MOLECULAR PHARMACOLOGY, 19:44–48

Ring Stacking in Solutions of Norepinephrine and the 4:1 Norepinephrine·ATP Complex

B. H. MOON,[1] V. S. PAI, AND E. W. MAYNERT

Department of Pharmacology, University of Illinois College of Medicine, Chicago, Illinois 60680

Received April 8, 1980; Accepted August 6, 1980

SUMMARY

MOON, B. H., V. S. PAI, AND E. W. MAYNERT. Ring stacking in solutions of norepinephrine and the 4:1 norepinephrine·ATP complex. *Mol. Pharmacol.* **19**:44–48 (1981).

In D_2O (pD 6.8), increasing concentrations of norepinephrine were accompanied by an upfield shift of the phenyl proton signal. At all concentrations the addition of 1 mole of ATP for 4 moles of the amine caused the signal to move further upfield. To delineate stacking from other electronic processes in solutions of norepinephrine, dimethyl sulfoxide (DMSO), a pure hydrogen bond acceptor, was utilized. In this solvent, increasing concentrations of norepinephrine were accompanied by a downfield shift of the phenyl proton signal, whereas phenylethylamine, a compound incapable of hydrogen bonding, showed no change. The downfield shift observed with norepinephrine was explained by increased hydrogen bonding between catechol OH groups in the more concentrated solutions. If hydrogen bonding were the only factor influencing the magnetic field, the replacement of DMSO by D_2O, a hydrogen bond donator and acceptor, should cause a continuous downfield shift of the phenyl proton signal. In 0.5 M norepinephrine the signal moved downfield until the D_2O reached 80 volumes/100 volumes but then reversed its course. The upfield shift was attributed to stacking of the aromatic rings. In all mixtures of DMSO and D_2O, the phenyl proton signal of norepinephrine was moved upfield by the addition of adenosine. These shifts were explained by the formation of complexes between the adenine and catechol rings. In D_2O, ATP produced a greater upfield shift than did adenosine. This observation indicated augmented ring stacking in the catecholamine·ATP complexes. In D_2O, increasing the temperature of the norepinephrine·ATP complex from 28° to 80° caused a downfield shift of the phenyl—H and adenine ^2H signals, whereas the adenine ^8H showed no change. The phenyl—H signal of norepinephrine alone was not affected by temperature. These observations are in harmony with a Dreiding model of the 4:1 norepinephrine·ATP complex in which the adenine ^2H is located between two catechol rings.

INTRODUCTION

Earlier research from this laboratory (1, 2) has established that norepinephrine reacts with ATP at pH 7.0 to form a complex containing the amine and the nucleotide in the molar ratio of 4:1. The bond formed between the catecholamine cation and the nucleotide anion appears to owe some of its stability to the formation of hydrogen bonds between the enthanolamine OH groups and P═O moieties of ATP, an interaction indicated by both infrared and NMR[2] spectroscopy (1, 3). Another factor which might serve to increase the stability of the complex is orientation of the aromatic rings in stacks. This phenomenon can be detected by upfield shifts of the proton magnetic signals generated by the rings (4–7).

MATERIALS AND METHODS

Crystalline $2Na^+$·ATP, norepinephrine·HCl, and adenosine were obtained from Sigma Chemical Company, St. Louis, Mo. NMR spectra were recorded with a Varian A-60 spectrometer equipped with a variable temperature probe which was calibrated to the accuracy of ±0.2°. Chemical shifts were measured in parts per million relative to benzene as an external standard by using Wilmad coaxial inner cells. Inasmuch as the methylene protons generating the norepinephrine peaks were found to be independent of solute concentration, the bulk magnetic susceptibility differences were considered negligi-

This research was supported by United States Public Health Service Grant NB-06312. A preliminary account of this work appeared in *The Pharmacologist* **12**:98 (1970) (Catecholamine storage. III.).

[1] Present address, Department of Pharmacology, Rush-Presbyterian-St. Luke's Medical Center, Chicago, Ill. 60612.

