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Fluorescence spectroscopic analysis of the hydrogen bonding properties of catecholamines, resorcinolamines, and related compounds with phosphate and other anionic species in aqueous solution‡

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Fluorescence spectroscopic analysis based upon the difference in fluorescent behaviour of several phenol, catechol and resorcinol derivatives in 0·2 M phosphate and water, at different pH values ranging from 7·4 to 1·0, produced evidence for the existence of complexes between phosphate anions and these compounds. This complex formation was also found with acetate, citrate and glutamate but not with sulphate and borate. A comparison of different ligand structures revealed that the complex formation observed with unsubstituted phenol, catechol or resorcinol was not influenced by ethyl substitution; however, ethylamine or ethanolamine substitution favoured the formation of the complex. The findings indicate the formation of 1 : 1 intermolecular complexes between mono- and dihydroxyphenethylamines and several anionic species with participation of two hydrogen bonds. The predictions of a theoretical model describing this interaction are confirmed by the experimental data. Association constants of isoprenaline with phosphate and glutamate were found to be 5·1 and 26·6 litres mol^{-1} respectively; for the complexes of *m*-tyramine and terbutaline with phosphate, association constants of 15·3 and 6·9 litres mol^{-1} respectively, were obtained.

Apart from ionic interactions, catecholamines possess an obvious ability to complex with anionic species via hydrogen bonding. Therefore, it is surprising that, apart from the hydrogen bonding of catecholamines to nucleotides, little is actually known about this property and the structural requirements involved. The significance of studies on the hydrogen bonding properties is readily apparent if the possible role of the complex formation of catecholamines in the storage of these compounds in chromaffin granules (see Njus & Radda 1978 for a review) and in synaptic vesicles (Rajan et al 1972, 1977), as well as in the binding to phospholipids (Hammes & Tallman 1971) is considered. In addition, there are numerous allusions to this property in the literature, reference being made to the models of Belleau (1966) and Bloom & Goldman (1966), suggesting its possible involvement in the interaction of catecholamines with adrenoceptors. However, even in regard to the formation of complexes of catecholamines with ATP in the presence or in the absence of divalent metal ions, there is no general agreement about the stoichiometry or the structure of these complexes (Granot & Rosenheck 1978 and references cited therein).

Recently, ^1H n.m.r. and i.r. spectroscopic evidence reported by Zaagsma (1979) indicated that the hydrohalide salts of β-adrenoceptor blocking aryloxypropanolamines were able to form complexes with participation of the anion. The anion accepts hydrogen bonds both from the β-hydroxyl group and the protonated amine function, which gives rise to a seven-membered ring structure. It was speculated that a similar hydrogen bonding to phosphate or carboxylate anions might be involved in the binding of these compounds to the β-adrenoceptors. In the present paper we report on the hydrogen bonding of catecholamines and related compounds with several anions in aqueous solution, as studied by fluorescence spectroscopy.

MATERIALS AND METHODS

Fluorescence in aqueous solution of all compounds was recorded with a Perkin-Elmer MPF-2A Spectrofluorimeter. The excitation wavelength was 280 nm, the emission peak which was characteristic for the

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hydroxylated aromatic nucleus appeared at 305 or at 310 nm.

Two solutions (2 μM) were prepared for each compound studied; one in distilled water, the other containing the complexing anion. At the onset of the experiment the pH of each solution was adjusted to 7·4 using 0·001–0·2 M NaOH. Samples of 400 μl were taken in quadruplicate and the fluorescence was recorded immediately. Subsequently the pH of each solution was lowered stepwise (approximately 0·5 pH unit at a time) using minimal amounts of 0·01–11 M HCl and the fluorescence was measured immediately again. For each measurement part of the emission spectrum between 300–320 nm was recorded, in no case was a wavelength shift of the emission peak observed when the pH of the solutions was changed.

In separate experiments it was ascertained that during the time-course of the experiments oxidation of catecholamines did not interfere with the results. Identical results were obtained when 0·2 mM sodium metabisulphite was included in the solutions (in the absence of divalent cations and at acid pH values, catecholamines are relatively stable).

Evaluation of results

Plots of the fluorescence intensity (taken as mm recorder pen deflection) versus pH were constructed for all the compounds listed and these were normalized by setting the maximum fluorescence in H_2O to 1·0. All values were corrected for blanks obtained with solutions containing only the anion.

