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EFFECT OF METAL CHELATING AGENTS ON THE STORAGE OF NOREPINEPHRINE *IN VITRO* BY CEREBRAL SYNAPTIC VESICLES*

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Abstract—The inhibitory effects of fourteen different metal chelating agents on the storage *in vitro* of [^{3}H]DL-norepinephrine (NE) by the isolated synaptic vesicles from rat brain were investigated with a view to explore the possible importance of metal chelation phenomena. The chelators under examination belong to the classes of polyamines, aminocarboxylic and hydroxy carboxylic acids exhibiting a wide range of metal-binding strengths. Values of IC_{50}, viz., the amount of the chelator which will inhibit 50 per cent of the storage of [^{3}H]DL-NE, were determined for the different compounds. The strengths of chelate formation of the "synaptosomal and vesicular metal ions", viz. Cu^{2+}, Fe^{2+}, Fe^{3+}, Zn^{2+}, Mg^{2+} and Ca^{2+}, with the above compounds were compared with their inhibition *in vitro* of the vesicular storage of [^{3}H]DL-NE in order to determine if a correlation existed between the two. On the basis of regression analyses of the data on the stabilities of the individual metal chelates (log K_{ML}) and the inhibitory effects of the different chelators (log IC_{50}/NE), significant correlation coefficients were obtained with the Cu^{2+} and Zn^{2+} amine systems, i.e. 0.98 with $P < 0.02$, and 0.88 with $P < 0.02$ respectively. In regard to the metal–aminoacids systems, Mg^{2+} gave the most satisfactory correlation coefficient, i.e. 0.91, $P < 0.01$; with Fe(II) = 0.84, $P < 0.02$ and the mixed-metal system Cu + Zn + Fe + Mg + Ca = 0.87, $P < 0.05$. The existence of such correlation is considered significant from the point of view of the metal coordination hypothesis for the vesicular binding and storage of NE.

This work was undertaken as a part of our continuing investigations on the possible importance of metal chelation phenomena in the activity of the neurotransmitter amines. Since the discovery of the co-occurrence of the metal ions, viz. Cu^{2+}, Fe^{2+}, Fe^{3+}, Zn^{2+}, Mg^{2+} and Ca^{2+}, in cerebral synaptosomes and synaptic vesicles [1–3], model system studies on the chelation of a number of biogenic amines and ATP with the above "synaptosomal and vesicular metal ions" [4–8] have indicated the existence of a gradation of thermodynamic stabilities for the metal–amine binding. Some interesting correlation between the order of the metal–amine-binding strengths and the affinity order *in vivo* of the amines for granule binding [9] was also noted [4–8]. The objective of the present study is to determine effects of polyfunctional chelating agents on the storage of norepinephrine *in vitro* by synaptic vesicles. This approach is based on the assumption that if the vesicle-bound monoamines, ATP and the proteins were to exist in a "mutually chelated" form with the co-occurring metal ions, then the interactions of polydentate chelating agents with the vesicles might conceivably bring about significant inhibition of their binding of NE.† Chelating agents with different molecular backbones having two or more metal-binding groups of varying basicities might be expected to show differences in their inhibitory effects on the monoamine storage. In this investigation the method of IC_{50} was used. IC_{50} is the concentration (or the amount) of the chelator which will inhibit 50 per cent of the storage of [^{3}H]NE with which it is in competition. In any case, under a given condition of constant concentration of [^{3}H]NE, the relative values of IC_{50} for the various chelating agents give us a measure of their relative inhibitory effect.

The chelating agents that are investigated in this study are illustrated structurally in Fig. 1. Dotted lines show metal-binding sites.

