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Interactions of Catecholamines with Adenosine Triphosphate in Solutions and Adrenal Medullary Granules

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SUMMARY

In potassium bromide pellets, 1 Eq of Mg^{++} shifted all four infrared phosphate bands of adenosine triphosphate to higher frequencies, whereas in aqueous solution (Irtran cuvette) only the P=O peak was displaced (from 1225 to 1240 cm^{-1}). These effects can be interpreted by hydration of the P=O moiety and chelation of the metal by the β- and γ-phosphate groups. At both pH 3 and 7, 4 Eq of norepinephrine shifted the P=O and $P^+—O^-$ peaks in KBr pellets to lower frequencies. The displacement of the $P^+—O^-$ band can be attributed to association of the amine cation with the ATP anion. Since this process would be expected to cause P=O absorption at a higher frequency, it appears probable that the hydroxyl groups of the ethanolamine moieties form hydrogen bonds with the P=O groups. This formulation is attractive on stereochemical grounds. Mixtures of ATP, norepinephrine, and Mg^{++} yielded spectra similar to those from the nucleotide and the metal alone. Thus, in solution, Mg^{++} has a greater affinity than norepinephrine for ATP. Nevertheless, the spectra of the soluble contents of bovine adrenal medullary granules as well as intact granules resembled those of mixtures of catecholamines and ATP devoid of metals. These observations provide the first direct evidence for catecholamine·ATP complexes in granules. However, they leave undetermined the possibility that alkaline earth metals may play a role in the storage of catecholamines.

INTRODUCTION

Although complexes between catecholamines and ATP have been widely considered, the only direct evidence for such compounds was obtained by Weiner and Jardetzky (1), who examined mixtures of epinephrine and the nucleotide in D_2O by means of ^1H nuclear magnetic resonance spectroscopy. Their principal observation was a slight broadening of the resonance peaks associated with the CH triplet and the CH_2 doublet of the catecholamine. These effects were interpreted as reflecting decreased relaxation times of the protons in the CH and CH_2 moieties. The diminished relaxation periods were attributed to a decreased rotational freedom in the ethanolamine moiety, resulting from its association with the phosphate groups of ATP. This formulation may be correct, but conclusions based entirely on relaxation times, as distinct from chemical shifts, must be regarded as tentative (2). Inasmuch as ATP reveals strong, discrete absorption bands in both KBr pellets and D_2O (3, 4), infrared spectroscopy appeared to offer a powerful method for studying the interaction of the nucleotide with catecholamines

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and alkaline earth metals. The initial observations were made on simple solutions. Later, attention was focused on adrenal medullary granules.

MATERIALS AND METHODS

All spectra were recorded with a Perkin-Elmer model 257 instrument. Lyophilization and the preparation of KBr pellets were accomplished by standard techniques (5). The infrared observations on aqueous solutions were made in Irtran-2 cuvettes with carefully matched spacers (0.025 mm); the reference cell contained pure H_2O. All phenethylamines were used as the hydrochlorides; ATP, as the disodium salt; and Ca^{++} and Mg^{++}, as the chlorides. Titration of the various solutions to pH 7.0, accomplished by the addition of concentrated NaOH, was the final step before lyophilization or direct spectroscopic examination. A Beckman Zeromatic instrument with a Thomas universal glass electrode was employed.

Bovine adrenal granules were isolated by the procedure of Hillarp (6). To obtain the infrared spectrum of the intact particles, the granules were washed twice with cold 3% KCl prior to lyophilization. Lysis of the granules was accomplished by adding 3% KCl-washed granules to 10 volumes of H_2O. The membranes, separated from the soluble constituents of the granules by centrifugation at 22,000 × *g* for 20 min, were washed with H_2O prior to lyophilization and spectroscopic examination.

RESULTS

ATP spectra. The initial experiments involved the measurement of the infrared spectra of ATP at pH 7 in KBr pellets and in aqueous solutions in Irtran-2 cuvettes. Both spectra contained four strong phosphate bands at almost the same frequencies (Fig. 1). The band with the shortest wavelength was attributed to O═P—O⁻ asymmetrical stretching (3, 7), but since it reflects primarily the P═O resonance species, it is labeled as such. Similarly, the second band, O═P—O⁻ symmetrical stretching, is designated P⁺—O⁻. The identification of the third and fourth bands is more straightforward. The former is derived from the terminal phosphate group, and the latter, from the P—O—P ester linkages. The spectrum of ATP in KBr