Theoretical considerations

Calculation of the association constant K from the fluorescent data. We assume a 1 : 1 complex formation

$$L + S \rightleftarrows C$$

between the fluorescent ligand L and the non-fluorescent solute S in water. Hence

$$K = \frac{[C]}{[L].[S]} \quad (1)$$

Equation (1) may be written as

$$K = \frac{[C]}{([L_t] - [C]).([S_t] - [C])} \quad (2)$$

where $[L_t]$ and $[S_t]$ represent total concentrations of ligand and solute respectively. Under the experimental conditions of this study $[S_t] \gg [C]$, therefore equation (2) simplifies to:

$$K = \frac{[C]}{([L_t] - [C]).([S_t])} \quad (3)$$

Fluorescence of a solution (F_{obs}) containing the 3 species L, S and C is given by:

$$F_{obs} = K_l([L_t] - [C]) + K_s([S_t] - [C]) + K_c[C] \quad (4)$$

where K_l, K_s and K_c are the fluorescence coefficients of the uncomplexed ligand, the solute and the complex respectively.

K_s is experimentally shown to be zero and by extrapolation it was found that K_c can be taken as zero. Substitution of these values into (4) and eliminating [C] from equation (3) and (4) yields

$$\frac{F_{tot}}{F_{obs}} = K[S_t] + 1 \quad (5)$$

where F_{tot} is equal to the fluorescence of L_t in distilled water. A similar derivation is given by Cowgill (1965).

Materials

Phenol, adrenaline bitartrate (Merck); 3-ethylphenol, 4-ethylphenol (Aldrich); resorcinol (J. T. Baker); catechol, *p*-tyramine HCl, isoprenaline HCl (Sigma); *m*-tyramine HCl (Cyclo Chemical); dopamine HCl (Fluka); terbutaline sulphate (Astra); salbutamol base (Allenburys); noradrenaline bitartrate (BDH); synephrine bitartrate, phenylephrine HCl, *N*-(t-butyl)noradrenaline sulphate (Th 1206), orciprenaline sulphate (Boehringer Ingelheim). All materials were of reagent grade.

RESULTS

The fluorescence yield of catecholamines in water does not change between pH 7 and pH 2. Above pH 7 a decrease in fluorescence is noted caused by the increased ionization of the hydroxyl substituents (Lewis 1954; Sunkel & Staude 1968; Granot 1976; Gannelin 1977), giving rise to a non-fluorescent species (Udenfriend 1962). The fluorescent behaviour of 2 μM noradrenaline in acidified water is shown in Fig. 1, in combination with the pH-dependent fluorescence in 0·2 M phosphate. At pH 7·4 the fluorescence of 2 μM noradrenaline in 0·2 M phosphate is suppressed by half compared with the fluorescence in water at the same pH. Upon lowering the pH of the phosphate solution with concentrated HCl, the fluorescence of noradrenaline increases. At pH 4 the fluorescence intensity is increased to 90% of the value in water.

The structural requirements for the observed fluorescence decrease were explored using the compounds listed in Table 1. All these studies were with

FIG. 1

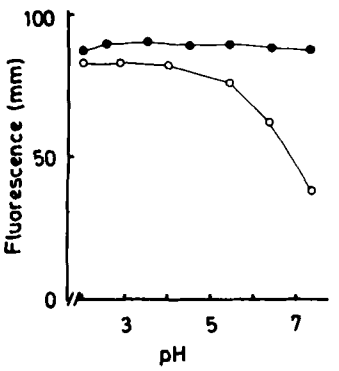
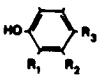


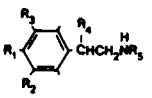
FIG. 1. Fluorescence yield of 2 μM noradrenaline in 0·2 M phosphate ○—○ and water ●—●, at different pH. Fluorescence yield is given as mm recorder-pen deflection after correction for blank values. The means from an experiment performed in quadruplicate are shown.

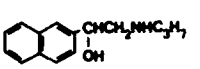
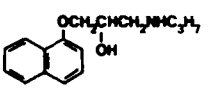
0·2 M phosphate and 2 μM of the fluorescent ligands. At pH 7·41 unsubstituted phenol, catechol and resorcinol show pronounced differences in the degree their fluorescence is suppressed in 0·2 M phosphate. The depression is largest with phenol (62%), followed by resorcinol (52%) and catechol (32%). Alkyl substitution into the nucleus as in 3-ethylphenol and 4-ethylphenol does not alter the fluorescence suppression observed with the parent compound. However, ethylamine substitution results in an increment in the observed fluorescence depression: cf. phenol (62%) versus *p*-tyramine (68%) or *m*-tyramine (73%) and catechol (32%) versus dopamine (53%). The β-hydroxyl group does not seem to have a significant influence since no difference was found between the fluorescence depression of *p*-tyramine and synephrine; however, minor differences were seen consistently between the values obtained on the same day with *m*-tyramine and phenylephrine and

Table 1. Relative fluorescence yield of 2 μM of monohydroxy- and dihydroxyphenyl derivatives in 0·2 M phosphate.



