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Interactions of Biogenic Amines with Organic Phosphate: A Proposed Model *in Vitro* for Study of Biological Interactions by ^{31}P Nuclear Magnetic Resonance

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SUMMARY

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The interaction between ^{31}P-containing substrates, 4-deoxypyridoxine phosphate and monomethyl phosphate, and a series of protonated biogenic amines has been quantitated by use of [^{31}P]-^{1}H nuclear magnetic double resonance and titration. The difference in the phosphorus chemical shifts with and without amine in deuterated water indicated that optimal biogenic amine–phosphate interaction appears to require the presence of both a catechol ring and a β-hydroxyl group in the ethylamine side chain. This is in good agreement with pharmacological measurements *in vitro* of biogenic amine retention by storage vesicles of rat heart adrenergic nerve terminals, indicating that phosphate could be involved in binding of biogenic amines in these vesicles. The model *in vitro* presented could provide a rapid, efficient method for biological evaluation of various drug-receptor systems involving phosphate-amine interactions.

INTRODUCTION

Microsomal fractions isolated from tissue homogenates of heart (1), brain (2, 3), and splenic nerves (4, 5) are known to contain catecholamine storage vesicles. Musacchio *et al.* (6, 7) demonstrated that retention of norepinephrine-related biogenic amines by the vesicular fraction varied significantly with the structure of the amine. It has been suggested that a catecholamine-ATP complex is responsible for the storage of the amines in the vesicles (8–10). Evidence for amine-phosphate ionic interactions has been reported in model systems *in vitro* (11–14). Recently we demonstrated (15) that 4-dPNP[1]- and MMP-amine interactions can be quantitatively measured by the use of ^{31}P NMR coupled with pH titration. Therefore we decided to extend this method to study the binding of a number of structurally related biogenic amines to the above phosphate-containing

Portions of this work have been published in preliminary form [(1976) *Fed. Proc.*, 35, 340].

[1] The abbreviations used are: 4-dPNP, 4-deoxypyridoxine phosphate; MMP, monomethyl phosphate.

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molecules. Apparent correlations between NMR results and binding studies *in vitro* are discussed.

MATERIALS AND METHODS

The experiments were performed on a Varian HA-100 spectrometer with a probe modified for the [^{31}P]-^{1}H double resonance experiment (16). A concentric capillary containing 10% tetramethylsilane in CCl_4 was centered in each NMR sample tube and used as an external ^{1}H lock. The ^{31}P irradiation frequency was derived from a Hewlett-Packard model 5100 frequency synthesizer and model 5110B synthesizer driver. The ^{31}P resonance frequency was determined as the frequency at which a maximum decoupling effect (collapse of the ^{1}H doublet into a singlet) of the 5′-methylene group (in 4-dPNP) or the methyl group (in MMP) was observed. To minimize errors resulting from a possible drift of the magnetic field, the ^{31}P resonance frequency was always measured relative to that of a standard 4-dPNP solution (at pH 3.00) following each sample run. The same measurements were repeated at least three times, and the values were averaged. The frequency difference between the reference sample and a titrated 4-dPNP (or MMP) sample at various pH values was referred to as ^{31}P chemical shift [$\delta(^{31}P)$] (see Fig. 1). An increase in $\delta(^{31}P)$ indicated a downfield shift. No correction was made for susceptibility.

Sample preparation. 4-dPNP was purchased from Calbiochem. MMP was synthesized by Andrulis Research Corporation, Bethesda, Md. Samples of the various amines were received from Drs. C. R. Creveling and J. Daly of the Laboratory of Chemistry, National Institute of Arthritis, Metabolism, and Digestive Diseases. To eliminate contamination of paramagnetic ions, the samples were passed over Dowex 50 ion-exchange resin. All the biogenic amines used were hydrochloride salts.

The sample concentration was kept constant at 0.04 M in D_2O throughout the study, except where otherwise stated. The pH of the solutions was measured with a Radiometer pH meter with an Instrumentation Laboratories No. 14200 electrode, which could be conveniently inserted into the 5-mm NMR tube. The pH meter was calibrated every few readings with standard buffer (Fisher) in the range being measured. To adjust the pH of the solution, a D_2O solution of DCl (1 or 0.1 M) or NaOD (1 or 0.1 M) was used. The pH of the solution was measured before and after each reading, the values generally agreeing within 0.03 pH unit. The average pH value was used for calculations. No correction was made for the differences between activities of hydrogen and deuterium ion at the glass electrode.

