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Insoluble Solid Complexes of Norepinephrine and Adenosine Triphosphate

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SUMMARY

Dilution with H_2O of aqueous solutions 0.5 M in norepinephrine HCl and 0.125 M in ATP (pH range, 3–7) yielded precipitates in 5–10% yield. In contrast, the products obtained by lyophilization of either the concentrated solutions or the filtrates from the precipitates readily dissolved in the original volume of H_2O. The insoluble solid obtained at pH 7 had the proper elementary composition and infrared and ^1H nuclear magnetic resonance spectra for a 4:1 norepinephrine·ATP complex. The precipitate produced at pH 3 was composed mostly of the 3:1 complex but appeared to contain a small amount of the 4:1 complex. In the preparation of these solids, ATP could be used as the $2Na^+$, Mg^{++}, or $2(CH_3)_4N^+$ salt, but no precipitates were observed when norepinephrine bitartrate or the hydrochlorides of epinephrine or a variety of other phenethylamines were substituted for norepinephrine HCl. The maximum solubility of the complexes in H_2O was estimated at 0.2 mM. This value was approximately doubled in 1.5% NaCl or KCl, and increased about 50 times in $MgCl_2$ or $CaCl_2$ of the same concentration.

Dilution of aqueous solutions containing norepinephrine and ADP precipitated a 3:1 complex at pH 7, and a 2:1 complex at pH 3. AMP also formed an insoluble complex.

The 4:1 norepinephrine·ATP complex was recovered unchanged upon neutralization of a solution in 0.1 N HCl. However, longer exposure to acidic conditions yielded the 3:1 norepinephrine·ADP complex. The 4:1 norepinephrine·ATP complex was more resistant to hydrolysis in 0.1 N HCl than was ATP or a mixture of norepinephrine and ATP.

Insoluble complexes of catecholamines and ATP could account for the calculated hypertonicity and the nondiffusibility of the amines in adrenal medullary granules.

INTRODUCTION

In the course of spectroscopic studies on mixtures of norepinephrine and ATP, it was noticed that the addition of 4 volumes or more of water to aqueous solutions 0.5 M in the amine and 0.125 M in the nucleotide resulted in the formation of precipitates. Analysis of these solids has now revealed that the product obtained at pH 7 contains norepinephrine and ATP in a 4:1 ratio, whereas the precipitate separated at pH 3 has a 3:1 amine to nucleotide ratio. This paper describes the properties of these compounds.

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MATERIALS AND METHODS

Norepinephrine HCl and the $2Na^+$ and Mg^{++} salts of ATP were purchased from Sigma Chemical Company. Epinephrine HCl was prepared by lyophilization of an

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aqueous solution of the amine containing 1 Eq of acid. The $2(CH_3)_4N^+$ salt of ATP was obtained from 2Na·ATP by exchange at 0° on a Dowex 50 column; ^1H nuclear magnetic resonance spectroscopy indicated that the ratio of $(CH_3)_4N^+$ to ATP was 2:1.

Infrared spectra were recorded with a Perkin-Elmer model 257 instrument, and ultraviolet spectra, with a Beckman DK-2 spectrometer. NMR measurements were made with a Bruckner 90-Hz instrument. Chemical shifts were measured from the center of a single peak or groups of peaks. The integration of proton signals was accomplished by tracing the graphical record and weighing individual peaks or constellations of peaks cut from the paper (1). All pH values were determined with a Beckman Zeromatic meter with a Thomas universal glass electrode. Elementary analyses were performed by the Schwarzkopf Microanalytical Laboratory. Emission spectroscopy was conducted at the Lilly Research Laboratories, Indianapolis, through the courtesy of Dr. I. H. Slater.

Inorganic phosphate derived from ATP or the 4:1 norepinephrine·ATP complex was measured by the colorimetric method of Fiske and SubbaRow (2). The results were corrected for a small amount of green color generated by the catecholamine. Control experiments proved that adenosine did not interfere with the method.