	$F_{pH\ 7.4}/F_{H_2O\,max}$	n*	$F_{pH\ 3-4}/F_{H_2O\,max}$**	n*	R_1	R_2	R_3
Phenol	0·38 ± 0·03†	2	0·77	2	H	H	H
3-Ethylphenol	0·41 ± 0·01	3	0·79	2	H	C_2H_5	H
4-Ethylphenol	0·43	1	0·83	1	H	H	C_2H_5
Resorcinol	0·48 ± 0·01	4	0·87	4	H	OH	H
Catechol	0·68 ± 0·01	3	0·98	3	OH	H	H



					R_1	R_2	R_3	R_4	R_5
p-Tyramine	0·32 ± 0·02	2	0·83	2	OH	H	H	H	H
m-Tyramine	0·27 ± 0·01	4	0·76	2	H	OH	H	H	H
Synephrine	0·32 ± 0·01	2	0·78	2	OH	H	H	OH	H
Phenylephrine	0·30 ± 0·01	3	0·72	3	H	OH	H	OH	H
Dopamine	0·47 ± 0·01	5	0·83	2	OH	OH	H	H	H
Noradrenaline	0·43 ± 0·01	3	0·88	2	OH	OH	H	OH	H
Adrenaline	0·42	2	0·90	1	OH	OH	H	OH	CH_3
Isoprenaline	0·44 ± 0·01	5	n.d.‡		OH	OH	H	OH	iC_3H_7
Th-1206	0·43 ± 0·01	2	n.d.‡		OH	OH	H	OH	tC_4H_9
Orciprenaline	0·37 ± 0·02	3	0·90	2	H	OH	OH	OH	iC_3H_7
Terbutaline	0·38 ± 0·02	6	0·93	2	H	OH	OH	OH	tC_4H_9
Salbutamol	0·45 ± 0·01	2	0·76	1	OH	CH_2OH	H	OH	tC_4H_9
Pronethalol	1·00	1	1·00	1					
Propranolol	1·00	1	1·00	1					

* number of observations.
† means ± s.e.m.
‡ not determined.
** The value given is the mean of the number of observations between pH 3–4. No s.e.m. is included because the observations were not made at the same pH.

between noradrenaline and dopamine, but as a whole these differences are not significant.

N-Alkyl substitution has no influence on the fluorescence depression as observed with the unsubstituted compound (cf. noradrenaline, adrenaline, isoprenaline, Th 1206).

It can be inferred from equation 5 that at a fixed concentration of S_t, F_{tot}/F_{obs} gives a relative measure for the association constant K. Thus, the values reported in Table 1 are a relative measure for the strength of the complex between the compounds listed and the anionic species involved.

In Fig. 2 the increase of fluorescence in 0·2 M phosphate on going from pH 7·4 to pH 1 is shown for phenol and catechol derivatives with or without a β-hydroxyl group in the ethylamine substituent. After a relatively constant fluorescence yield between pH 5 and pH 2 for all compounds, there is a second rise for the phenol derivatives, while that of the catecholamines declines again below pH 2.

FIG·2

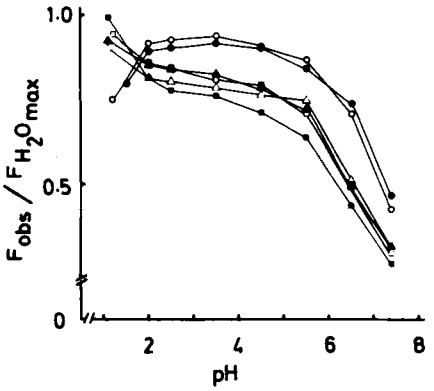


FIG. 2. Relative fluorescence yield in 0·2 M phosphate at various acidities for noradrenaline ○—○, dopamine ●—●, phenylephrine □—□, *m*-tyramine ■—■, synephrine △—△, *p*-tyramine ▲—▲, all at 2 μM. Data for each compound are the mean from two experiments each performed in quadruplicate.