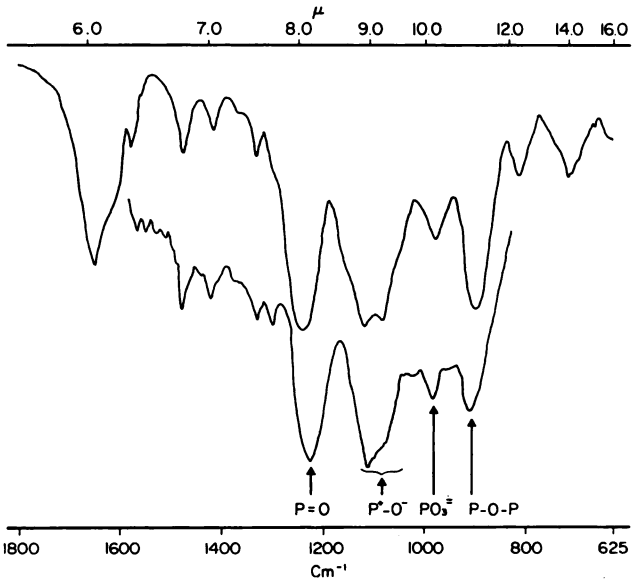

FIG. 1. *Infrared spectra of ATP in a KBr pellet and in aqueous solution*
The lower curve is that of a 0.125 M solution (pH 7.0) examined in an Irtran-2 cuvette. The upper curve is that of a lyophilized solution incorporated into a KBr pellet.

TABLE 1

Phosphate absorption frequencies of ATP in the presence of Mg^{++} or norepinephrine

Four molar equivalents of norepinephrine or 1 Eq of $MgCl_2$ were added to 0.125 M ATP in water. The resulting solutions, adjusted to pH 3.0 or 7.0, were examined directly in Irtran cuvettes or were lyophilized and incorporated into KBr pellets.

Conditions	Mixture	P=O	$P^+—O^-$	PO_3^-	P—O—P
		cm^{-1}	cm^{-1}	cm^{-1}	cm^{-1}
KBr, pH 7	ATP	1240–1250	1085 1120	980	895–900
	ATP + Mg^{++}	1250	1095–1100 1125–1130	990	920–925
	ATP + norepinephrine	1230–1235	1075–1080 1105–1110	980	895–900
	ATP + Mg^{++} + norepinephrine	1250	1110–1115	995	920–925
KBr, pH 3	ATP	1240–1250	1075 1120	990–995	895
	ATP + Mg^{++}	1250–1260	1090–1100 1145	990–995	900–915
	ATP + norepinephrine	1225–1240	1065 1110–1115	990–995	895
H_2O, pH 7	ATP	1225	1085 1115	985	910
	ATP + Mg^{++}	1240	1085 1115	985	910

pellets has been published before (3), but that in H_2O has not.

Chelation of Mg^{++}. The addition of an equimolar quantity of $MgCl_2$ to ATP (final pH, 7.0) shifted all the phosphate bands observed in KBr pellets to higher frequencies (Table 1). The numerical values are in good agreement with those of Epp *et al.* (3). The justification for offering new data on this question is that they are needed for precise quantitative comparisons. In contrast to the solid-state spectra, Mg^{++} had a more restricted effect on ATP absorption in aqueous solution. The data in Table 1 show that only the P=O band was shifted to a higher frequency. Inasmuch as ^{31}P NMR spectroscopy has provided strong evidence that Mg^{++} is chelated by the β and γ-phosphate groups of ATP (8), it is reasonable to assume that the infrared shifts described above were produced by the process of chelation. It may be noted that all the phosphate bands of the chelate had lower frequencies in solution than in KBr pellets. This general shifting of absorption bands can be attributed to solvation of the phosphate groups by H_2O.

Interaction with catecholamines. Norepinephrine also caused changes in phosphate absorption in ATP, but these were in the opposite direction from those induced by Mg^{++}. The interaction of the catecholamine with the nucleotide was examined only in KBr pellets, because solvation effects in aqueous solution tend to obscure the fine structure of the absorption spectrum. At pH 7.0 the P=O and $P^+—O^-$ bands showed significant reductions in frequency, but the other bands were not changed (Table 1). Similar results were obtained at pH 3, a condition which reduces the negative charge of ATP, and its capacity to bind catecholamines, from 4 to 3 units.

In order to discover the relative affinities

TABLE 2

Phosphate absorption frequencies of adrenal medullary preparations

Bovine adrenal medullary granules, isolated by gradient-density centrifugation and washed twice with 3% KCl, were lysed in 10 volumes of H_2O. The membranes, separated from the soluble contents by centrifugation at 22,000 × *g* for 20 min, were washed with H_2O. Intact slices of medulla, whole granules, membranes, and the soluble granular constituents were lyophilized prior to incorporation into KBr pellets.

Preparation	P=O	$P^+—O^-$	PO_3^-	P—O—P
	cm^{-1}	cm^{-1}	cm^{-1}	cm^{-1}
Soluble granular contents	1230–1240	1065–1070 1110	980–990	900–905
Whole granules	1230–1240	1065–1075 1105–1110		900
Intact tissue	1230–1240	1070–1075 1105–1110		900–905
Granule membranes	1230–1240	1060–1070		

of Mg^{++} and norepinephrine for ATP, a ternary mixture at pH 7 was examined in a KBr pellet. The frequencies of the P=O, PO_3^-, and P—O—P bands (Table 1) were practically identical with those found in preparations containing only ATP and Mg^{++}. However, the $P^+—O^-$ doublet merged into a single band. These results leave no doubt that ATP binds Mg^{++} more strongly than norepinephrine.

