentate EDTA ligand does not displace the entire water from the inner coordination sphere of iron(III), while the anion $[Fe(H_2O)edta]^-$ is a more effective relaxant than might have been expected. Indium(III) has a coordination number of 7 in its EDTA complex[108] and 8 in its DTPA complex.[111]

(ii) For all the structurally investigated crystalline normal complexonates ML and ML_2 with a coordination number greater than or equal to the denticity of the ligand, all the electron-donating ligand atoms are coordinated to the cation. Not a single case has been found in which the water molecule competed with the deprotonated carboxy-group for a site in the coordination sphere of the cation, i.e. a situation where the water molecule would have entered into the composition of the coordination sphere of the metal while one of the deprotonated carboxylate groups remained 'free'. On the basis of these data, one may predict, with a high degree of probability, the degree of shielding of the cation by the ligand on the basis of the structure of the molecule of the chelating agent itself.

Here it is appropriate to mention the lively discussion concerning the possibility of the retention of the structure of the complexonate when it passes from the crystalline phase to an aqueous solution.[112-118] Systematic radiospectroscopic[107,119-122] and EXAFS studies[123] in recent years have shown that the principal features of the structures of the complexonates, in particular the denticity of the ligand, are retained on dissolution. Thus the prediction of the structures of both simple complexonates and of the chelating component of the conjugates of bifunctional complexones in solution, based on X-ray diffraction structural data, is entirely correct.

V. Certain problems in the search for new effective ligands

In the general case, the isolation and analysis in a 'pure form' of any factors influencing the stability constants K_{ML} of polydentate, including bifunctional, complexones are extremely difficult. Thus any variation of the denticity of the ligand is as a rule coupled with the simultaneous variation of several parameters: the electrostatic charge, the size of the chelate ring, the ratios of the numbers of electron-donating atoms of different nature, etc.

Procedures such as variation of the denticity and of the dimensions of the rings potentially capable of being formed and the introduction into the structure of the chelating agents of elements of rigidity as well as heteroatoms, which ensure the selectivity of action in relation to the cation under consideration, are most widely used.[1]

For example, the introduction of the phosphonate group instead of the carboxylate group sharply increases the selectivity of the chelating agent in relation to copper(II) ions (Fig. 4). The tropicity (selectivity) in relation to bone tissue frequently

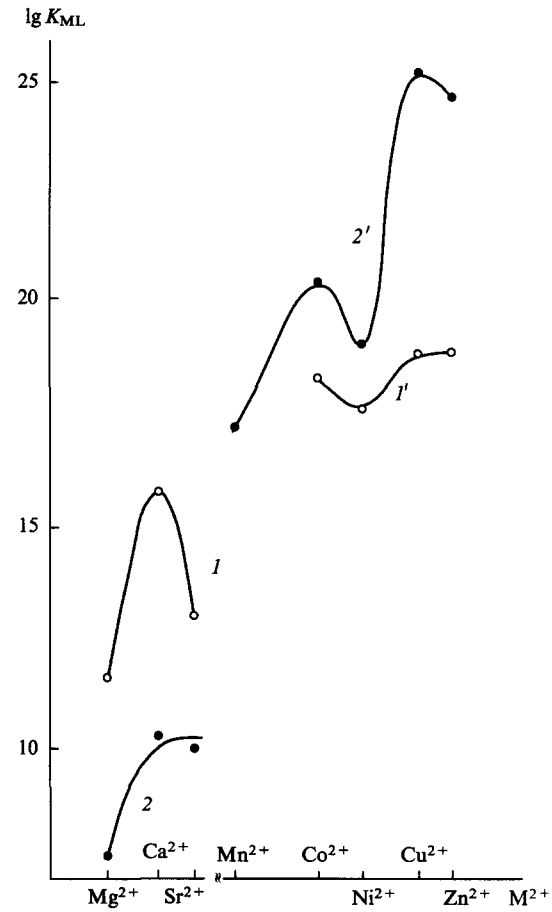


Figure 4. Relative changes in the stability constants in the series of alkaline earth (curves *1* and *2*) and 3*d* cations (curves *1'* and *2'*) for DOTA (curves *1* and *1'*) and its methylenephosphono-analogue (curves *2* and *2'*).[1,89,92,93]