In Table 2 it is shown that the observed fluorescence depression is not an unique property of the phosphate anions (see also Discussion). The pH-dependent fluorescence behaviour of 2 μM *m*-tyramine at pH 7·4 and pH 3·0 in 0·2 M sodium citrate or sodium acetate is similar to that seen in 0·2 M phosphate; however, no depression is found with 0·2 M sodium sulphate, implying that the anionic character of a solute is not the only determinant for a fluorescence depression. The fluorescence of isoprenaline in 0·15 M glutamate is almost completely suppressed around pH 7·4; it gradually increases

Table 2. Fluorescence of 2 μM *m*-tyramine in solutions of various composition. Relative fluorescence yield is defined in methods.

	Relative fluorescence yield pH 7·4	pH 3·0
0·2 M Na-citrate	0·31	0·78
0·2 M Na-acetate	0·31	0·78
0·2 M Na-borate*	0·9	0·9
0·2 M Na-sulphate	0·9	0·9
Krebs-bicarbonate solution	1·0	1·0

* Na-borate is almost fully protonized at pH 7·4.

with decreasing pH until a maximal fluorescence is reached at pH 4·0 (Fig. 3). This increase corresponds to an increased protonization of the γ-carboxylate group in glutamate (pK_a 4·2). Complex formation of isoprenaline with the α-carboxylate group of glutamate (pK_a 2·21) or glycinate (pK_a 2·35) could not be established. We observed only a depression of the fluorescence of isoprenaline in 0·15 M glutamate or 0·2 M glycinate below pH 3. This is similar to the observed fluorescence behaviour of catecholamines in 0·2 M phosphate with low pH.

FIG. 3

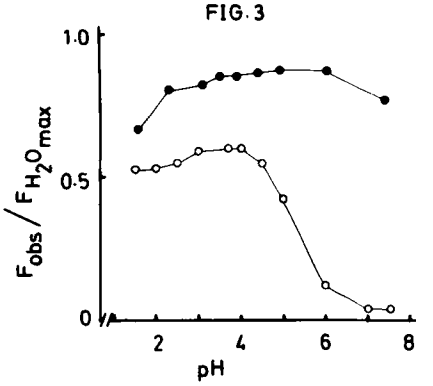


FIG. 3. Relative fluorescence yield of 2 μM isoprenaline at various acidities in: 0·15 M glutamate ○—○; 0·2 M glycinate ●—●. Each curve is the mean from two experiments each performed in quadruplicate.

The influence of the phosphate concentration upon the observed fluorescence decrease was studied and the results were plotted according to equation 5. Fig. 4 shows the data for *m*-tyramine, terbutaline and isoprenaline in phosphate solution and also of isoprenaline in glutamate solution. In Table 3 the calculated association constants are given together with the correlation coefficients and intercepts on the F_{max}/F_{obs} axis obtained. When the concentration of phosphate is increased above 0·5 M, a deviation from linearity is observed, F_{max}/F_{obs} increases rapidly because F_{obs} approaches zero (not shown).

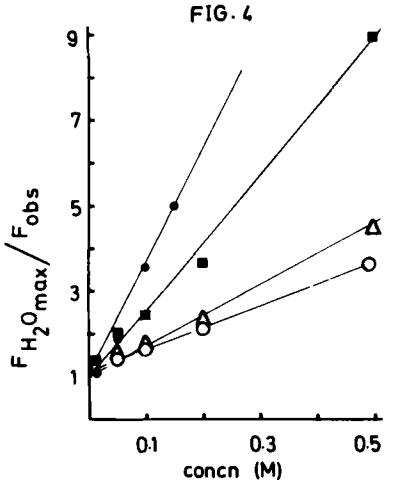


FIG. 4. Normalized fluorescence ($F_{H_2O_{max}}/F_{obs}$) dependence on the concentration of the complexing anion. Isoprenaline–phosphate ○—○; isoprenaline–glutamate ●—●; *m*-tyramine–phosphate ■—■; terbutaline–phosphate △—△. For details see the text and Table 3. Data are the means from two experiments each performed in quadruplicate.

DISCUSSION

In our opinion, the observed fluorescence decrease of catecholamines and related compounds in 0·2 M phosphate solution at pH 7·4 compared with the fluorescence in water is best explained by assuming the formation of a complex between these compounds at the HPO_4^{2-} species.

