NMR Studies of Catecholamines. Interactions with Adenine Nucleotides and Divalent Metal Ions in Aqueous Solution

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Abstract: A proton magnetic resonance survey of the interactions between catecholamines and adenine nucleotides in the absence and in the presence of divalent metal ions is presented. The induced chemical shifts reveal that at pD ~7 a binary catecholamine–nucleotide complex is formed mainly through purine and catechol ring stacking, which is augmented by an interaction between the positive ammonium group and the negative phosphate moiety. Hydrogen bonds are also formed between the hydroxyls of the ring and the side chain of catecholamines and suitable acceptor sites in the nucleotide molecules. Associations via ring or chain interactions alone are much weaker than when both are involved. This observation coupled with the findings that catecholamines do not self-associate, and interact very similarly with the three adenine nucleotides, suggest that 1:1 or 2:1 amine–nucleotide complexes might be mainly formed. With divalent metal ions a ternary metal–nucleotide–catecholamine complex is formed, in which the nucleotide is strongly bound to the metal ion whereas the amine weakly associates to the nucleotide with no direct interaction with the metal ion. Catecholamines in the cationic state do not interact with metal ions in the absence of nucleotides. The association constants of the dopamine adducts with either ATP or MgATP and CoATP are found to be of the same order in accordance with the suggested models for the complexes.

Catecholamines are stored with ATP at relatively high concentrations in vesicles of the adrenal medulla and of the terminal varicosities of sympathetic nerves. Early observations that catecholamines and ATP occur in the vesicles at a fixed molar ratio of approximately 4:1[1-5] have led to the suggestion that both participate in a nondiffusible storage complex (apparently together with soluble protein). More recent studies[6-10] have shown, however, that the catecholamine/ATP ratio may be as high as 7–12, particularly in nerve vesicles. Thus, it was assumed that part of even most of the catecholamines might not be stored as a complex with ATP. Another type of storage mechanism involving a ternary metal–ATP–catecholamine complex has been postulated by Colburn and Maas[11] on the basis of their findings that divalent metal ions also occur in significant amounts in the storage vesicles of catecholamines.

Several spectroscopic studies have confirmed the possibility of formation in aqueous solution of either a binary catecholamine–ATP complex[12-15] or a ternary complex involving a divalent metal ion.[13,14,16] However, neither the stoichiometry nor the structure of these complexes has been clarified. It is the aim of the present studies to elucidate these points by a more systematic investigation of the interactions of catecholamines with adenine nucleotides and divalent metal ions in aqueous solutions. Reported here is a ^{1}H NMR survey of the associations of aromatic amines, e.g., β-phenylethylamine (PEA), tyramine (TA), dopamine (DA), L-norepinephrine (NE), and L-epinephrine (E) with adenosine, AMP, ADP, and ATP, in the absence and in the presence of Mg^{2+} or Co^{2+}. The DA–ATP system was found to be experimentally favorable and was the one mainly used in the study of the characteristics of the catecholamine complexation. Since complex formation between aromatic molecules is expected to affect mainly the chemical shifts, the latter will be of our primary interest in the present work. Our study, though of a qualitative nature, has some implications concerning the mode of interaction of catecholamines with nucleotides.

Experimental Section

Materials. Adenine nucleotides as sodium salts and phenolic amines as hydrochlorides (except for epinephrine which was acid free) of the highest grade were obtained from Sigma Chemical Co. and were used without further treatment. It has been assumed, on the basis of the relatively weak association of Na^+ with adenine nucleotides,[17] which involves mainly the P_β group of the phosphate chain of ADP and ATP,[18] that the sodium ions present in the experimental solutions would not perturb the interactions between catecholamines and nucleotides. This should be even more so in the presence of divalent metal ions. This assumption has been verified by treating ATP with cationic exchange resin (AG 50W-X4, hydrogen form; Bio-Rad Laboratories) to remove the sodium ions. Next several solutions with different concentrations of dopamine were prepared and the addition of NaCl was monitored by ^{1}H NMR measurements. No significant effect, within the experimental error, was detected for Na^+/ATP molar ratios up to 6:1. The metal salts $Co(No_3)_2 \cdot 6H_2O$ and $MgCl_2 \cdot 6H_2O$ were obtained from Merck and were dried in vacuo with slight heating (to prevent decomposing) before use. Experimental solutions were made up by dissolving the materials in D_2O (99.7%). The pD values were adjusted by the addition of concentrated DCl and NaOD and were corrected for isotope effects. Unless otherwise specified, experiments were carried out at pD 6.7–6.9.