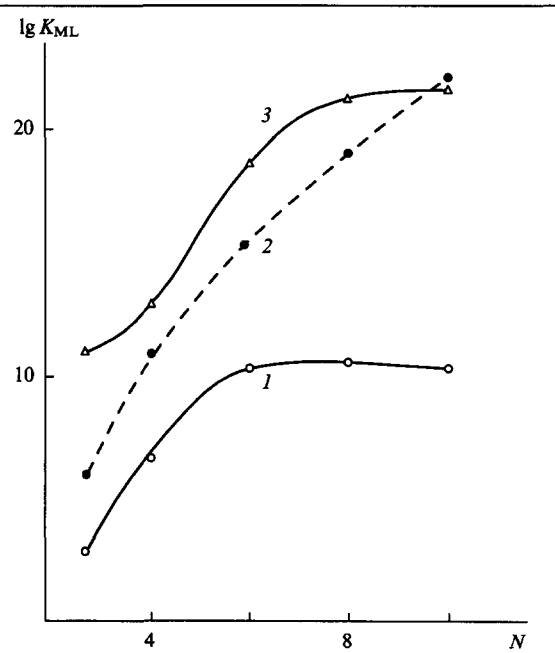


Figure 5. Dependence of the stability constants of the M^{n+} complexonates on the potential denticity of the ligand (*N*); M = Ca (curve *1*), La (curve *2*), or Cu (curve *3*). Data[85] for IDA, NTA (NTA or H_3nta = nitrilotriacetic acid), EDTA, DTPA, and TTHA (TTHA or H_6ttha = triethylenetetraminehexaacetic acid), considered as models of tri-, tetra-, hexa-, octa-, and deca-dentate chelating agents, were employed.

increases under these conditions in the complex formed.[124,125] An increase in the denticity and the charge of the ligand (Figs 5 and 6) as a rule renders this ligand more selective in relation to lanthanides, which have large coordination numbers.[126-129]

At the same time effects which are difficult to explain are also observed. For example, there is no equivalent increase in the stability of the complexes of the barium ion on passing from the hexadentate EDTA to the octadentate DTPA, although the size of the ion is not less than that of the lanthanide ions.

The creation of a chelating agent for a specific cation frequently extends over many decades. As an example, we may consider the development of events in the construction of the molecule of the optimum chelating agent for the iron(III), gallium(III) and indium(III) ions.

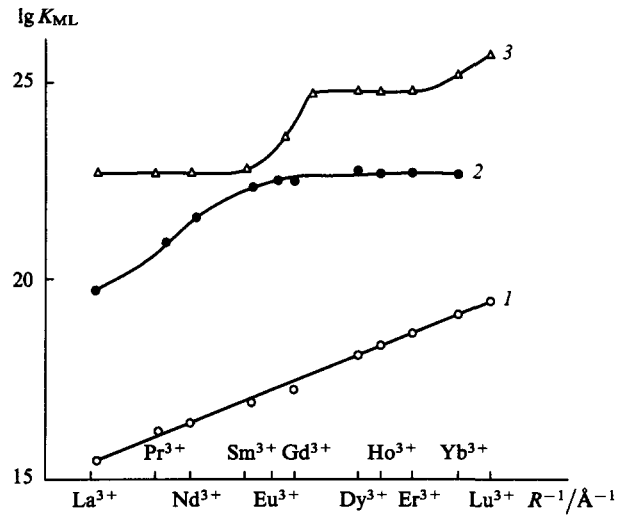
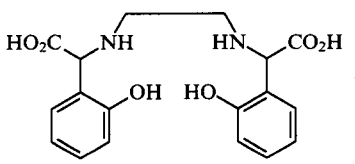
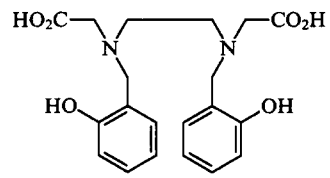


Figure 6. Dependence of the stability constant of the chelating agent on the ionic radius of the lanthanide (R):[85,88,89,93] (*1*) EDTA; (*2*) DTPA; (*3*) DOTA.