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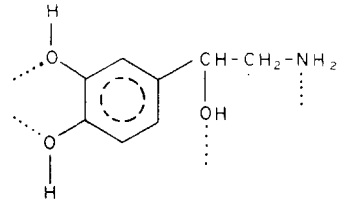
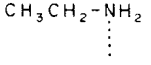
† Abbreviations used in the text: NE, DL-norepinephrine; [^{3}H]NE, DL-norepinephrine, DL-bitartrate[7-^{3}H(N)]; EDA, ethylenediamine; DETA, diethylenetriamine; DTPA, diethylenetriamine-penta-acetic acid; EDTA, ethylenediaminetetra-acetic acid; EGTA, [ethylene-bis-(oxyethylenenitrilo)] tetra-acetic acid; TTHA, triethylenetetraminehexa-acetic acid; EDDHA, ethylenediaminedi-(*O*-hydroxyphenyl-acetic acid); IMDA, iminodiacetic acid; ALA, alanine; CYS, cysteine; SSA, sulfosalicylic acid; and MA, mandelic acid.

EXPERIMENTAL

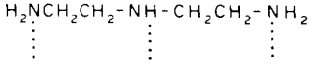
The synaptic vesicles were isolated from whole rat brain by a modification of the procedure described by Johnson *et al.* [10], which is in turn a modification of that of Whittaker *et al.* [11]. The essential steps of the procedure are as described below. Male Sprague–Dawley albino rats (200–250 g) were decapitated and the whole brain was rapidly (10–20 sec) removed to ice-cold 0.32 M sucrose. The pooled brains (3–4 g) were mechanically homogenized in 40 ml of 0.32 M sucrose in a Potter–Elvehjem tissue



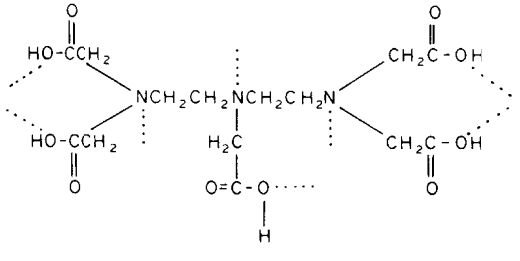
(I) Norepinephrine (NE)



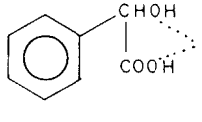
(II) Ethylamine (EA)



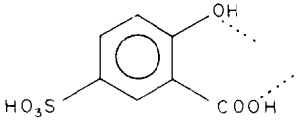
(III) Diethylenetriamine (DETA)



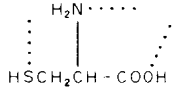
(IV) Diethylenetriaminepentaacetic Acid (DTPA)



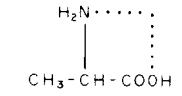
(V) Mandelic Acid (MA)



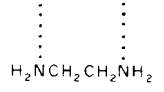
(VI) Sulfosalicylic Acid (SSA)



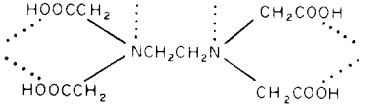
(VII) Cysteine (CY)



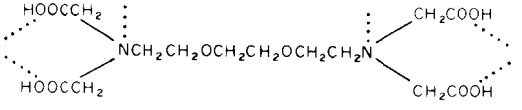
(VIII) Alanine (AL)



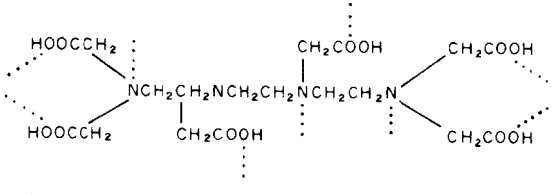
(IX) Ethylenediamine (EDA)



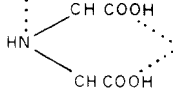
(X) Ethylenediaminetetraacetic Acid (EDTA)



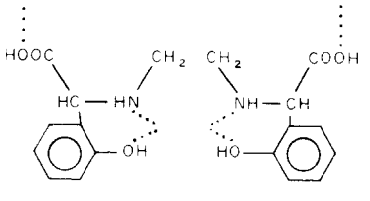
(XI) [Ethylenebis(oxyethylenenitrilo]tetraacetic Acid (EGTA)



(XII) Triethylenetetraminehexaacetic Acid (TTHA)



(XIII) Iminodiacetic Acid (IMDA)



(XIV) Ethylenediaminedi-(O-Hydroxyphenylacetic Acid) (EDDHA)