[2] The abbreviations used are: NMR, nuclear magnetic resonance; DMSO, dimethyl sulfoxide; NE, norepinephrine.

0026-895X/81/010044-05$02.00/0

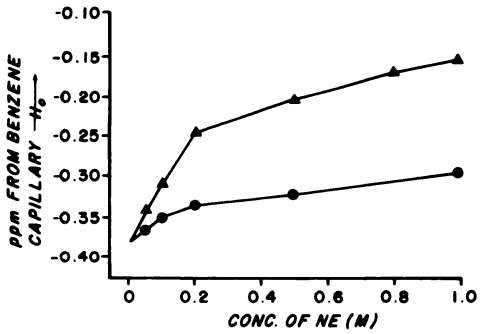

FIG. 1. *Chemical shifts in the phenyl proton NMR signal of norepinephrine with changes in concentration and the addition of ATP*

D_2O solutions (pD 6.8), 1.0 M in norepinephrine (●——●) or 1.0 M in norepinephrine and 0.25 M in ATP (▲——▲), were measured and then diluted with D_2O.

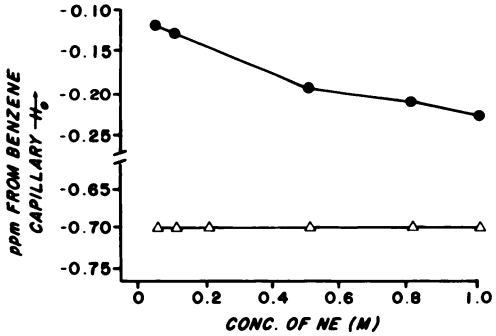

FIG. 2. *Chemical shifts in the phenyl proton NMR signal with changes in the concentration of norepinephrine·HCl* (●——●) *or phenylethylamine HCl* (△——△) *in DMSO*

ble, and no corrections of chemical shifts were made. In the variable-temperature study, dioxane was used as an internal standard. All pH measurements were made on a Beckman Expandomatic meter equipped with a Thomas universal glass electrode. Solutions of norepinephrine·HCl and $2Na^+$·ATP in D_2O were neutralized with NaOD. A pD of 6.8 was assigned to solutions with meter readings of 6.4 (8).

RESULTS

The first experiment involved a comparison in D_2O at pD 6.8 of the phenyl proton resonance in norepinephrine and 4:1 molar mixtures of the amine and ATP. The results in Fig. 1 show that increasing concentrations of norepinephrine from 0.05 to 1.0 M were accompanied by an upfield shift from −0.37 to −0.30 ppm. In the presence of ATP the phenyl proton signals were consistently located at higher magnetic fields. For example, in 0.5 M norepinephrine the presence of ATP caused an upfield shift amounting to 0.13 ppm.

The increased magnetic fields associated with increased concentrations of norepinephrine are consistent with augmented vertical stacking of the planar rings as the molecules are brought closer together (6, 7). However, the participation of electronic effects such as increased hydrogen bonding of catechol OH groups to H_2O, a process which would increase the negativity of the electron cloud, had to be considered (9). The large upfield shift produced by the addition of ATP could be explained by the formation of catecholamine·nucleotide complexes and consequent orientation of the rings in vertical stacks. However, the data are consistent with other formulations. For example, hydrogen bonding of the catechol OH groups to the phosphate groups of ATP might cause a large increase in the negativity of the electron cloud. Thus, it appeared advisable to devise experiments to delineate NMR effects of ring stacking from those induced by other electronic changes. This can be done by utilizing a solvent such as DMSO, which is a pure hydrogen bond acceptor (5, 7).