The main argument for the existence of such a complex is the pH dependence of the observed fluorescence decrease and the parallel between this observation and the presence of the species HPO_4^{2-} (pK_a 12·33) in the solution. In our experimental set-up the largest suppression of fluorescence was measured at pH 7·4, at which pH value 70% of the phosphate anions are present as HPO_4^{2-} whereas at pH 4·0 less than 1% is present as the HPO_4^{2-} and 99% in the $H_2PO_4^-$ (pK_a 7·21) form. Thus, the observation that at around pH 4·0 the fluorescence in 0·2 M phosphate is still not equal to that in water indicates that around pH 4·0 complex formation between the $H_2PO_4^-$ species and the fluorescent ligand might be possible. With monohydroxyphenethylamines an additional increase in fluorescence occurs below pH 2·0 up to the same level as obtained in acidified H_2O; on the pH scale this corresponds with the conversion of the species $H_2PO_4^-$ to H_3PO_4 (pK_a 2·23).

Table 3. Association constants of isoprenaline, terbutaline and *m*-tyramine with phosphate or glutamate at pH 7·4.

	K(litres mol^{-1})	Correlation coefficient*	Intercept*
Isoprenaline—phosphate	5·11	0·998	1·11
Isoprenaline—glutamate	26·59	0·995	0·89
Terbutaline—phosphate	6·93	0·999	1·02
m-Tyramine—phosphate	15·30	0·997	1·01

* Correlation coefficient and intercept on the ordinate of the straight lines plotted in Fig. 3, obtained with linear regression analysis.

In Table 1 we showed that complex formation is not restricted to catecholamines or resorcinolamines, but is found with the parent compounds phenol, catechol and resorcinol as well. Ethyl substitution into these compounds does not interfere with complex formation as seen from the data obtained with 3-ethylphenol and 4-ethylphenol.

The fluorescence of phenol is lost upon complex formation because the hydrogen bond between the OH-group and the HPO_4^{2-} anion increases the polarization of the OH-bond. This is also thought to occur with catechol and resorcinol; however, the complex formation between catechol and resorcinol with HPO_4^{2-} is less than with phenol (Table 1). There is no correlation between the pK_a values of these three structures (see Table 4) and the loss of fluorescence as indicated in Table 1.

Table 4. pK_a values of the aromatic hydroxyl groups in phenol, catechol and resorcinol and some monomethyl derivatives. (From Sunkel & Staude 1968.)

	pK_{a1}	pK_{a2}
Phenol	9·99	
Catechol	9·25	12·37
Resorcinol	9·20	11·27
4-CH_3-phenol	10·26	
4-CH_3-catechol	9·67	12·77
5-CH_3-resorcinol	9·30	11·66

It might be speculated that steric factors associated with the structure of the complexing anion play an important role in the complex formation, as indicated by the absence of any effect upon the fluorescent properties of *m*-tyramine by SO_4^{2-}; the S-O bond is 1·4 Å whereas the P-O bond is 1·6 Å (Huheey 1975). However, the exact parameters governing the observed fluorescence loss of the various compounds could not be determined.

The increase in fluorescence depression upon introduction of an ethylamine or an ethanolamine substituent, as seen most clearly with catechol, might be explained by the formation of an energetically favourable intermolecular complex between the fluorescent ligand and HPO_4^{2-}. Since the closest approach between the protonated amine group and the hydroxyl substituents in the phenyl ring is 4·8 Å (Bustard & Egan 1971) and the effective H-bonding distance is known to range from 2·61 to 2·89 Å (Hearn et al 1973), intramolecular hydrogen bonding in catecholamines and related compounds must be solvent- or solute-mediated.

We propose the formation of a complex between catecholamines and related compounds with HPO_4^{2-} as shown in Fig. 5a. The existence of such a 1:1 complex between catecholamines and HPO_4^{2-} is also supported by the very good fit of the experimental data according to equation 5, which was derived assuming the formation of 1:1 complexes between the fluorescent ligand and the complexing anion.

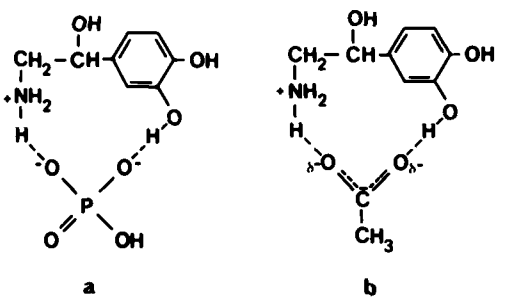


FIG. 5. Proposed structures of the hydrogen bonded complexes of noradrenaline with HPO_4^{2-} (Fig. 5a) and with the acetate anion (Fig. 5b).