Fig. 1. Structural diagrams of fourteen chelating agents.

homogenizer fitted with a Teflon pestle of specific clearance (250 μm). Homogenization was for 1 min at 860 rev/min. The homogenate was centrifuged at 1,000 g for 10 min to precipitate blood, unbroken cells, and debris. The initial supernatant, S_1, was decanted and saved. The precipitate was washed with 20.0 ml of 0.32 M sucrose and centrifuged at 1,000 g for 10 min. The second supernatant, S_2, was combined with S_1 and centrifuged at 10,000 g for 20 min. The resulting supernatant was decanted and discarded. The pellet, P_3, was resuspended in 20 ml of ice-cold 0.32 M sucrose and centrifuged at 10,000 g for 30 min. The P_3 pellet corresponds to the "mitochondrial fraction" of Whittaker *et al.* and consists largely of mitochondria, intact synaptosomes, myelin fragments plus cellular debris. After centrifugation, the supernatant S_4 was discarded and the pellet, P_4, was "osmotically shocked" by resuspension in 30 ml of ice-cold, triple-distilled water, followed by mechanical homogenization for 2 min as already described. The homogenate was then centrifuged at 15,000 g for 30 min. The final supernatant, S_5, largely contains released synaptic vesicles, and small cellular debris. The S_5 fraction was divided into three 10-ml aliquots and each carefully layered onto a discontinuous sucrose density gradient consisting of 15 ml of 1.0 M, 5 ml of 0.80 M, 5 ml of 0.60 M, and 7.5 ml 0.40 M sucrose previously prepared. The gradients were centrifuged in an SW-25 swinging rotor in a Beckman model L centrifuge at 25,000 rev/sec (61,000 g) for 2 hr at 4°. The high-speed centrifugation produces a hazy, bluish-white band within the 0.40 M sucrose layer which may be siphoned using a pasteur pipette and held at 4° until diluted with triple-distilled water to 0.3 M sucrose and utilized directly in uptake experiments. An aliquot was saved for protein analysis. All experiments are begun within 1 hr of the dilution of the purified synaptic vesicle preparation.

In order to minimize any possible variations between the preparations of the synaptic vesicles used in the storage studies, a number of precautions were taken. The age, weight and number of animals used for any given series of experiments were kept as constant as possible. The rats were of the same sex and ranged in weight from 200 to 250 g. Protein analyses were carried out on each preparation. The control storage of [^{3}H]NE (per mg of protein) under the experimental conditions was found to vary by 10–20 per cent between the preparations. Experiments where the variation was greater than 20 per cent were discarded.

Chelating agents. Aqueous solutions of the chelating agents were prepared by dissolving the reagent grade compounds in double-distilled water. The pH values of the solutions were adjusted to 7–7.2 using NaOH or HCl by means of a glass–calomel electrode system.

NE storage. The norepinephrine storage experiments were based on the methods originally described by Colburn and Maas [1, 2] in which isolated mouse synaptic vesicles were incubated at 4° in a 0.32 M sucrose medium containing tritium-labeled norepinephrine and various chelators. In general, the greater the metal-binding strength, the greater might be the inhibition of storage of tritiated norepinephrine by the isolated synaptic vesicles, provided the transport characteristics of the chelating agents across the vesicular membranes are nearly similar.

In a preliminary series of storage experiments, solutions of DL-norepinephrine ([^{3}H]NE) (0.45 nmole) were prepared to which additions were made of either nonlabeled DL-NE or of potential inhibitors, viz. the chelating agents EA, EDA, DETA, DTPA and NE. The structural formulas of these and other compounds used in this study are illustrated by structures I–XIV. (It is seen that the various chelating agents furnish one through eight potential coordinate binding sites for the metal ions.) The uptake was initiated by addition of a 1.0-ml vesicle suspension in a total volume of 1.2 ml containing the different chelators and gently shaken at 30° for 45 min. The total amount of the chelator in the medium was varied from 0 to 10^4 nmoles. After incubation the uptake was terminated by rapid vacuum filtration using 0.2 μm pore size, celotate filters (Millipore Corp.). The retained vesicles and filters were not washed, but placed directly into a toluene-based scintillation fluid and counted for tritium label. For blanks, we used incubation mixtures with tritiated NE, but no vesicles. Also included in each set of experiments were tritiated NE to show the maximal (100 per cent) binding, and sufficient nonlabeled NE for partial (i.e. 50 per cent binding) as well as complete inhibition (0 per cent binding).