In DMSO, increasing concentrations of norepinephrine caused a downfield shift in the phenyl proton signal which ranged from −0.12 ppm at 0.05 M to −0.22 ppm at 1.0 M (Fig. 2). In contrast, phenylethylamine exhibited no alterations in its phenyl signal as the concentration was varied. The shifts in magnetic resonance observed with norepinephrine can be interpreted in terms of electronegativity of the catechol oxygen atoms (Fig. 3). In very dilute solutions, practically all of the catechol OH groups are bonded to the oxygen atoms of DMSO. This process increases the negativity of the electron cloud of the catechol ring. As the concentration of norepinephrine is increased, the molecules of this compound are brought together close enough to form hydrogen bonds between catechol OH groups. This interaction reduces bonding to the solvent and thereby diminishes the electronegativity of the electron cloud. The constant phenyl proton signal observed with phenylethylamine was expected, since the aromatic ring in this compound is devoid of groups which can form hydrogen bonds with the solvent.

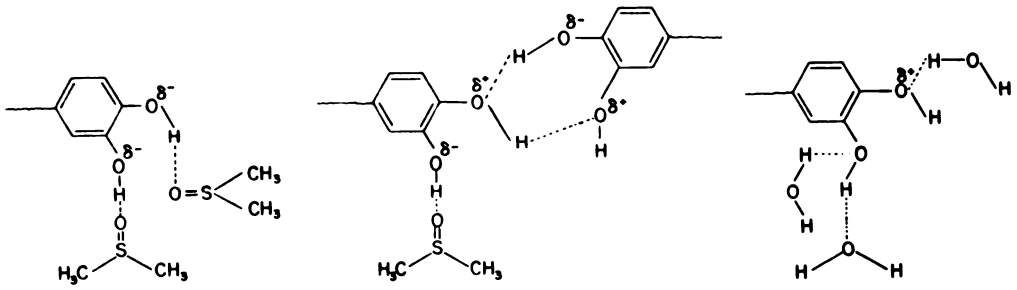

FIG. 3. *Structural formulae designed to illustrate the effects of hydrogen bonding on the electronegativity of the π-electron cloud of the catechol ring of norepinephrine*

The structure on the *left* depicts maximal negativity in dilute solutions in DMSO. The formula in the *center* portrays the hydrogen bonding between catechol OH groups expected to occur in concentrated solutions in DMSO. The structure on the *right* depicts the catechol OH group as a hydrogen bond acceptor in aqueous solutions.

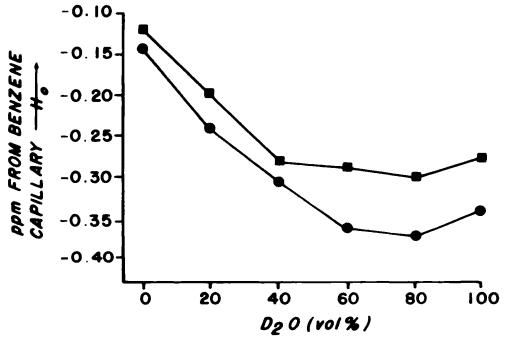

FIG. 4. *Chemical shifts in the phenyl proton NMR signal of 0.5 M norepinephrine (●——●) or 0.5 M norepinephrine and 0.125 M adenosine (■——■) in mixtures of DMSO and D_2O*

The next series of experiments involved the gradual replacement of DMSO by D_2O in solutions kept 0.5 M in norepinephrine. This procedure causes three changes. First, it substitutes a hydrogen-donating and hydrogen-accepting solvent for a pure acceptor. Second, it leads to a kind of hydrogen bonding of the catechol OH groups which has a greater electronic effect than intermolecular interactions of norepinephrine (Fig. 3). Finally, it diminishes electronegativity through a direct action of D_2O on the electron clouds of the aromatic rings (10). Thus, as the concentration of D_2O is increased and that of DMSO is decreased, if only hydrogen bonding influenced the phenyl proton signal in norepinephrine, a continuous downfield shift would be expected. The experimental results (Fig. 4) show that the signal moved downfield until the D_2O reached a concentration of 80 volumes/100 volumes but then reversed its course. Inasmuch as this change in direction of the signal cannot be attributed to hydrogen bonding, it must represent some other phenomenon such as ring stacking.