NMR Spectra. The ^{1}H NMR spectra were recorded on a Bruker HFX-10 spectrometer operating at 90 MHz, with an internal deuterium lock. A trace of dioxane in the solution served as an internal reference for the shift measurements. Up-field or down-field shifts (expressed in Hz) are denoted by positive or negative signs, respectively. The experimental uncertainty in the chemical shifts is estimated as 0.5–1.0 Hz for the diamagnetic shifts and up to 3% for the paramagnetic shifts. All the measurements were performed at an ambient probe temperature of 27 °C.

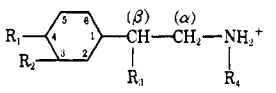
The assignment of the ring and side-chain protons of the studied aromatic amines[19,20] is given in Table I. Note that H_5, H_6, H_β, and H_α refer to amine protons, while H_1', H_2, and H_8 refer to nucleotide protons.

Results

Self-Association of Catecholamines. The chemical shifts of DA, NE, and E were measured as functions of their concentration, in the range 0.03–0.5 M for DA and NE and 0.05–0.24 M for E. Small down-field shifts, 0.5–2.5 Hz, were observed. Since self-association involving interaction between aromatic rings produces up-field shifts in the proton resonance, the results indicate that in the pD used in the experiments there is practically no self-association of catecholamine molecules in aqueous solutions.

Interaction of Aromatic Amines and Adenine Nucleotides. The mutual effects on the shifts in 1:1 mixtures of aromatic amines and ATP are summarized in Table II. It is seen that complex formation results in up-field shifts for all of the protons. Evidently a stacking interaction takes place between the

Table I. The Structure of the Studied Compounds



	R_1	R_2	R_3	R_4
β-Phenylethylamine (PEA)	H	H	OH	H
Tyramine (TA)	OH	H	H	H
Dopamine (DA)	OH	OH	H	H
Norepinephrine (NE)	OH	OH	OH	H
Epinephrine (E)	OH	OH	OH	CH_3

Table II. Chemical Shifts Induced by Complex Formation in 1:1 Mixtures of ATP and Aromatic Amines[a]

Amine	Amine protons			ATP protons		
	5	β	α	8	2	1′
PEA	13.0	8.5	6.0	3.5	3.5	5.0
TA	15.5	10.0	6.5	5.0	5.0	5.5
DA	17.0	11.0	7.0	6.0	6.5	6.0
NE	10.0	5.5	5.5	5.5	5.0	5.0
E	10.0	3.5	3.0	4.5	4.0	4.0

[a] [Amine] = [ATP] = 0.1 M; pD 6.9; 27 °C.

purine ring of ATP and the ring of the aromatic amines. The larger shifts obtained for the ring protons of the amines relative to those of the purine are compatible with the larger effect of the purine ring current.[21]

The effects on the chemical shifts of the interactions of DA and NE with adenosine and adenine nucleotides, for several relative concentrations, are summarized in Table III. Note that while the interaction is considerably enhanced upon passing from the nucleoside to the nucleotides, it is nearly equivalent for AMP, ADP, and ATP (for catecholamine/nucleotide molar ratio between 0.5 and 4.0), as reflected in the shifts of the catecholamine protons. A similar effect is observed with the shifts of the ADP and ATP protons. AMP seems to be an exception probably due to its more rigid conformation.[22]

The effect of pD on the chemical shifts of DA and ATP in a 4:1 mixture is shown in Figure 1. The strongest interaction between DA and ATP is observed in the region of pD 7. At acidic pD there is a slight decrease in the shifts. This effect may be attributed to the ring protonation of ATP. A protonated purine ring was found to have a smaller stacking ability than a deprotonated one.[23] At pD higher than 7 clearly the DA-ATP complex dissociates. Note that the chain protons of DA are completely shifted to their unbound positions, whereas some residual shift on the ring protons remains. A small decrease of the ammonium deprotonation constant is observed, which may be attributed to the association between the ammonium group and the phosphate chain.