RESULTS

Preparation of norepinephrine·ATP complexes. The original method for obtaining solid complexes was to adjust to pH 7.0 a solution 0.5 M in norepinephrine HCl and 0.125 M in 2Na·ATP and add it dropwise with stirring to 4–20 volumes of H_2O at room temperature. In the course of a few minutes the milky suspension was resolved into an amorphous, white precipitate and a clear supernatant solution. The solid, collected by sedimentation in a clinical centrifuge, was washed several times with H_2O, then with acetone, and finally with ether. After drying in a vacuum desiccator the product had a grayish-yellow tint. The yield ranged from 5 to 10%. Somewhat larger yields (10–15%) were obtained by the slow addition, with stirring and continuous adjustment of pH, of 0.5 M norepinephrine HCl to 0.005 M 2Na·ATP. When either of these procedures was conducted at pH 3, a solid of similar appearance but different chemical composition (see below) was obtained in about the same yield as at pH 7. All these methods produced precipitates when the Mg^{++} or $2(CH_3)_4N^+$ salts of ATP were substituted for the $2Na^+$ salt, but failed when norepinephrine bitartrate or the hydrochloride of epinephrine, dopamine, tyramine, or phenylethanolamine was used in place of norepinephrine HCl.

Analyses. In the identification of the solid complexes, the primary method was infrared analysis, although ultraviolet and NMR spectroscopy and elementary analysis were also utilized. The usual procedure in infrared analysis was to compare in KBr pellets the spectra of the solids with those of lyophilized solutions containing norepinephrine HCl and 2Na·ATP in various quantities. On the basis of their phosphate absorption frequencies (see ref. 3) and their great solubility in H_2O, the lyophilized products probably represent mixtures of NaCl and simple norepinephrine salts of ATP. Figure 1 shows that the insoluble solid obtained at pH 7 yielded a spectrum practically identical with that of a 4:1 norepinephrine-ATP solution taken to dryness. Likewise, the spectrum of the solid that precipitated at pH 3 was almost the same as that of a lyophilized solution of the amine and the nucleotide in a molar ratio of 3:1 (Fig. 2). However, the relative intensities of the norepinephrine N—H and the adenine C=N bands suggested the presence of a small amount of the 4:1 complex. Figure 3 depicts the alterations in these bands caused by changes in pH and the molar ratio of catecholamine and ATP. In accordance with theory (4), the C=N stretching band had a higher frequency at the lower pH (1695–1700 cm^{-1} vs. 1650–1660 cm^{-1}). This shift can be attributed to protonation of the adenine ring (5).

Ultraviolet spectra of the solid complexes were measured in aqueous solution at pH 6.5. Both substances yielded a single broad absorption band, which represents contribu-

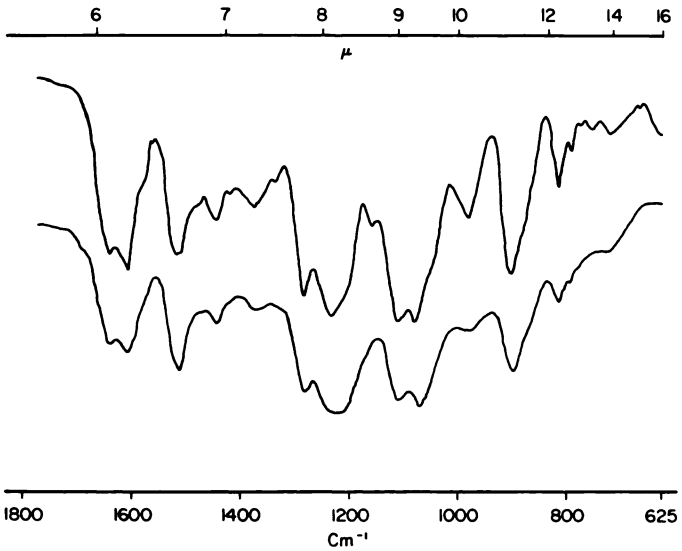

FIG. 1. *Infrared spectra of KBr pellets containing a lyophilized pH 7.0 solution 0.5 M in norepinephrine HCl and 0.125 M in 2Na·ATP (upper curve) and solid obtained by dilution with H_2O of a solution of the ame composition (lower curve)*

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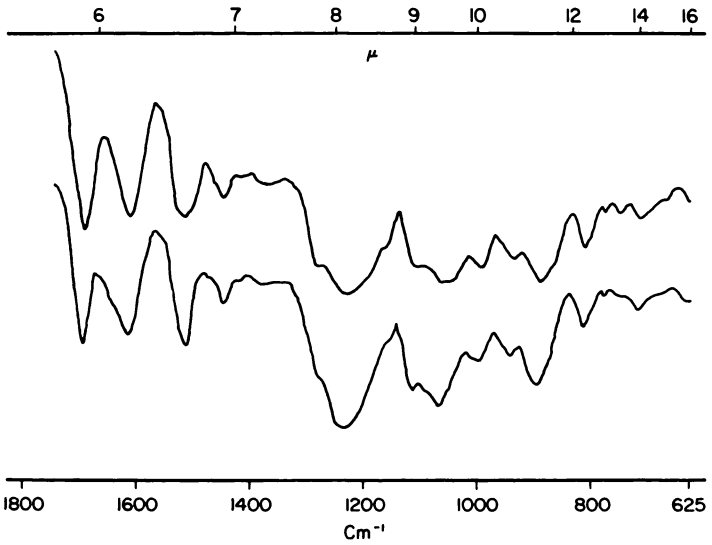