In an alternate series of experiments, the following chelators were investigated: EDA, NE, EDTA, EGTA, TTHA, EDDHA, IMDA, ALA, CYS, SSA and MA. In this, the millipore filtration step for the termination of the [^{3}H]NE uptake was replaced by an alternate procedure. Details of this series of uptake experiments are described below. Incubations were carried out at 30° for 45 min. Cellulose nitrate centrifuge tubes (3 in. × 5/8 in., made for the Beckman No. 40 rotor) were used as incubation vessels. All incubation vessels contained 0.286 nmole of tritiated norepinephrine (obtained as NET-048 from New England Nuclear Co.) in 0.020 ml and one of the following: (a) 0.100 ml of unlabeled norepinephrine, (b) 0.100 ml chelator (potential inhibitor at various concentrations), or (c) 0.100 ml of triple-distilled water.

The uptake of tritiated norepinephrine was initiated by the addition of 2.00 ml of the synaptic vesicle preparation (in 0.3 M sucrose). The total volume of all incubation vessels was 2.12 ml. The uptake of tritiated norepinephrine was terminated after 45 min by precipitation of the synaptic vesicles by centrifugation at 27,000 g for 20 min at 4°. The supernatant was discarded and the pellet containing the synaptic vesicles saved. The centrifuge tubes were inverted and allowed to drain onto paper for 15 min, then the inside of each tube was wiped out with a piece of absorbent paper wrapped around an applicator stick; special care was taken not to disturb the pellet. One ml of 0.1 N NaOH was added to each tube and each tube was mixed on a vortex to dissolve the pellet. The contents of each tube was placed directly into Bray's scintillation fluid and counted for the tritium label. Counting was performed by a Nuclear-Chicago Unilux II liquid scintillation counter equipped with a barium channels ratio mode for the estimation of quenching factor. Problems with color quenching and instability of quenching fluid were not encountered.

Table 1. Inhibition of binding of [^{3}H]NE by chelating agents*

Compounds	IC_{50}† Amounts (nmoles)	Ratio: (Chelating Agent)/NE
EA	3.0×10^3	2.0×10^3
EDA	13.5	9
DETA	28	18
DTPA	80	52
NE	1.3	0.9

* Binding data were obtained by using the millipore filtration technique.

† Amount of inhibitor necessary to inhibit 50 per cent of the uptake of 1.55 nmoles [^{3}H]NE.

It should be pointed out that the concentrations of NE used in the study were very small. In view of the extremely small amounts of the tissue fractions, the amounts of NaOH used were small. Furthermore, the time lapse between sample preparation and measurement was very small. Hence no color development was observed.

Table 2. Inhibition of [^{3}H]NE binding by chelating agents

Compounds	IC_{50}* Amounts (nmoles)	Ratio: Chelating agent/NE
NE	0.27	0.93
EDA	2.7	9.4
EDTA	2.0	7.0
TTHA	11	37
EGTA	20	70
EDDHA	105	362
IMDA	250	862
CYS	5.7×10^3	2.0×10^4
SSA	7.8×10^3	2.7×10^4
ALA	8.5×10^4	2.9×10^5
MA	No inhibition at 2×10^3 nmoles	

* Amount of inhibitor necessary to inhibit 50 per cent of the uptake of 0.29 nmoles [^{3}H]NE.

In each experiment, synaptic vesicles were incubated at 30° for 45 min in 0.3 M sucrose with 0.286 nmole of tritiated DL-norepinephrine and increasing amounts of unlabeled chelator (potential inhibitor). In each set of experiments, control (100 per cent binding) tubes were included. In these controls, 0.286 nmole [^{3}H]NE was incubated with the synaptic vesicle preparation, and 0.100 ml water replaced the chelator. For blanks, 6000 nmoles of unlabeled NE in 0.100 ml replaced the chelator. This amount of unlabeled NE is sufficient to inhibit essentially all storage of [^{3}H]NE. These blank tubes thus serve as an index of non-specific binding of NE (0 per cent synaptic vesicle binding).