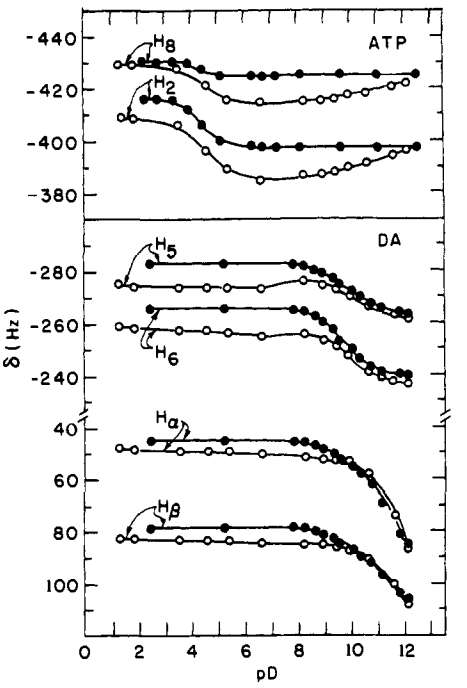


Figure 1. The pD dependence of the proton shifts of 0.2 M dopamine and of 0.05 M ATP taken alone (filled circles) and in their 4:1 mixture (open circles). The shifts are referred to dioxane at 90 MHz, 27 °C.

Table III. Chemical Shifts Induced by Complex Formation of Dopamine (DA) and Norepinephrine (NE) with Adenosine and Adenine Nucleotides[a]

	Molar ratio	Catecholamine protons				Nucleotide protons		
		5	6	β	α	8	2	1′
DA + adenosine	1:1	7.5	8.0	4.0	1.0	1.5	3.0	1.5
DA + AMP	1:1	15.5	18.5	10.5	6.5	3.0	5.0	5.5
DA + ADP	1:1	16.0	18.5	10.5	6.5	6.5	6.5	6.5
DA + ATP	1:1	17.0	19.0	11.0	7.0	6.0	6.5	6.0
DA + ATP + adenosine	1:1:1	18.0	19.5	11.0	7.0	6.5[b]	10.5	7.5
DA + ATP + AMP	1:1:1	22.5	25.5	14.0	9.0	7.5[b]	10.5	7.0
DA + ATP + ADP	1:1:1	23.0	25.5	14.0	9.0	7.5[b]	10.0	7.0
DA + ATP	1:2	23.0	25.5	14.0	9.0	6.5[b]	9.5	6.0
DA + adenosine	4:1	4.5	4.5	2.5	~0	2.0	3.0	1.0
DA + AMP	4:1	8.5	10.0	6.0	4.0	4.0	6.0	6.0
DA + ADP	4:1	8.5	10.0	6.5	4.0	10.5	10.0	11.0
DA + ATP	4:1	9.5	11.0	7.0	4.5	11.5	10.5	11.0
NE + adenosine	1:1	3.0	1.5	c	~0	1.0	2.0	~0
NE + AMP	1:1	10.0	8.0	c	4.5	2.5	3.0	3.5
NE + ADP	1:1	10.0	8.0	c	5.0	5.0	5.0	4.5
NE + ATP	1:1	10.5	9.0	c	5.5	5.5	5.0	5.0
NE + adenosine	4:1	1.5	1.0	c	~0	1.5	2.5	1.0
NE + AMP	4:1	5.5	3.5	c	2.5	4.0	5.0	6.0
NE + ADP	4:1	5.5	3.5	c	2.5	9.5	8.5	9.0
NE + ATP	4:1	5.5	3.5	c	2.5	10.5	9.5	9.0

[a] The basic concentration unit is 0.1 M; pD 6.9; 27 °C. [b] The effect here is due also to nucleotide stacking. [c] Obscured by the HDO signal.

Table IV. The Effect of Ribose 5′-Phosphate and Ethylamine on the Chemical Shifts of the Dopamine-ATP Adduct[a]

	Molar ratio	Dopamine protons				ATP protons		
		5	6	β	α	8	2	1′
DA + ATP	1:1	17.5	19.0	11.0	7.0	6.0	6.5	6.0
DA + ATP + ribose 5′-phosphate	1:1:1.3	16.0	17.5	10.0	6.5	6.5	6.5	6.5
DA + ATP + ethylamine	1:1:1.5	16.0	17.5	10.0	6.0	6.5	7.0	6.0
DA + ATP	3:1	13.5	15.5	10.5	6.5	9.0	9.0	8.5
DA + ATP + ribose 5′-phosphate	3:1:2.5	11.0	13.0	9.5	5.0	7.5	8.0	7.5
DA + ATP	1:3	26.0	29.0	16.5	10.0	4.0	4.0	3.5
DA + ATP + ethylamine	1:3:4	24.5	27.5	*b*	8.5	3.5	4.0	3.0

[a] The basic concentration unit is 0.1 M; pD 6.9; 27 °C. [b] Obscured by an ethylamine signal.