FIG. 2. *Infrared spectra of KBr pellets containing a lyophilized pH 3.0 solution 0.375 M in norepinephrine HCl and 0.125 M in 2Na·ATP (upper curve) and the solid obtained by dilution with H_2O of a pH 3 solution 0.5 M in norepinephrine HCl and 0.125 M in 2Na·ATP (lower curve)*

tions of both the adenine ring and the catechol groups. A comparison of the pH 7 solid with that of a 4:1 mixture of norepinephrine and ATP revealed slight differences in the absorption maxima and minima (261 vs. 263 nm and 245 vs. 242 nm, respectively). However, alkalinization caused the absorption maxima of the two solutions to shift to longer wavelengths at the same rate. This change can be attributed to the oxidation of the catechol moieties. A comparison of the pH 3 solid with a 3:1 mixture of norepinephrine and ATP revealed much the same similarities and differences.

The pH 7 solid was subjected to ^1H

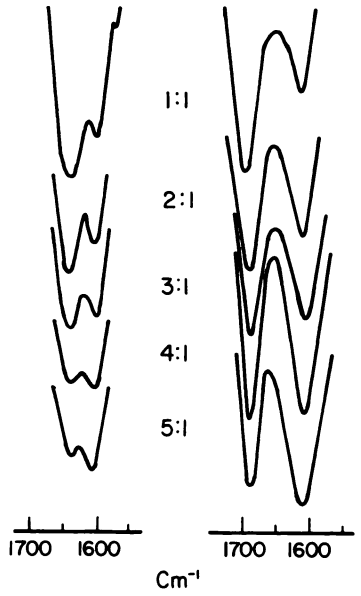

FIG. 3. *Infrared absorption bands used to estimate molar ratios of norepinephrine and ATP in complexes of amine and nucleotide*

Between 1 and 5 Eq of norepinephrine HCl were added to 0.125 M 2Na·ATP. After adjustment of the pH, the solutions were lyophilized and pressed into KBr pellets. The bands to the left of the numerical ratios were obtained at pH 7.0, and those on the right, at pH 3.0. Notice that the decrease in pH shifted the adenine C=N absorption from 1650–1660 cm^{-1} to1695–1700 cm^{-1} and that the norepinephrine N—H band (1600–1610 cm^{-1}) increased proportionally to its concentration in the solution.

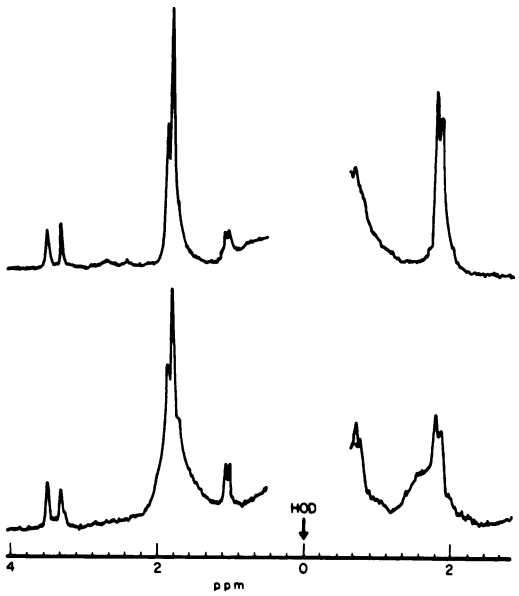

FIG. 4. *^1H NMR spectra in 0.1 M DCl of a 4:1 mixture of norepinephrine and ATP (upper curve) and the pH 7 norepinephrine·ATP complex (lower curve)*

The concentration of the complex was 100 mg/ml. The mixture was 0.336 M in norepinephrine HCl and 0.084 M in 2Na·ATP.