Based on X-ray crystallographic data, Hearn et al (1973) showed a complex between HPO_4^{2-} and ephedrine, with the HPO_4^{2-} anion hydrogen bonded to the protonated amine function and the β-hydroxyl group. Our data do not argue against an involvement of the β-hydroxyl group in hydrogen bonding with a HPO_4^{2-} anion (in fact very small, but consistent differences in fluorescence decrease were observed between dopamine and noradrenaline and between *m*-tyramine and phenylephrine, in favour of a role for the β-hydroxyl group in hydrogen bonding) but the spectrofluorimetric method may be inappropriate to detect such an involvement because the fluorescence due to the aromatic moiety presumably is not influenced by HPO_4^{2-} hydrogen bonded to the β-hydroxyl group and the pronated amine function (cf. the data obtained with propranolol and pronethalol in Table 1).

Optical techniques have been used only occasionally for the study of the complex forming properties of catecholamines. An interaction of adrenaline with adenine, thymine and uracil was reported by Al-Obeidi & Borazan (1976) using u.v.-spectroscopic data, but, considering the very high concentrations used (0·2 M and higher), this is most probably a stacking interaction. Pai & Maynert (1972) using i.r.-spectroscopic data provided evidence for the involvement of the catechol hydroxyl groups as well as the β-hydroxyl group in hydrogen bonding with ATP. Maynert et al (1972) showed that at pH 7 a 4:1 noradrenaline–ATP complex was formed and at pH 3 a 3:1 complex. Complex formation between catecholamines and $MgHPO_4$ was reported by Seifter et al (1972); they isolated catecholamines as the $MgHPO_4$ salt, but they did not demonstrate this species to exist in aqueous solution.

The interaction between catecholamines and nucleotides has also been studied by ^{1}H-n.m.r. and ^{31}P-n.m.r. Weiner & Jardetzky (1964) and Jardetzky (1964), on the basis of relaxation times of the CH and CH_3 protons of adrenaline proposed an interaction of the β-hydroxyl group but not of the ring hydroxyl groups of adrenaline with the phosphate groups of nucleotides. However, Colburn & Maas (1965), Hammes & Tallman (1971), Katz et al (1974, 1977) and Muro et al (1971) produced evidence for the involvement of the catechol hydroxyl groups as well as the β-hydroxyl group of catecholamines in the interaction with phosphate groups of different nucleotides. Granot (1977, 1978a, b) provided the most detailed study of the interaction of catecholamines with ATP. His data indicated the existence of catecholamine: ATP complexes with stoichiometries of 1:1 and 2:1 in the absence of metal ions. These complexes were stabilized by ring association, vertical stacking and hydrogen bond formation between catecholamine hydroxyls and the nitrogen atoms of the purine ring, without participation of the phosphate groups. In the presence of divalent metal ions ternary complexes are formed between catecholamines, ATP and the metal ion without a direct interaction between the catecholamine and the metal ion or the phosphate moiety.

Thus it appears that there is no general agreement in the literature on the role of the hydroxyl groups in catecholamines in the complexing of these compounds with phosphate. It shows that the experimental conditions and in particular the absence or presence of metal ions play a decisive role in deter-

mining whether hydrogen bonding at a particular hydroxyl group in the catecholamine molecule takes place. Our data indicate unequivocally a participation of the aromatic hydroxyl groups in the hydrogen bonding of catecholamines to phosphate in the absence of metal ions.

Hydrogen bonding between catecholamines and phosphate or carboxylate anions has been suggested as a possibility for the binding of catecholamines with adrenoceptors (Bloom & Goldman 1966; Belleau 1966; Plauchithiu et al 1978; Zaagsma 1979). We propose the structure as depicted in Fig. 5b for the formation of a complex between catecholamines and glutamate or acetate.

In conclusion, we have demonstrated the hydrogen bonding between several anionic species (HPO_4^{2-}, acetate, glutamate) and catecholamines, resorcinolamines and related compounds in aqueous solution. The exact number of species of complexes possible between catecholamines and HPO_4^{2-} could not be determined, but based on the assumption of an 1 : 1 intramolecular complex formation a function was derived which gives a very good description of the experimental data. Optimal interaction is found with those compounds possessing only one hydroxyl group and an ethylamino substituent into the phenyl nucleus.

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