It should be pointed out that the inhibition studies by EDA and NE were carried out by both methods described above, viz. millipore filtration and centrifugation, in order to determine if the results obtained by the two methods are similar and comparable. The data from the two series are presented separately in Tables 1 and 2.

RESULTS

Figure 2 represents the typical IC_{50} curves for the chelators, i.e. EA, EDA, NE and DTPA. Values of the per cent storage of [^{3}H]NE are plotted against the various amounts of the chelators, i.e. EA, EDA, DTPA and NE. The data obtained by employing the millipore filtration technique were used for tracing the curves in Fig. 2. Values of IC_{50} were obtained from these curves at the 50 per cent storage mark, and the data are presented in Table 1.

In the second series of experiments, values of IC_{50} for EDA and cold NE were redetermined by using the centrifugation method for the termination of [^{3}H]NE uptake (Table 2). All the subsequent experiments *in vitro* on the inhibitory effects of the other chelators, i.e. ALA, CYS, SSA, EDTA, TTHA, EGTA, IMDA and EDDHA, were carried out using the cen-

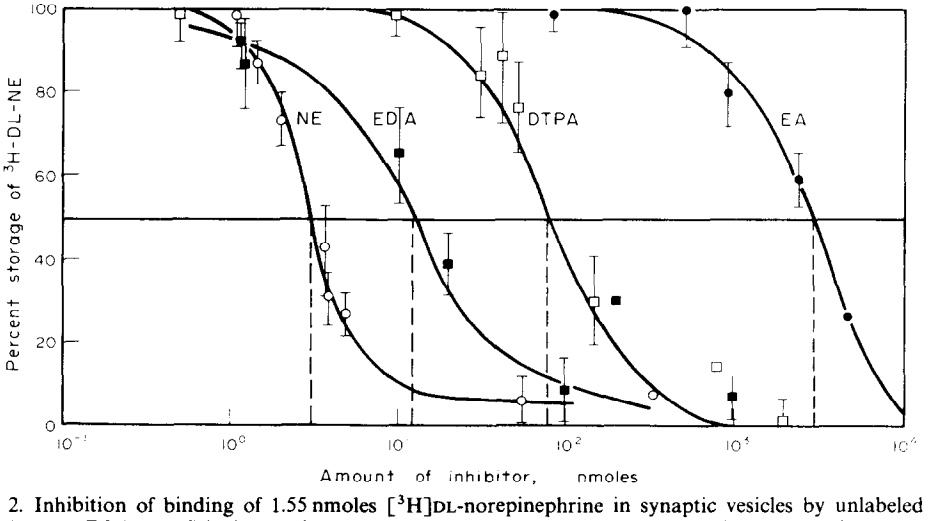


Fig. 2. Inhibition of binding of 1.55 nmoles [^{3}H]DL-norepinephrine in synaptic vesicles by unlabeled NE, DTPA, EDA and EA (incubation volume = 1.2 ml; sucrose = 0.32 M; each point in the graph is the average of four experiments).

Table 3. Metal chelate stabilities of some amines and their inhibition *in vitro* of [^{3}H]NE storage

Chelators	IC_{50} *in vitro** (ratios)	Metal chelate stabilities (log K_{ML})†				
		Cu(II)	Zn(II)	FE(II)	Mg(II)	Ca(II)
EA	2×10^3	11.5				
DETA	18	20.9	14.5(β_2)	10.4(β_2)	<1	<1
EDA	9.4	20.0(β_3)	12.9(β_3)	9.6(β_3)	<1	<1
NE	0.93	27.9(β_2)	17.0(β_2)	10(β_2)	2.9	2.2

* The IC_{50} ratio represents the ratio of the amount of the chelating agent to that of NE at 50 percent inhibition. β_2 and β_3 represent the overall stability constants for the metal chelates having metal:ligand ratios of 1:2 and 1:3 respectively.