NMR spectroscopy in 0.1 M DCl (Fig. 4). Resonance peaks, expressed in parts per million with reference to HOD, were observed at −3.53, −3.35, −1.88 (multiplet), −1.10 (doublet), +0.66 (multiplet), and +1.79 (doublet). These peaks were identified as adenine H-8, adenine H-2, phenyl H, ribose H-1′, ribose H-2′, 3′, 4′, 5′, 5″, and norepinephrine CH_2, respectively. The norepinephrine CH peak was obscured by the HOD peak. The respective integration ratios of adenine H-8, adenine H-2, phenyl H, and norepinephrine CH_2 were 1:1:13:7.5. The theoretical integration values for a 4:1 norepinephrine·ATP complex are 1:1:12:8. The experimental data are in excellent agreement with theory in view of the fact that the insolubility of the compound required that the observations be made in dilute solution.

The pH 7 solid had the elementary composition expected in a 4:1 norepinephrine·ATP complex.

$$C_{42}H_{60}O_{25}N_9P_3$$

Calculated: C 42.61, H 5.10, N 10.64, P 7.88
Found: C 42.87, H 5.23, N 10.60, P 7.06

When it was subjected to emission spectroscopy, only Al, Mg, Cu, Fe, and Ba could be detected. None of these elements amounted to more than 10 ppm. Further evidence that the functioning of the complex does not depend on the presence of metals was provided by the observation that the solid could be precipitated from 25 mM EDTA.

Solubility of the complexes. The most remarkable property of the solid complexes was their insolubility in H_2O. A slow rate of dissolution, coupled with instability in very dilute solutions, precluded precise measurements of solubility constants. However, on the basis of ultraviolet spectroscopy, the

TABLE 1

Phosphate absorption frequencies in ADP and its solid complexes with norepinephrine

The solid complexes, precipitated at pH 7.0 or 3.0, or the lyophilized products from 0.125 M solutions of ADP (pH 7.0 or 3.0) were incorporated into KBr pellets.

Preparation	pH	P=O	$P^+—O^-$	PO_3^-	P—O—P
		cm^{-1}	cm^{-1}	cm^{-1}	cm^{-1}
ADP	7	1220–1225	1100–1110	975 1005	915
3:1 norepinephrine·ADP	7	1200–1205	1070 1105	1000	915
ADP	3	1220–1230	1075 1100	960	910–915
2:1 norepinephrine·ADP	3	1205–1210	1065 1095	960	910–915

TABLE 2

Liberation of orthophosphate by the 4:1 norepinephrine·ATP complex

Two milligrams of the complex or equivalent amounts of 2Na·ATP or norepinephrine HCl, or both, were dissolved in 2 ml of 0.1 N HCl and heated at 100° for the specified periods. The original and final pH values of all solutions were 1.54 ± 0.01. Orthophosphate was determined by the method of Fiske and SubbaRow (2). The results were corrected for a small absorption attributable to norepinephrine.

Preparation	Weight	Orthophosphate yield		
		0 min	2 min	5 min
	mg	%	%	%
2Na·ATP	1.053	1.0	31	58
2Na·ATP norepinephrine HCl	1.053 1.389	0.5	31	58
4:1 norepinephrine·ATP	2.000	1.0	8.5	31

maximum solubility was estimated at 0.2 mM. This value was approximately doubled in 1.5% NaCl or KCl, and was increased about 50 times by the same concentration of $MgCl_2$ or $CaCl_2$. In connection with the solubilizing effects of the alkaline earth metals, it is interesting that high concentrations of Mg^{++} (e.g., 0.5 M) prevented precipitation of the complexes from mixtures of norepinephrine and ATP. Increased concentrations of H^+ or OH^- also greatly augmented the solubility of both solids. Marked insolubility in benzene, chloroform, ether, acetone, and ethanol was noted but not quantified.

Complexes of other nucleotides. Dilution with H_2O of solutions 0.125 M in 2Na·ADP or Na·AMP and 0.5 M in norepinephrine HCl also led to the precipitation of amorphous solids at both pH 7 and 3. Infrared spectroscopy indicated that ADP bound 3 molecules of norepinephrine at pH 7, and 2 molecules at pH 3. Surprisingly, AMP appeared to form the same product at both pH values. Whether this complex contained 1 or 2 molecules of the amine could not be decided, because of inconsistencies in the relative intensities of the adenine C=N and norepinephrine N—H bands. The phosphate band frequencies of ADP and its complexes are presented in Table 1. These data suggest that norepinephrine reacts with the phosphate anions to form ionic complexes stabilized by hydrogen bonding between the ethanolamine hydroxyl groups and the P=O groups (see ref. 3).