The insolubility of ATP in DMSO precluded the systematic study of norepinephrine·ATP complexes in mixtures of this solvent and D_2O. Therefore, adenosine was substituted for ATP. The data (Fig. 4) show that, under all conditions investigated, 0.125 M adenosine caused an upfield shift in the phenyl proton signal generated by 0.5 M norepinephrine. Increasing concentrations of D_2O caused a sharp downfield shift up to 40 volumes/100 volumes and a more gradual decline up to 80 volumes/100 volumes. Higher concentrations of D_2O resulted in an upward shift. These results indicate increased ring stacking in the presence of the nucleotide.

If the augmented ring stacking reflects organization of the aromatic rings of the catecholamine and the nucleotide in the form of intermolecular complexes, downfield shifts of the phenyl proton signal should accompany increases in temperature that increase dissociation of the complex. The experimental approach was to compare D_2O solutions of norepinephrine alone and in the 4:1 ratio with ATP at temperatures of 28° and 80°. As shown in Table 1, the phenyl proton signal from a mixture 1 M in norepinephrine and 0.25 M in ATP was shifted 0.12 ppm downfield at the higher temperature. In contrast, a solution containing the same concentration of norepinephrine but no nucleotide gave the same signal at both 28° and 80°. At the lower temperature, a 1:2 dilution of the catecholamine·ATP mixture had a phenyl proton signal 0.05 ppm below that of the concentrated solution, and the downfield shift produced by heat amounted to only 0.05 ppm. These data indicate decreased complexation in dilute solutions.

Extension of the measurements to the adenine ^2H and ^8H signals was undertaken to provide information on the interaction of the catechol rings with these regions of the nucleotide. As shown in Table 1, elevation of the temperature of solutions of the 4:1 norepinephrine·ATP complex caused a downfield shift of the ^2H signal amounting to 0.10 to 0.13 ppm. In contrast, the ^8H resonance was not changed. Control experiments on the effect of elevated temperature on the same concentrations of ATP in the absence of the catecholamine revealed downfield shifts of the ^2H but not the ^8H signal. The magnitude of the changes in the ^2H resonance was only slightly smaller than that observed in the 4:1 norepinephrine·ATP complex. This finding was anticipated in view of published work (5) on vertical stacking in solutions of purines.

At 28°, dilution of ATP from 0.25 M to 0.125 M resulted in a 0.08 ppm downfield shift of the ^2H signal, whereas similar treatment of a solution containing 4 Eq of norepinephrine showed no change (Table 1). Likewise, the catecholamine CH_2 resonance observed in the complex was not shifted by a 1:2 dilution. These findings indicate that the catecholamine·nucleotide complexes have greater stability than do intermolecular associations of ATP.

DISCUSSION

The phenyl proton NMR signal generated by norepinephrine is probably influenced by many factors, includ-

TABLE 1

Temperature-induced chemical shifts of various proton resonances in norepinephrine, ATP, and a 4:1 complex

The observations were made in D_2O, pD = 6.8, and are expressed in parts per million relative to dioxane, used as an internal standard. All of the values reported are averages of several observations agreeing within ±0.01 ppm.

ATP concentration	NE concentration	ATP—^2H		ATP—^8H		NE—ΦH		NE—CH_2	
		28°	80°	28°	80°	28°	80°	28°	80°
M	M								
0.25		−4.47	−4.57	−4.82	−4.80				
0.125		−4.55	−4.60	−4.83	−4.82				
	1					−3.27	−3.27	+0.37	+0.35
	0.5					−3.25	−3.27	+0.37	+0.35
0.25	1	−4.45	−4.58	−4.80	−4.82	−3.08	−3.20	+0.43	+0.35
0.125	0.5	−4.45	−4.55	−4.78	−4.77	−3.13	−3.18	+0.43	+0.37

ing the donation and acceptance of hydrogen bonds, electrostatic repulsion between cationic heads, complex formation with proton-donating solvents, and the stacking of aromatic rings. Under experimental conditions designed to reduce continuously the negativity of the electron cloud of norepinephrine (viz., a solvent change from DMSO to D_2O), the signal reversed its course. The only factor which could readily account for the upfield shift of the magnetic field is ring stacking.