Table V. Paramagnetic Shifts Induced by Co^{2+} in the Proton Spectra of Amines in the Presence and in the Absence of ATP[a]

Amine	Amine protons			HDO
	5	β	α	
Ethylamine		~0	~0	−44.0
Ethylamine + ATP		+27.5	+22.5	−11.0
PEA	~0	~0	~0	−40.0
PEA + ATP	+75.0	+136.0	+146.0	−8.0
TA	−4.5	−1.5	−1.5	−43.0
TA + ATP	+95.0	+159.0	+154.0	−8.0
DA	−7.5	−4.5	−5.0	−43.0
DA + ATP	+128.0	+189.0	+180.0	−8.0

[a] [Amine] = [ATP] = 0.1 M, [Co] = 60 mM; pD 6.9; 27 °C.

Table VI. Paramagnetic Shifts Induced by Co^{2+} in the Proton Spectra of Dopamine (DA) and Norepinephrine (NE) in the Presence of Adenosine and Adenine Nucleotides[a]

	Catecholamine protons			
	5	6	β	α
DA + adenosine	1.0	1.5	~0	1.0
DA + AMP	16	21	19	13
DA + ADP	74	83	104	66
DA + ATP	55	73	111	102
NE + adenosine	1.5	1.0	1.0	1.5
NE + AMP	16	22	14	13
NE + ADP	83	101	125	81
NE + ATP	63	92	117	119

[a] [Amine] = [nucleotide] = 0.1 M, [Co] = 20 mM; pD 6.8; 27 °C.

Competitive Effects of Ethylamine and Ribose Phosphate. In order to elucidate the importance of the nucleotide and the amine side chains for complex formation, ribose 5′-phosphate and ethylamine were used as the appropriate derivatives lacking ring moieties. First, 1:1 (0.1 M) mixtures of DA + ribose 5′-phosphate and ethylamine + ATP were examined. No effect on the proton resonances was observed. This does not necessarily mean that there is no interaction between these molecules, but indicates that the observed proton shifts of aromatic amines bound to nucleotides are due only to ring-current effects. Next the competition between ribose 5′-phosphate, ethylamine, and DA + ATP was investigated. Since ribose 5′-phosphate or ethylamine does not affect the shifts of DA or ATP alone, their addition to a DA-ATP solution is expected to produce down-field shifts, due to disruption of the DA-ATP complex (if indeed ribose 5′-phosphate or ethylamine can compete with ATP or DA, respectively). The results, given in Table IV, reveal only small effects of the competing compounds.

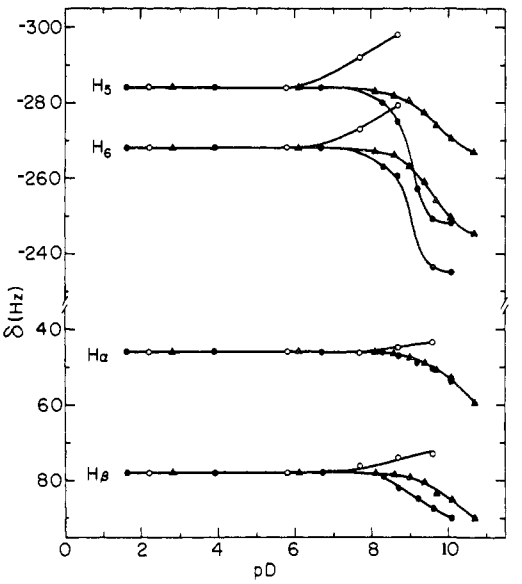


Figure 2. The pD dependence of the proton shifts of 0.1 M dopamine: alone (triangles); with 0.19 M Mg^{2+} (filled circles); with 11 mM Co^{2+} (open circles).