Stability of the complexes. In order to determine the stability of the solid complexes, 2 mg of the pH 7 norepinephrine·ATP complex were treated with 1 ml of 0.1 N HCl under a variety of conditions. After 10 min at room temperature the addition of sufficient $NaHCO_3$ to bring the pH to 7.0 caused almost quantitative precipitation of the unchanged complex. A similar solution

prepared by dissolving norepinephrine and ATP in 0.1 N HCl did not deposit a precipitate upon neutralization. Exposure of the complex to acid for 48 hr led to the formation of the 3:1 norepinephrine·ADP complex, which was isolated in good yield. Neutralization of the product obtained by heating the reaction mixture at 100° for 1 hr caused the separation of a new substance, which lacked phosphate bands and the C=N absorption band of the adenine ring. This compound was not studied in detail, but it was thought to be 3,5,6-trihydroxyindole. When it was treated with acetic anhydride in pyridine it yielded 3,5,6-triacetoxyindole, identified by comparison of its infrared and ^{1}H NMR spectra with those of the product obtained by the acetylation of noradrenochrome (6, 7). An attempt to prepare an acetylated derivative of the pH 7 complex by shaking it for 3 days with a mixture of acetic anhydride and pyridine also yielded 3,5,6-triacetoxyindole.

In order to determine whether the binding of catecholamines stabilizes the triphosphate moiety of ATP, the generation of orthophosphate by the nucleotide and the complex was compared. As shown in Table 2, exposure to 0.1 N HCl at 100° converted 31% of the phosphorus of ATP to orthophosphate in 2 min, whereas 5 min were required to obtain the same yield from the complex. The marked difference in rate during the first 2 min and the subsequent 3 min suggests that the early period of heating was occupied largely by disorganization of the complex to expose the triphosphate moiety.

DISCUSSION

The accompanying paper (3) provided infrared spectroscopic data indicating the existence in solution of ionic complexes between norepinephrine and ATP, but evidence that all the negative charges in a nucleotide can be neutralized has been lacking. The isolation of the ATP· and ADP·norepinephrine complexes described above repairs this deficiency. These complexes differed from simple salts of catecholamines and ATP in their marked insolubility in water. This property was shown not to depend on the incorporation of metals. Another impressive feature of the 4:1 norepinephrine·ATP complex was its resistance to acid-catalyzed hydrolysis of the triphosphate moiety. Neither the insolubility nor the enhanced stability of the complex has been fully elucidated, but it appears likely that a combination of hydrogen bonding and ring stacking may be involved. Hydrogen bonding between the ethanolamine hydroxyl groups and the P=O moiety has been detected by infrared spectroscopy. Moreover, work still in progress (8) has demonstrated staggered stacking between the catechol rings and the adenine H-2 moiety.

The limitation of the formation of insoluble catecholamine·adenine nucleotide complexes to norepinephrine HCl cannot be completely explained. It seems possible that the bitartrate anion prevented precipitation by competing with ATP for the norepinephrine cation. The failure of epinephrine HCl could reside in a weaker affinity of the cation for the anionic sites, owing to steric hindrance by the methyl group. The inability of dopamine to form insoluble complexes can be attributed to the lack of a β-hydroxyl group and the consequent impossibility of forming hydrogen bonds with the P=O moieties of the nucleotides. The failure of phenylethanolamine HCl to substitute for norepinephrine HCl suggests that the catechol hydroxyl groups are important in conferring insolubility on the complexes. The question of mechanism must await further investigation.

The formation of precipitates by dilution of aqueous solutions with water can be harmonized with the findings of Berneis and co-workers (9) that in high concentrations mixtures of catecholamines and nucleotides involve macromolecular aggregates. Disruption by dilution is a well-established property of colloidal micelles (10). However, the exact relationship of the insoluble norepinephrine·nucleotide complexes to the aggregates of Berneis *et al.* is obscure. It is worth noting that epinephrine and dopamine readily form micelles, but not solid precipitates.

Whether solid complexes of nucleotides and catecholamines occur in adrenal medullary granules is not known. As far as norepinephrine is concerned, the conditions within these subcellular particles would seem suitable for the precipitation of ATP com-

plexes. If formed, an insoluble solid would serve even better than colloidal micelles to reduce both hypertonicity and diffusibility. It is conceivable that the postulated nondiffusible condition of biogenic amines in some subcellular particles (11) may involve solid complexes of nucleotides.

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