RESULTS AND DISCUSSION

In the analysis of NMR spectra, the formation of intermolecular complexes is often reflected by a change in chemical shifts (and/or relaxation times). A change in chemical shift will occur when either the charge density or the magnetic anisotropic shielding at a given nucleus is different in the complexed from the noncomplexed state. Addition of amines to either 4-dPNP or MMP produces a negligible change in their proton titration curves (i.e., plot of proton chemical shift vs. pH[2]). This finding rules out significant intermolecular association at the pyridine ring (in 4-dPNP) or methyl group (in MMP). The presence of amines, however, causes a horizontal displacement (i.e., a change in the pK value) of the ^{31}P titration curve,[3] which follows the change in the equilibrium between monobasic and dibasic phosphate ions (Fig. 1). The lack of observed vertical displacement suggests no direct magnetic aniostropic effect on the ^{31}P chemical shift of the complex. Therefore this horizontal displacement could provide a basis for comparison of the binding strength of amines to the phosphate-containing substrates. It produces a maximum difference in the ^{31}P chemical shifts in the proximity of the pK_a of the phosphate group. The larger the displacement, the greater the difference in $\delta(^{31}P)$ of these two curves. Hence an alternative means for comparing

[2] For an NMR titration curve, see refs. 17–19.

[3] For a ^{31}P titration curve of nicotinamide mononucleotide, see ref. 20.

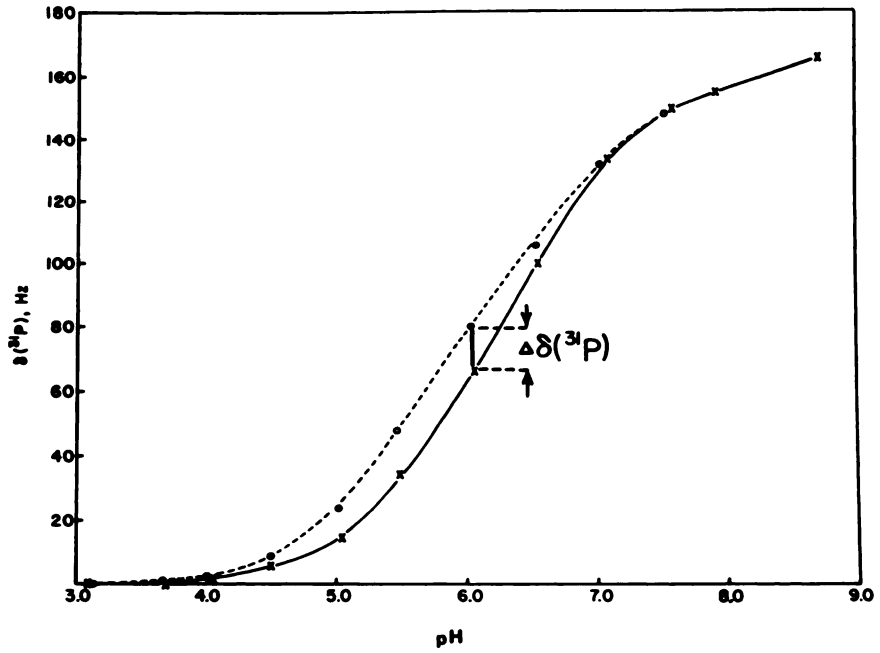


FIG. 1. *pH dependence of ^{31}P shift*

δ (^{31}P) values were measured at 23,487 G for a 0.04 M solution of 4-dPNP in D_2O without (×——×) and with (O- - -O) amine, at an organic phosphate to amine ratio of 1:3. A solution of 10% tetramethylsilane in CCl_4 was used as external ^1H lock.

the binding strength of amines to the phosphate-containing substrates is to measure the difference in $\delta(^{31}P)$ of the phosphate group close to its pK_{a_2} region (pH = 6.1) in the absence and presence of identical amine concentrations. The difference in $\delta(^{31}P)$ at the same pH is defined as

$$\Delta\delta(^{31}P) = \delta(^{31}P) \text{ with amine} - \delta(^{31}P) \text{ without amine} \quad (1)$$

In our previous studies (15) the $\Delta\delta(^{31}P)$ values were found to depend upon the nature and concentration of the amine[4] and the ionic strength of additional cations present (see legend to Table 1).