Interaction of Aromatic Amines with Divalent Metal Ions. Effect of Nucleotides. The Co^{2+} induced shifts for ethylamine and aromatic amines, in the absence and presence of ATP, are given in Table V. In the absence of ATP small down-field shifts are observed, whereas with ATP large up-field shifts are induced, indicating the formation of a ternary metal-ATP-amine complex. The ATP resonances were considerably broadened and shifted, indicating, as expected, a strong binding to the metal ion, comparable to that in the CoATP chelate.[23,24]

The effects of Co^{2+} on the chemical shifts of DA and NE in 1:1 mixtures with adenosine and adenine nucleotides are given in Table VI. The induced shifts follow the sequence ATP ~ ADP > AMP ≫ A. The negligible effect with adenosine is expected on the basis of its low affinity to divalent metal ions.[25] The smaller effect with AMP may be explained on the basis of electrical charge considerations; AMP^{2-} is completely neutralized by the divalent metal ion, thus its ability to bind positively charged catecholamine molecules is appreciably reduced.

The pD titration curves of dopamine in DA + M^{2+} solutions (M = Mg, Co) are shown in Figure 2. Clearly while there is no association of the metal ions to DA in its cationic state (at acidic pD), a strong interaction is observed as soon as deprotonation of DA begins. With Co^{2+} the DA resonances are shifted and broadened beyond detection. The diamagnetic Mg^{2+} does not produce significant shifts; however, another effect of the metal ions is observed, an increase of the acidity of the catechol hydroxyl group. This is due to the competition

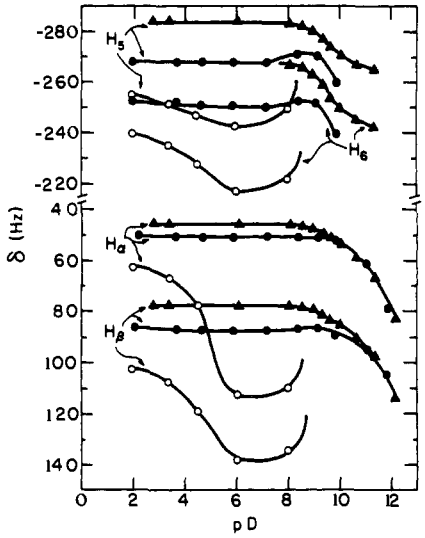


Figure 3. The pD dependence of the proton shifts of 0.1 M dopamine in a 1:1 DA/ATP mixture: metal free (triangles); with 0.1 M Mg^{2+} (filled circles); with 10 mM Co^{2+} (open circles).

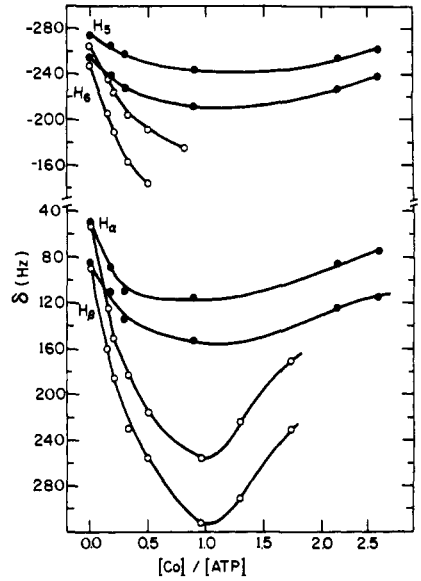


Figure 4. The effect of Co^{2+} on the proton shifts of 0.1 M dopamine in 1:1 (open circles) and in 4:1 (filled circles) DA/ATP mixtures at pD 6.7.

between the metal ion and the proton (deuteron) for this binding site.

The pD titration curves of DA in M^{2+}-ATP-DA solutions (M = Mg, Co) are shown in Figure 3. As the pD is raised (up to ~7) the induced shifts increase, reflecting the strengthening of interaction between DA and ATP (see also Figure 1). At higher pD the observed effects are similar to those in the DA + M^{2+} system (cf. Figure 2), i.e., strong association of the anionic catecholamine molecule to the metal ion, and an increase in the catechol acidity.