At the end of the 1950s and the beginning of the 1960s, it was found that the combination in the complexone molecule of fragments of ethylenediamine-*N,N'*-diaacetic acid and phenol, which happens in *N,N'*-ethylenebis[2-(*o*-hydroxyphenyl)glycine] (H_2ehpg),



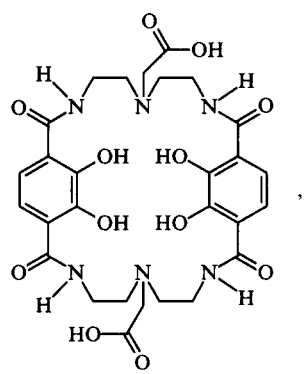
increases sharply the stability of the iron(III) complex ($\lg K_{ML}$ = 33.9) compared with the universal chelating agents such as EDTA ($\lg K_{ML}$ = 25.0).[130,131] The success achieved initiated the synthesis of a series of preparations with contrasting properties.[132-137] However, according to L'Eplattenier et al.,[138] the relative positions of the donor atoms in the ligand considered generated a steric hindrance to their simultaneous involvement in coordination. Later X-ray diffraction studies on the complexes $[Mg(H_2O)_6][Gaehpg]_2 \cdot 3H_2O$, $[Co(H_2O)_6][Coehpg]_2 \cdot 4H_2O$, $Na_2[Cuehpg] \cdot 5.5H_2O$, as well as the iron(III) complex[139-140] showed that these fears were groundless: in all the complexes investigated, the ligand exhibits the maximum possible denticity of six by coordination of both nitrogen atoms, the two oxygen atoms from each carboxylate group, and the two oxygen atoms of the deprotonated hydroxy-groups. Nevertheless, the sterically free complexone ethylenediamine-*N,N'*-di(2-hydroxybenzyl-*N,N'*-diaacetic acid (H_2hbed),



made it possible to achieve the highest attained stability for an iron(III) complex: $\lg K_{ML}$ = 39.68.[138] The corresponding constants for indium(III) and gallium(III) also proved to be some of the highest (39.66 and 39.57).[141] These studies stimulated in their turn the synthesis of a series of bifunctional ligands based on H_2hbed.[68,142]

Subsequent experiments on the modification of similar structures led to the following results. The replacement of the carboxy-groups in H_2hbed by phosphono-groups made it possible to increase the selectivity of the complexone in relation to the copper(II) ions in the series of 3*d* elements.[143] Although another modification of H_2hbed, involving the replacement of the phenol moiety by a pyridine group, led to the formation of iron(III) complexes less stable than the corresponding compound derived from H_2hbed ($\lg K_{ML}$ = 36.91), it made it possible to increase its solubility and also its acceptability to the human organism.[143,144]

Contrary to expectations, attempts to synthesise on the basis of H_2hbed the macrocyclic complexone 4,17-bis(carboxymethyl)-10,11,23,24-tetrahydroxy-8,13,21,26-tetraoxo-9,10,11,12 : 22,23, 24,25-dibenzo-1,4,7,14,17,20-hexaazacyclohexacosane,[145]



did not lead to a further increase in the stability of the iron(III) complexes, although the stability constant proved to be extremely high — $\lg K_{ML}$ = 37.6 (25 °C, I = 0.1).

It is noteworthy that a high stability of the complex is by no means always the main aim. The structure of the chelating agents is frequently dictated by purely biological or methodological features. For example, as a consequence of the low stoichiometric chelate : protein ratios, the cyclic dianhydride of DTPA proved to be entirely effective for the creation of bifunctional conjugates with radioactive cations.[34] However, magnetic-resonance contrasting requires complexes with a high chelating agent : protein ratio. Under these conditions, the given chelating agent is less suitable,[146] since an increase in the relative content of the dianhydride of DTPA entails a sharp increase in the number of intermolecular covalent bonds involving it and in the number of heterogeneous products formed on conjugation; under these conditions, the reproducibility and technological applicability of the synthesis fell.

This obstacle can apparently be overcome by varying the macromolecular component of the conjugate. For example, when albumin was replaced by polysaccharides, it was possible to obtain conjugates with DTPA and gadolinium(III) virtually without intermolecular cross links.[147]

Another problem is the permeability of the cell membrane to the conjugate. Its solution requires the optimisation of a large

number of parameters. It has been shown[148,149] that the membranes are satisfactorily permeable to electrically neutral complexes. Electrical neutrality also reduces the osmotic pressure. However, this is accompanied by a decrease in the solubility of the complexonate itself[1] and also in the effective denticity of the complexone, which leads as a rule to a decrease in the thermodynamic stability of the complex and hence to an increase in its toxicity owing to the removal from the complex of a large amount of the cation-label in a 'free' state.