In a solution of D_2O, increasing concentrations of norepinephrine produced an upfield shift in the phenyl proton signal. That this response cannot be attributed simply to a greater number of aromatic rings per unit volume of solvent is indicated by the observation that, in DMSO, phenylethylamine showed no change in phenyl proton resonance as the concentration was varied. In DMSO, norepinephrine exhibited a continuous downfield shift as the concentration was increased. Inasmuch as ring stacking is minimal in infinitely dilute solutions, this downfield shift cannot be explained by reduced stacking and must be attributed to other electronic alterations. The primary process would appear to be intermolecular hydrogen bond formation between catecholamine OH groups, an interaction which diminishes the electronegativity of the π-electron cloud as compared with the situation where all of the OH groups are bonded to the solvent. Undoubtedly, bonding between catechol groups brings the phenyl rings closer together, but such association would lead to negligible ring stacking if the major species were planar, such as that depicted in Fig. 3. Whether vertical stacking of aromatic rings is more prominent in aqueous solutions of norepinephrine cannot be decided on the basis of the present data. However, an important feature of water is its strong tendency to associate with itself and thereby cause solute-solute interaction. Under such conditions, ring stacking may occur to a greater extent than in solvents such as DMSO.

The adenosine-induced upfield shift of the phenyl proton signal of norepinephrine indicates that this nucleoside enhances the orientation of the catechol rings in vertical stacks. The underlying mechanism would appear to be intercalation of the purine ring between catechol rings. The ability of purine derivatives to form complexes with other aromatic compounds has been observed previously (11).

Compared with adenosine, ATP had a larger effect on the phenyl NMR signal. For example, in 0.5 M solutions of norepinephrine in D_2O, 0.125 M ATP shifted the phenyl signal upfield by 0.12 ppm, whereas an equivalent amount of adenosine changed it by only 0.07 ppm. Thus, ATP appeared to induce more vertical stacking of catechol rings than did adenosine. This finding suggested that ionic bonding between amine cations and nucleotide anions (1) as well as hydrogen bonding between ethanolamine OH groups and P═O moieties (2) in mixtures of norepinephrine and ATP orient the ring systems of the two components appropriately for participation in ring stacking. Therefore, attention was given to constructing a model incorporating all of these features.

The stereochemical feasibility of ring stacking in the 4:1 norepinephrine·ATP complex is illustrated in Fig. 5. In this model constructed from Dreiding components, the adenine ring is juxtaposed between two catechol rings. On the opposite side of the molecule, the other two catechol rings also assume an appropriate position for π-complex interaction. This model incorporates nicely the other features mentioned above. The four cationic heads of norepinephrine are located near enough to the four P—O^- bonds of ATP to form ionic bonds. Likewise, the ethanolamine OH groups are situated appropriately to form hydrogen bonds with the P═O groups.

A new point revealed by this model was that the adenine ^2H is shielded by the catechol ring, whereas the adenine ^8H is not. Experimental support along these lines was obtained by the observation that heating the 4:1 mixture of norepinephrine·ATP caused the adenine ^2H and phenyl proton signals to move downfield, whereas the adenine ^8H signal was not affected. The model shown in Fig. 5 represents an advance over earlier formulations (3, 12–15) of catecholamine·ATP complexes in that it incorporates ring stacking and excludes metals. Infrared spectroscopy of intact adrenal medullary granules (1) has proved that the concentration of divalent metals in the vicinity of the complexes must be quite low. Although Weiner and Jardetzky (3) denied ring stacking, their data as well as those of other investigators (13, 14, 16) show ATP-induced upfield shifts in the phenyl proton signal of catecholamines. In the past, little attention has been given to distinguishing ring stacking from other electronic interactions that could cause upfield shifts in the phenyl proton resonance. Daniels *et al.* (13) and Tuck and Baker (14) have regarded ring stacking as a feature of catechol-