The addition of Mg^{2+} to a 3.5:1 molar ratio DA-ATP solution ([Mg] ≃ [ATP], pD 6.9) resulted in small shifting (2-3 Hz) of both the DA and the ATP resonances down-field, i.e., in the direction of their unbound positions, indicating the disruption to some extent of the DA-ATP complex. The paramagnetic shifts induced by the addition of Co^{2+} to 1:1 and 4:1 DA/ATP solutions are shown in Figure 4. The strong effect on the DA resonances for [Co] < [ATP] demonstrates the strong binding of ATP to the metal ion which in turn affects the catecholamine. In the excess of Co^{2+} over ATP, the DA shifts are reversed, resembling the metal ion association with DA alone (cf. Figure 2).

It is interesting to note the effect of the cobalt ions on the solvent (HDO) chemical shifts (cf. Table V). In solutions of aromatic amine alone, at pD <7, the water signal is shifted by Co^{2+} down-field by an amount compatible with the formation of a regular cobaltous aquo complex.[26] At pD >7, however, the water signal is shifted back, up-field (and becomes narrower), indicating that water molecules are expelled from the cobalt inner-coordination sphere as the amines become bound to the metal ion. In the CoATP complex three water molecules are directly coordinated to the metal ion.[27] The observed shift of the HDO signal in cobalt-ATP-amine solutions (cf. Table V) is then lower than expected (~20 Hz for [Co] = 60 mM). This may be attributed to the contribution of the up-field shifted exchangeable protons of the aromatic amine bound in the ternary complex. These protons exchange rapidly with the water protons, and hence produce an up-field shift of the coalesced signal. This hypothesis was supported by the observation that although the shift was smaller, the HDO signal was broader than expected for three coordinated water molecules.

Association Constants for the DA-ATP and DA-Metal-ATP Complexes. The dependence of the catecholamine shifts, in the presence of nucleotides or nucleotides + metal ions, on their relative concentrations is shown in Figures 5-7. A weak complexation behavior is observed, i.e., a slow approach toward saturation, which is achieved at high molar ratios. In order to estimate the association constants, DA was titrated with an excess of ATP or with 1:1 M^{2+}-ATP (M = Mg, Co) so as to ensure that a 1:1 complex with DA would be the major species formed. Under the experimental conditions ATP can be regarded as being completely bound to the metal ion. The induced shifts of DA were analyzed assuming the formation of a 1:1 complex in the equilibrium

$$A + B \rightleftharpoons AB \qquad K = [AB]/[A][B]$$

where A is DA and B is either ATP or the metal-ATP adduct. The induced shifts ($\Delta\delta$) are given by

$$\Delta\delta = ([AB]/A_0)\delta_0$$

where δ_0 is the saturation shift and A_0 is the total concentration of DA. The average constants obtained were 15, 12, and 11 M^{-1} for complexation with ATP, MgATP, and CoATP, respectively.

Discussion

The observation of up-field shifts indicates that the interaction between aromatic amines and nucleotides involves ring association through vertical stacking. This is in contrast with an earlier suggestion by Weiner and Jardetzky,[12] that no such interaction takes place in the complex formation. The magnitudes of the induced shifts follow the sequence $\Delta\delta(H_5) > \Delta\delta(H_\beta) > \Delta\delta(H_\alpha) > \Delta\delta(H_1') \sim \Delta\delta(H_2) \sim \Delta\delta(H_8)$. The similarity of the $\Delta\delta$ values for the nucleotide protons (and for H_5 and H_6 of DA and NE) suggests that the stacked rings are approximately centered. The $\Delta\delta$ values for the amines decrease in an expected way, being smaller for the chain protons, which are further away from the ring. The increase in the shifts induced by ATP upon going from PEA to TA to DA (Table II) indicates that the phenolic-hydroxyl groups strengthen the interaction between the associating rings probably due to the ability of the hydroxyl protons to approach closer the adenine ring and to form hydrogen bonds with its nitrogens. The β-hydroxyl group of NE and E, however, has an opposite effect, i.e., to decrease the proton shifts. Hydrogen bond formation between this group and the ribose or the phosphate moieties of ATP has been suggested on the basis of the more pronounced changes of the proton relaxation rates of E (relative to DA)

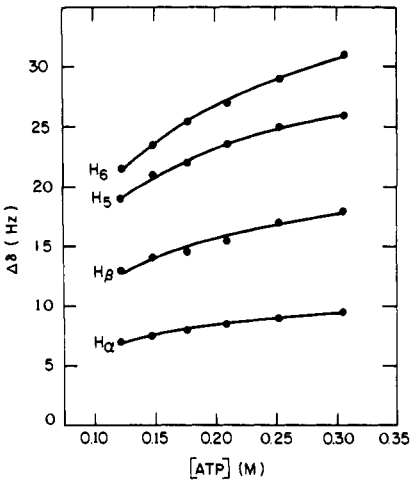


Figure 5. The proton shifts induced by increasing concentrations of ATP in the spectrum of 0.047 M dopamine at pD 6.9.