For example, the gadolinium complex of DTPA proved to be an extremely effective contrasting agent in NMR tomography by virtue of its high stability constant.[150] However, as a consequence of the high negative charge of $[Gddtpa]^{2-}$, this preparation is poorly assimilated through the gastrointestinal track[151] and can be used only in the form of injections. The replacement of DTPA by its dimethylamide derivative made it possible to obtain an electrically neutral gadolinium complex with a significantly higher permeating capacity but a lower thermodynamic stability in relation to dissociation into the 'free' ligand and the metal ion. However, subsequent studies showed that the toxicity of the complex not only did not increase but actually fell (Table 3). This is due to the fact that the stability of the complexes of the dimethylamide of DTPA with ions competing with gadolinium (calcium, zinc) diminishes to an even greater extent and under in vivo conditions the selectivity in the binding of gadolinium and the lethal dose increase.[18]

Table 3. Results of toxicological studies on gadolinium complexes (intravenous administration to mice).[18,151]

Ion investigated, complex	$\Delta \lg K_{ef}$ [a]	LD_{50}/ mmol kg^{-1}
$[Gd^{3+}aq]$	–	0.4 [b]
Na_2Hdtpa - BMA [c]	–	0.16
Na[Cadtpa - BMA]	–	16.6
[Gddtpa - BMA]	9.04	14.8 (0.7)
Na_2[Gddtpa]	7.04	5.6 (0.2)
	–	10.0 [b]
[Gddtpa - BP] [d]	5.32	2.8 (0.1)
Na_2[Gdedta]	4.23	0.3 (0.1)

[a] The difference between the logarithms of the effective stability constants of the gadolinium and zinc complexes with the given ligand at pH 7.4 was adopted as $\Delta \lg K_{ef}$.
[b] Intravenous administration to rats.
[c] dtpa - BMA = DTPA *N,N″*-bis(methylamide).
[d] dtpa - BP = *N,N″*-bis-(2-pyridylmethyl)-DTPA.

It is remarkable that an X-ray diffraction study of the gadolinium(III) complex of the dialkylamide of DTPA[152] did not reveal a decrease in the denticity of the complexone compared with the DTPA itself;[153] a coordination number of 9 in the case of gadolinium is achieved as a result of the presence of the octadentate ligand and one water molecule. Admittedly, the average metal - oxygen distances in the amide fragments proved to be greater than in the acetate fragments. The problem whether this structure is retained on dissolution of the complex still remains open.

* * *

On the whole one may conclude that the predictions of the thermodynamic and kinetic stabilities of the conjugate based on estimates of the properties of simple model chelates have proved to be fairly reliable. In particular, the high stability constants ($\lg K_{ML} > 20$) of the indium(III) complex of EDTA and the gadolinium(III) complex of DTPA make it possible to obtain effective indium- and gadolinium-containing diagnostic and therapeutic preparations, in which the chelating fragment is based on EDTA and DTPA derivatives.[154-157] The approach based on the kinetic stability of CDTA and DOTA complexonates proved no less fruitful. Thus the conjugates based on the CDTA and DOTA ligands retain the kinetic features characteristic of their classical analogues.[158,159]

On the other hand, successful bioconjugates could not be created on the basis of complexones such as IDA and NTA, which form complexes with low and moderate stabilities ($\lg K_{ML} < 17$).

However, one should note that, even in the case of the classical chelating compunds, it is not always possible to predict the optimum effect in vivo. For example, in selecting the ligand for the extraction of plutonium from the human organism it was found that the DTPA-based preparation was just as effective as the tetracarboxycatecholate ligand, which forms with plutonium complexes more stable by a factor of 10^{10} than those formed by DTPA.[160]

Bifunctional complexones constitute a vigorously developing field in the chemistry of chelate compounds. It may be expected that in the immediate future the search for chelating agents and conjugates with a specific tropicity in relation to particular organs and tissues will assume a greater importance.

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