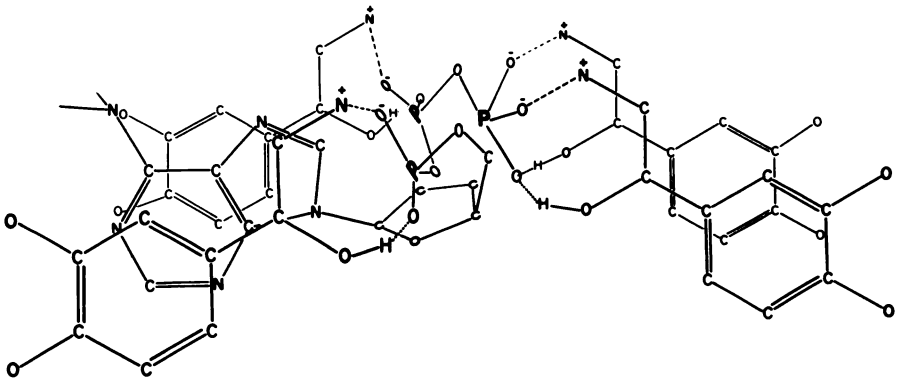

FIG. 5. *A Dreiding model of the 4:1 norepinephrine·ATP complex compatible with data on ring stacking, charge neutralization interaction, and the formation of hydrogen bonds between ethanolamine OH groups and the P═O moieties*

amine·ATP complexes, but their models incorporate metals. Daniels *et al.* (13) suggested that the phenyl ring was located over adenine ^{8}H, but this arrangement is certainly the result of added manganese. This group (13) interpreted NMR measurements on adrenal medullary granules as indicating ring stacking, but Sharp and Richards (17) denied this. Granot and Fiat (18) stated that the stacked rings in catecholamine·ATP complexes are approximately centered, but these authors did not test the stereochemical feasibility of this arrangement.

If the model shown in Fig. 5 is the thermodynamically favored form in 4:1 mixtures of norepinephrine and ATP, hydrophobic forces consequent to ring stacking might play a substantial role in stabilization of the complex. Weiner and Jardetzky (3) speculated that the dissociation constant for the ionic bonds in catecholamine·ATP complexes would be approximately 10^{-1}. They did not consider a role for ring stacking, and it remains possible that formation and breaking of ionic bonds may not extensively disrupt the complex. On the other hand, Steffen *et al.* (19) concluded from fluorescence measurements that nucleotides do not markedly hinder the motility of catecholamines. Other lines of research have shown that mixtures of catecholamines and ATP form high molecular weight aggregates that are sedimentable by ultracentrifugation (20), exhibit low osmotic pressure (21), and resist diffusion through lipid barriers (21).