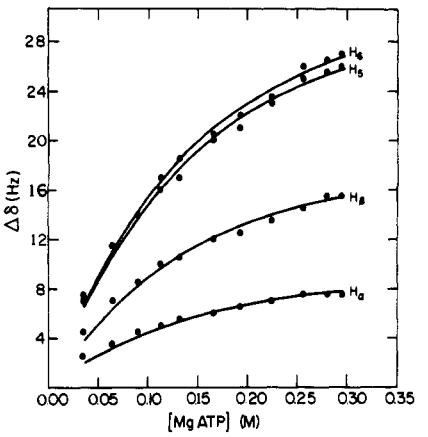


Figure 6. The proton shifts induced by increasing concentrations of MgATP in the spectrum of 0.1 M dopamine at pD 6.9.

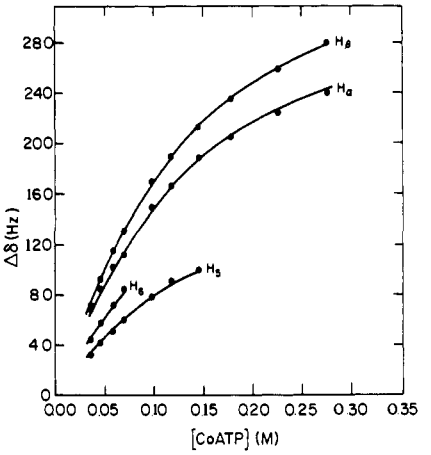


Figure 7. The proton shifts induced by increasing concentrations of CoATP in the spectrum of 0.093 M dopamine at pD 6.9. (At the higher Co^{2+} concentration range, the HDO signal obscured the H_5 and H_6 resonances.)

bound to ATP[12] and of the shifting of the IR frequency of the P=O band of ATP by E but not by DA.[14] It seems that such a hydrogen bond may lead to a different distribution among the rotamers of NE and E which will be observed as a chemical shift.

The weaker interaction of catecholamines with adenosine relative to that with nucleotides (cf. Table III) indicates that, apart from ring stacking, an electrostatic interaction between the positive side chain of the amines and the negatively charged phosphate chains of the nucleotides has an important role in the complex formation. The competition study revealed that molecular association through chain interaction alone is however much weaker than that involving ring stacking as well. The disruption of the DA-ATP complex at pD higher than 7 (Figure 1), where the ammonium group is deprotonated[20] and its interaction with the phosphate moiety canceled, provides further evidence for the role of electrostatic chain interactions in the catecholamine association with nucleotides. The residual shift on the ring protons at basic pD is thus the result of an association through ring stacking alone and is indeed comparable with the effect of adenosine (cf. Table III).

The near equivalence of the interactions of AMP, ADP, and ATP with catecholamines (cf. Table III) implies the formation of similar complexes. At the experimental pD (~7) AMP is doubly ionized and can interact with two positively charged molecules. The rings of the two catecholamine molecules would stack to the purine ring one at each side. It appears highly probable that the same occurs with ADP and ATP. Since the catecholamines do not self-associate it seems that no more than two catecholamine molecules associate directly with the purine ring. The condition that the molecules would interact also via electrostatic chain attraction can also be fulfilled with the three nucleotides. Tentatively our results are thus compatible with the formation, in aqueous solutions, of mainly 1:1 or 2:1 catecholamine-nucleotide complexes. In the presence of a large excess of catecholamines, ADP and AMP can potentially (on the basis of ionic charge considerations) bind one or two additional molecules through the electrostatic chain interaction alone. However, since this interaction is weak, complexes with higher than 2:1 stoichiometry would constitute only a minor fraction. Daniels et al.[15] proposed a model for a 4:1 epinephrine-ATP complex in which two molecules of epinephrine are directly stacked on both sides of the purine ring, while the other two are stacked on top of the first. This model seems improbable as judged on the basis of the present results.