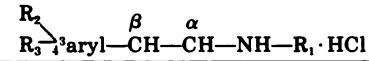
A 3:1 phosphate to amine ratio provided conveniently large $\Delta\delta(^{31}P)$ values. Therefore interactions between 4-dPNP or MMP and various biogenic amines (Table 1) were measured at this ratio. Norepinephrine (*1*) causes the largest change in $\delta(^{31}P)$ value [$\Delta\delta(^{31}P)$ = 27.0 Hz for 4-dPNP and 22.0 Hz for MMP], suggesting that among these amines norepinephrine exhibits the strongest interaction with the phosphate-containing substrates. Such interaction is weakened when the β-hydroxyl (*2*), 3-hydroxyl (*4*), or 4-hydroxyl (*3*) group of norepinephrine is substituted by a hydrogen atom, as indicated by the decrease in the $\Delta\delta(^{31}P)$ value. This decrease is even more effective when two or more of the hydroxyl groups are substituted (compare *1* and *5*). This effect can also be observed in other related compounds (e.g., compare *6* and *7*, *2* and *5*, *8* and *9*, *10* and *12*). The general decrease in $\Delta\delta(^{31}P)$ value as a result of the absence of hydroxyl groups in the biogenic amines suggests that these groups are also involved in interactions with the phosphate-containing substrate, presumably through hydrogen bonding. Infrared (12) and ^1H relaxation (11) studies on the catecholamine-ATP system are consistent with this interpretation. Methoxylation at position 3 or 4 of the catechol ring lowers the $\Delta\delta(^{31}P)$ value significantly (compare *2* and *11*, *2* and *10*, *5* and *12*). These values

[4] A concentration dependence study of $\Delta\delta$ (^{31}P) values for the interaction of *l*-norepinephrine and 4-dPNP provided an apparent association constant of 8.0 M^{-1}.

TABLE 1

Interactions between biogenic amines and organic phosphate measured by $\Delta\delta(^{31}P)$ values

$\Delta\delta(^{31}P)$ is defined in Eq. 1; values were measured at 23,487 G and at a phosphate (0.04 M) to amine ratio of 1:3. Results are the averages of three or more determinations at pH 6.00 for 4-dPNP and at pH 6.15 for MMP. The possible standard error is ±1.0 Hz. The salt effect due to Na^+ was found to be negligible and was not accounted for, since the Na^+ concentrations in the phosphate samples both with and without amines were equal (0.04 M).



No.	Name	R_1	R_2	R_3	α	β	$\Delta\delta(^{31}P)$	
							MMP	4-dPNP
							Hz	*Hz*
1	*l*-3,4-β-Trihydroxy-PEA[a]	H	OH	OH	H	OH	22.0	27.0
2	3,4-Dihydroxy-PEA	H	OH	OH	H	H	17.5	21.5
3	3-β-Dihydroxy-PEA	H	OH	H	H	OH	18.0	21.5
4	4-β-Dihydroxy-PEA	H	H	OH	H	OH	15.5	20.0
5	4-Hydroxy-PEA	H	H	OH	H	H	15.0	16.5
6	*l*-3,4-β-Trihydroxy-*N*-methyl-PEA	CH_3	OH	OH	H	OH	19.0	24.5
7	3,4-Dihydroxy-*N*-methyl-PEA	CH_3	OH	OH	H	H	15.0	20.0
8	3,4-Dihydroxy-α-methyl-PEA	H	OH	OH	CH_3	H	13.5	18.0
9	4-Hydroxy-α-methyl-PEA	H	H	OH	CH_3	H	11.5	11.5
10	3-Hydroxy-4-methoxy-PEA	H	OH	OMe	H	H	13.5	20.0
11	4-Hydroxy-3-methoxy-PEA	H	OMe	OH	H	H	12.5	12.0
12	4-Methoxy-PEA	H	H	OMe	H	H	10.0	8.5

[a] PEA = phenethylamine. *1* = *l*-norepinephrine; *2* = dopamine; *3* = *m*-octopamine; *4* = octopamine; *5* = tyramine; *6* = *l*-epinephrine; *7* = *N*-methyldopamine; *8* = α-methyldopamine; *9* = α-methyltyramine; *10* = 4-*O*-methyldopamine; *11* = 3-*O*-methyldopamine; *12* = 4-*O*-methyltyramine.

further suggest the participation of the hydroxyl group in intermolecular hydrogen bonding and emphasize the greater importance of the 3-hydroxyl group in biogenic amine–phosphate interactions. The larger $\Delta\delta(^{31}P)$ value generally observed for 4-dPNP, compared with MMP, is probably due to the additional interactions dependent on the steric environment of the phosphate group. Introduction of a bulky methyl group at a nitrogen or at the α-position of the biogenic amines also leads to a decrease in the $\Delta\delta(^{31}P)$ value in comparison with the unsubstituted compound (compare *1* and *6*, *2* and *7*, *2* and *8*). The reduction in $\Delta\delta(^{31}P)$ value as a result of methylation is presumably due to the steric effect, which weakens the amine-phosphate interaction.

A relationship between amine structure and the strength of binding to the phosphate group is clearly discernible, since no correlation between the pK_a of the amines and $\Delta\delta(^{31}P)$ values has been observed. For optimal interaction the biogenic amine requires the catechol structure, the β-hydroxyl group, the absence of a methoxyl group(s), and the absence of bulky *N*-methyl or α-methyl groups.