† Martell and Sillen [12]; K_{ML} represents the thermodynamic equilibrium constants for the respective metal–amine binding reactions, viz. Cu(II) + NE $\rightleftharpoons$ Cu(II)NE, K_{ML} = [Cu(II)NE]/[Cu(II)][NE]).

trifugation technique. The IC_{50} values obtained for these systems are included in Table 2.

After obtaining the IC_{50} values for each of the chelating agents, the ratios of the amount of chelator necessary to inhibit 50 per cent of the NE uptake to that of NE with which it is in competition were determined for each of the systems. Values of the IC_{50} and the ratios are also included in Tables 1 and 2.

DISCUSSION

A comparison of the relative effectiveness of the different chelating agents in inhibiting the storage of NE *in vitro* by the vesicles is of interest. The lower the values of IC_{50} (and the ratios of the chelating agent to NE), the greater will be its competitive tendency for the inhibition of the storage. Colburn and Maas [1, 2] reported that 3.2 μmoles EDA considerably inhibited the binding of the 0.6 nmole NE in mouse brain vesicles at 0°. In addition to essentially confirming the observations of Colburn and Maas [1, 2], our data have extended the studies to a number of different chelating agents and have provided complete inhibitory data.

By using the two different methods (viz. millipore filtration and centrifugation), nearly identical IC_{50} values were obtained for EDA and NE systems (compare Tables 1 and 2). Hence in the discussion of the inhibitory effects of the different chelators, the data obtained by using the two methods were compared.

In examining the possible correlation with the metal chelate stabilities, it is appropriate to discuss the inhibitory effects as a function of the types of chelators used, viz. amines and aminocarboxylic acids, particularly in view of the relatively greater ease of transport of the amines across the vesicular membrane than the amino acids.

A comparison of the data *in vitro* on the amines, viz. EA, EDA, DETA and NE, with their metal-binding strengths (Table 3) indicates that the monodentate amine, EA (with one metal-binding group), having the lowest metal complexing affinity has shown the smallest inhibitory effect, i.e. $IC_{50} = 3 \times 10^3$ nmoles, and ratio = 2×10^3. The polydentate amines (with more than one metal-binding group) EDA, DETA and NE have shown substantially larger inhibition of [^{3}H]NE storage which appears to be consistent with their high metal chelating abilities (Table 3).

Among the carboxylic acids, the polydentate chelators having more than two metal-binding groups, viz. EDTA, DTPA, TTHA and EGTA, have in general shown significantly larger inhibition (Table 4) than ALA, CYS and SSA. It appears that the presence of the diamine (or polyamine) backbone might be important in bringing about an effective inhibition. For example, EDTA, DTPA and TTHA are more effective than IMDA, ALA and CYS.

In view of the fact that significant concentrations of all the five different metal ions (i.e. Cu, Fe, Zn, Ca and Mg) were found to occur in the vesicles, a

Table 4. Metal-binding strengths of some carboxylic acids and their inhibitory effects on [^{3}H]NE storage*

Compounds	Inhibition *in vitro* (IC_{50}/NE)	Metal chelate stabilities (log K_{ML})†					
		Cu(II)	Zn(II)	Fe(II)	Fe(III)	Mg(II)	Ca(II)
EDTA	7.0	18.8	16.4	14.3		8.5	11.0
DTPA	52	27.9	22.8	16.6		9.3	10.7
TTHA	37	15.3(20.3)‡		17.1	29.4	8.5	10.1
EGTA	70	17.7	12.9	11.9	20.5	5.2	11.0
EDDHA	362	21.4	14.2		39.7	10.5	9.3
IMDA	862	16.2(β_2)	12.2(β_2)	10.1(β_2)		3.7	2.7
CYS	2.0×10^4		18.2(β_2)	11.8(β_2)		<4	
SSA	2.7×10^4	16.3(β_2)	10.6(β_2)	9.9(β_2)	25.1(β_3)		2.2
ALA	2.9×10^5	15.1(β_2)	9.5(β_2)	7.3(β_2)	10.4	1.9	1.2
MA	No inhibition	2.9	2.4				1.4

* The terms IC_{50} ratios, β_2, β_3 and K_{ML} are as described in Table 2.