Catecholamines in their cationic state (the predominant species at pD $\lesssim$7) do not interact with metal ions. However, strong binding via ionized phenolic hydroxyl groups is observed at pD >7. The down-field shifts induced by Co^{2+}, which are generally ascribed to the effect of the contact interaction between the unpaired electrons of the metal ion and the protons of the ligand,[26,28] as well as the expulsion of water molecules from the cobaltous aquo complex at pD >7, in the presence of catecholamines, imply a direct (inner-sphere) coordination. The direct binding of Co^{2+} to the ionized phenolic hydroxyls is also reflected in the more pronounced effect on the ring protons relative to the side-chain protons. Ethylamine and PEA which lack the hydroxyl binding sites do not interact with Co^{2+}. The larger effect on DA relative to TA (Table V) is compatible with the smaller ionized fraction of the latter at the experimental pD.[20]

Catecholamines in the cationic state however do interact with metal-nucleotide adducts (particularly with ADP or ATP) to form a ternary complex. The induced paramagnetic up-field shifts may be ascribed to a nondirect pseudocontact interaction between Co^{2+} and the catecholamines, implying that the latter are outer-sphere coordinated. This reasoning was further confirmed by titrating DA with 1:1 Ni^{2+}-ATP. The proton shifts of DA were found to follow, within the experimental error, the shifts observed with MgATP. Hence no net paramagnetic shifts were induced by the NiATP adduct. As mainly contact shifts are expected with Ni^{2+}, the results clearly indicate that there is no direct interaction between the metal ion and the catecholamine molecule. Since cationic amines do not interact with divalent metal ions, the association of the former in the ternary complex has to be through inter-

action with the nucleotide (which is by itself strongly bound to the metal ion). The relative magnitudes of the paramagnetic shifts (Table V) DA > TA > PEA ≫ ethylamine indeed reflect their affinity to association with ATP. The titration of DA + ATP with Co^{2+} (Figure 4) also shows that the interaction of DA with the metal ion proceeds through association with ATP. As the Co^{2+} concentration exceeds that of ATP, downfield shifts are induced indicating a direct binding of DA to the excess cobalt. Contrary to the metal-free complex, the shifts of the side-chain protons in the ternary complex are larger than those of the ring protons. These shifts come from two sources: the purine ring current, and the paramagnetic effect of the metal ion which is much stronger. Both decay with the distance as $1/r^3$. The observed net effect implies that the amine chain protons are closer to the metal ion. Indeed this is expected to be the case, since the metal ion bound to the phosphate moiety of the nucleotide would be near the amine side chain, also interacting with this group, and further away from the amine ring, stacked to the purine ring which is outer-sphere coordinated to the metal ion.[27]

The results thus indicate the formation of two types of ternary metal-nucleotide-catecholamine complexes. In the first type, the catecholamine, in its cationic state, is associated with the nucleotide without a direct association to the metal ion. In the second type, the ionized catecholamine directly binds to the metal ion. In both complexes the nucleotide is strongly bound to the metal ion. The second type, while involving stronger chelating of the amines, is apparently of minor importance because of the small fraction of ionized catecholamines species at pD ~7. Comparing the induced shifts of DA in the complexes with ATP and MgATP (cf. Figures 5 and 6) it seems that the catecholamine molecule associates with ATP in the ternary complex very similar to its association in the binary metal-free complex. The resemblance of the association constants of the DA-ATP and the DA-metal-ATP complexes is in accord with this suggestion.

The weak overall association of catecholamines with nucleotides implies that whenever catecholamines are in excess in aqueous solution, an appreciable fraction would not be bound. It appears that divalent metal ions have no significant effect on this association. Moreover, the binding of the metal ion to ATP may inhibit the formation of higher complexes of catecholamines with the latter. Our results thus pose a question whether complex formation between catecholamines and nucleotides or metal-nucleotide adducts in an aqueous medium can indeed be responsible by itself for the storage of catecholamines. Study of the role of other cell components as well as the effect of the medium in the storage vesicles would therefore be of importance for gaining better understanding of the storage mechanism of catecholamines.

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