REFERENCES

1. Pai, V. S., and E. W. Maynert. Interactions of catecholamines with adenosine triphosphate in solutions and adrenal medullary granules. *Mol. Pharmacol.* **8**:82–87 (1972).
2. Maynert, E. W., B. H. Moon, and V. S. Pai. Insoluble solid complexes of norepinephrine and adenosine triphosphate. *Mol. Pharmacol.* 8:88–94 (1972).
3. Weiner, N., and O. Jardetzky. A study of catecholamine nucleotide complexes by nuclear magnetic resonance spectroscopy. *Naunyn. Schmiedebergs Arch. Exp. Pathol. Pharmakol.* **248**:308–318 (1964).
4. Jardetzky, C. D., and O. Jardetzky. Investigation of the structure of purines, pyrimidines, ribose nucleosides and nucleotides by proton magnetic resonance. II. *J. Am. Chem. Soc.* **82**:222–229 (1960).
5. Chan, S. I., M. P. Schweizer, P. O. P. Ts'o, and G. K. Helmkamp. Interaction and association of bases and nucleosides in aqueous solutions. III. A nuclear magnetic resonance study of the self-association of purine and 6-methylpurine. J. *Am. Chem. Soc.* **86**:4182–4188 (1964).
6. Schweizer, M. P., S. I. Chan, and P. O. P. Ts'o. Interactions and association of bases and nucleosides in aqueous solutions. IV. Proton magnetic resonance studies of the association of pyrimidine nucleotides and their interaction with purine. *J. Am. Chem. Soc.* **87**:5241–5247 (1965).
7. Schweizer, M. P., A. D. Broom, P. O. P. Ts'o, and D. P. Hollis. Studies of inter and intramolecular interactions in mononucleotides by proton magnetic resonance. *J. Am. Chem. Soc.* **90**:1042–1055 (1968).
8. Glasoe, P. K., and F. A. Long. Use of glass electrodes to measure acidities in deuterium oxide. *J. Phys. Chem.* **64**:188–190 (1960).
9. Becker, E. D. *High Resolution NMR: Theory and Chemical Applications.* Academic Press, New York, 70 (1969).
10. Kleinfelter, P. C. A nuclear magnetic resonance study of OH . . . π hydrogen bonding. *J. Am. Chem. Soc.* **89**:1734–1735 (1967).
11. Hruska, F. E., C. L. Bell, T. A. Victor, and S. S. Danyluk. Medium effects on the nuclear magnetic resonance spectra of purines. *Biochemistry* **7**:3721–3727 (1968).
12. Pletscher, A., M. Da Prada, H. Steffen, B. Lutold, and K. H. Berneis. Mechanisms of catecholamine accumulation in adrenal chromaffin granules. *Brain Res.* **62**:317–326 (1973).
13. Daniels, A. J., R. J. P. Williams, and P. E. Wright. The character of the stored molecules in chromaffin granules of the adrenal medulla: a nuclear magnetic resonance study. *Neuroscience* **3**:573–585 (1978).
14. Tuck, L. D., and J. K. Baker. A study of the interactions among adenosine triphosphate, epinephrine and magnesium ions. *Chem. Biol. Interactions* **7**: 355–366 (1973).
15. Colburn, R. W., and J. W. Mass. Adenosine triphosphate-metal-norepinephrine ternary complexes and catecholamine binding. *Nature (Lond.)* **208**:37–41 (1965).
16. Thyrum, P. T., R. J. Luchi, and H. L. Conn, Jr. The complex formation of procaine and procaineamide with adenosine triphosphate. *J. Pharmacol. Exp. Ther.* **164**:239–251 (1968).
17. Sharp, R. R., and E. P. Richards. Analysis of the carbon-13 and proton NMR spectra of bovine chromaffin granules. *Biochim. Biophys. Acta* **497**:14–28 (1977).
18. Granot, J., and D. Fiat. Adenine nucleotides and divalent metal ions. III. Aqueous solution. *J. Am. Chem. Soc.* **99**:4963–4968 (1977).
19. Steffen, H., M. Da Prada, and A. Pletscher. Fluorescence properties of catecholamines in isolated storage organelles of adrenal medulla. *Biochem. Biophys. Acta* **338**:561–571 (1974).
20. Berneis, K. H., A. Pletscher, and M. Da Prada. Phase separation in solutions of noradrenaline and adenosine triphosphate: influence of bivalent cations and drugs. *Br. J. Pharmacol.* **39**:382–389 (1970).
21. Berneis, K. H., M. Da Prada, and A. Pletscher. Catecholamine transport through a lipid barrier. *Nature (Lond.)* **248**:604–606 (1974).

Send reprint requests to: Dr. B. H. Moon, Department of Pharmacology, Rush University, Rush-Presbyterian-St. Luke's Medical Center, 1753 West Congress Parkway, Chicago, Ill. 60612.