† Martell and Sillen [12].

‡ Stability constant for a 2:1, Cu(II): TTHA chelate.

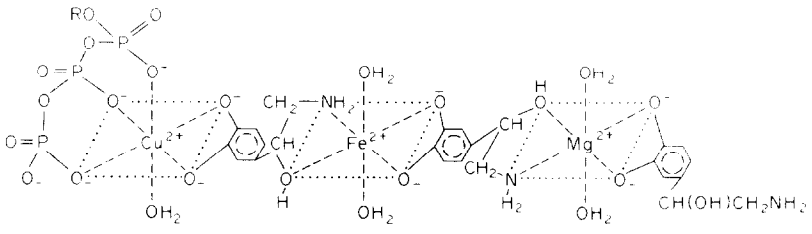


Fig. 3. Model of a mixed-metal polynuclear chelate with ATP and norepinephrine.

correlation analysis involving the inhibitory effects of the different chelating compounds, $\log_{10}$ (IC_{50} for the ligand/NE) and the stabilities of their chelates with the individual metals (log of the chelate stabilities, i.e. log K_{ML}) as well as different combinations of metals might prove useful in obtaining an insight into the mode of occurrence of the metals in the amine-storage sites and their possible involvement in the amine-storage mechanism. It is conceivable that in the amine storage sites, a combination of two or more metals might be incorporated, being coordinately bound by ATP, vesicular biomolecules and norepinephrine (NE). In other words NE might be bound to more than one of the vesicular metals in accordance with the hypothetical scheme presented in Fig. 3.

Correlation analyses for the individual metals were carried out by using the values of the stability constants (log K_{ML}) of their chelates with the different chelating agents and their corresponding inhibitory effects, i.e. log (IC_{50} for the ligand/NE). In the cases of the combinations of the different metals, their overall stabilities, obtained by the product of the stability constants of the individual metal chelates (or the sum of the log of the individual stability constants), were employed. In this treatment, the overall stability, "β_3" of a "mixed-metal chelate system" consisting of three metals, viz. M_1, M_2 and M_3, and a chelator L may be represented as follows:

$$M_1 + M_2 + M_3 + L \rightleftharpoons M_1M_2M_3L$$

$$\beta_3 = \frac{[M_1M_2M_3L]}{[M_1][M_2][M_3][L]}.$$

In the correlation analyses, a value for β_3 for each of the different combinations of the metals for any chelator, L, was estimated by assuming that the following approximation, i.e.

$$\beta_3 = K_1 \cdot K_2 \cdot K_3,$$

holds for the mixed-metal system where K_1, K_2 and K_3 represent the stability constants of the chelates of the metals M_1, M_2 and M_3 with L determined at similar conditions of ionic strength and temperature. Then,

$$\log \beta_3 = \log K_1 + \log K_2 + \log K_3$$

Hence, values of log β_3 for the stabilities of the mixed-metal systems and the corresponding IC_{50} values for the individual chelators were used in the correlation analyses.

Data from the amine and aminoacid systems were separately treated for their correlation. On the basis of a treatment of the data of the individual metals, it was found that Mg^{2+} gave the most satisfactory correlation coefficient, i.e. $r = 0.91$, with $P < 0.01$ for all the aminoacids investigated in this study. Fe^{2+} gave, $r = 0.84$, $P < 0.02$ and the mixed-metal system, i.e. Cu + Zn + Fe + Mg + Ca, $r = 0.87$, $P < 0.05$. In the case of the amine systems, Cu^{2+} and Zn^{2+} gave $r = 0.98$, $P < 0.02$ and $r = 0.88$, $P < 0.02$ respectively.

It should be pointed out that the results of the above correlation analyses might at best be taken to strongly suggest the importance of metal coordination phenomena in the vesicular binding and storage of norepinephrine. Having been attempted for the first time, it provides an interesting stimulation for further detailed investigations of the metal chelation hypothesis for the biogenic amine activity.

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