Musacchio *et al.* determined the percentage of biogenic amines retained in storage vesicles isolated from microsomal fractions of rat heart adrenergic nerve terminals (6) and related their subcellular distribution to their release induced by drugs (7). It was found that greater retention of an amine by the particulate fraction correlated with greater resistance to depletion by drugs such as reserpine, tyramine, and guanethidine. Their results indicate a relationship between amine structure and binding strength. Table 2 compares our results and those of Musacchio *et al.* (6). In the biological study, norepinephrine showed the highest percentage retention in storage vesicles suggesting that its binding to the storage sites is by far the strongest. Dopamine, *m*-octopamine, and

TABLE 2

Comparison between retention of biogenic amines by storage vesicles and $\Delta\delta(^{31}P)$ values

No.	Name	Retained in microsomal fraction[a]	$\Delta\delta(^{31}P)$	
			4-dPNP	MMP
		%	*Hz*	*Hz*
1	Norepinephrine	48 ± 1.1	27.0	22.0
2	Dopamine	38 ± 2.2	21.5	17.5
3	*m*-Octopamine	30 ± 3.4	21.5	18.0
4	Octopamine	25 ± 1.4	20.0	15.5
5	Tyramine	10[b]	16.5	15.0
9	α-Methyltyramine	6 ± 0.5	11.5	11.5

[a] Results of Musacchio *et al.* (6). All values were determined as percentages of [^{3}H]norepinephrine found in the microsomal fraction 1 hr after injection, compared with the sum of the microsomal and soluble fractions obtained as the supernatant from the microsomal fraction.

[b] Measured 4 min after injection of [^{3}H]tyramine. After the customary 1 hr, there was no measurable radioactivity left from [^{3}H]tyramine in the microsomal fraction.

p-octopamine, each of which lacks one of the three hydroxyl groups of norepinephrine, were much more easily released. The lower percentage retention of α-methyltyramine than that of tyramine suggests that the presence of a bulky α-methyl group in the biogenic amine will reduce its binding. For the amines listed, the $\Delta\delta(^{31}P)$ values of both phosphate-containing model substrates show good agreement with the biological data.

The chromaffin granules of the adrenal medulla are known to have an unusually high content of ATP (21). Since both catecholamines and ATP are depleted from the granules by prior treatment with reserpines (22, 23), it has been suggested that a catecholamine-ATP complex is responsible for the storage of amines in the granules (8, 9). Smythies *et al.* (10) proposed that the storage site of catecholamines in the adrenal medulla (and perhaps elsewhere) is a complex of ATP, Ca^{2+}, Mg^{2+}, and protein. Helle (24) found that ATP is firmly bound to a soluble protein of the adrenal chromaffin granules (chromogranin), which later was reported (25) to have a high content of glutamine (and asparagine).

The remarkable parallel between results obtained by our ^{31}P NMR method and pharmacological measurements by others (6, 7) tends to support the hypothesis, mentioned above, that the phosphate group may be related in some way to an amine binding site within the storage vesicles (8–10, 24, 25). Although this parallel could be coincidental, the observation that both 4-dPNP and MMP conform to it, in spite of structural dissimilarities, tends to reduce the likelihood of such a coincidence. While the storage site-biogenic amine interaction *in vivo* is stereospecific, $\Delta\delta(^{31}P)$ values found *in vitro* were very similar for the *d*(−) and *l*(+) forms. This lack of stereospecificity is not surprising, since the molecules involved in the interactions are structurally simple without restricted rotations or exaggerated steric hindrance. *In vivo*, a protein matrix could provide a specific steric orientation which might favor the *l*(+) over the *d*(−) form.

The present study raises some as yet unanswered questions. One of these is the need to analyze the data in "terms of complete set of equilibria requiring measurements as a function of both pH and amine concentration for each amine."[5] This could permit estimation of the stability constants which determine the pK shift caused by addition of the amine to the phosphate solution. Determination of the stability constants for the possible complexes formed could in turn validate our results, which suggest a much stronger dianionic complex than a monoanionic one. Other questions regarding the model presented refer to its validity when AMP or ATP is substituted for 4-dPNP and MMP.

[5] Quoted from the remarks of one reviewer.

Further validation of the ^{31}P NMR model *in vitro* could provide information on structure-activity relationships for drug designers in systems where phosphate-amine interactions play a major role in drug-receptor interactions. This study also suggests the possibility of extending the method to complex systems (enzymes, proteins) where electrometric titration could not provide pK determinations. However, the study is no more than a first step in the lengthy and tedious process of designing methods *in vitro* for assessment of important biological interactions.

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