Adrenal medullary preparations. The availability of a method for distinguishing between the binding of norepinephrine and Mg^{++} to ATP invited an examination of the soluble contents of adrenal medullary granules. The phosphate frequencies observed in the supernatant solution of lysed bovine granules (Table 2) were remarkably similar to those in mixtures of norepinephrine and ATP, and quite different from those in mixtures containing Mg^{++}. The spectra of lyophilized whole granules and intact medullary tissue were quite similar to those of the supernatant fraction of lysed granules. Although the data from these more highly organized preparations include contributions from membrane phospholipids, the absence of an absorption band at 1105–1110 cm^{-1} in granule membranes suggests that the band with this frequency observed in granules and medullary tissue may arise from the association of ATP with a catecholamine. The low frequency of the P—O—P band provides additional evidence that the ATP in these preparations is not complexed with alkaline earth metals.

In view of the fact that bovine adrenal granules contain more epinephrine than norepinephrine and more Ca^{++} than Mg^{++} (9), the interactions of epinephrine and Ca^{++} with ATP were also examined. However, technical difficulties precluded precise results. The experiments with epinephrine were handicapped by the insolubility of this compound at pH 7. The main obstacle encountered with Ca^{++} was the extreme hygroscopicity it conferred on KBr pellets. It is interesting that none of the preparations from adrenal medullary tissue was deliquescent.

DISCUSSION

The results presented above reveal that Mg^{++} and norepinephrine shifted some of the phosphate absorption bands of ATP in different directions. The shifts induced by Mg^{++} can be interpreted to a limited extent. In KBr the P=O moiety can be regarded as having considerable double-bond character. However, in aqueous solution, where it is extensively hydrated, it takes on more of the character of $P^+—O^-$ and thus absorbs light of lower frequency (1225 cm^{-1} vs. 1240–1250 cm^{-1}). By localizing the negative charges on the β and γ-phosphate oxygen atoms, Mg^{++} reduces resonance in the hydrated form (Fig. 2). Thus, the absorption of light is shifted to a shorter wavelength or a higher fre-

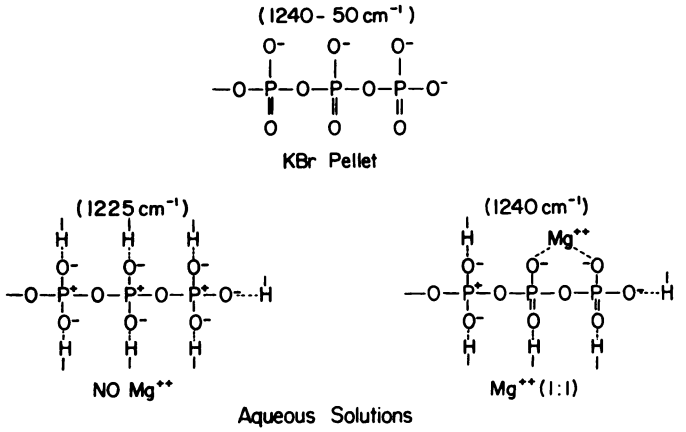

FIG. 2. *Structural formulae designed to explain influence of hydration and chelation of Mg^{++} on infrared absorption frequencies of P=O band of ATP*

quency (1240 vs. 1225 cm^{-1}). This frequency is still lower than that observed in nonchelated ATP in KBr pellets (1240–1250 cm^{-1}), because in aqueous solution the chelate is hydrated. In the solid state the shift in the P=O band produced by Mg^{++} was relatively smaller than in H_2O, because in the nonchelated nucleotide the P=O moiety already possesses marked double-bond character.

The Mg^{++}-induced shifts in the $P^+—O^-$ and PO_3^- bands do not seem amenable to a simple explanation. It is rather surprising that alterations in PO_3^- absorption were not more prominent. The very large increase in the frequency of the P—O—P band observed in KBr pellets (895–900 cm^{-1} to 900–925 cm^{-1}) can be attributed to restricted rotation and secondary alterations in the stretching characteristics of this bond in the chelate. In aqueous solution the P—O—P band of ATP had a much higher frequency than in KBr pellets (910 cm^{-1} vs. 895–900 cm^{-1}). Moreover, in water, Mg^{++} did not change this frequency. These observations suggest that the hydrated form of ATP assumes a configuration similar to that of the chelate.

In KBr pellets the effect of norepinephrine on the infrared spectrum of ATP was to shift the P=O and $P^+—O^-$ absorption bands to lower frequencies. The alteration in $P^+—O^-$ absorption can be explained on the basis of diminished ionic character. For example, a change in the pH of ATP from 7 to 3, a process which reduces ionization of the phosphate groups, shifted the $P^+—O^-$ band at 1085 cm^{-1} to 1075 cm^{-1} (Table 1). Likewise, association of catecholamine cations with phosphate anions to form complexes would reduce the ionic character of the $P^+—O^-$ species. At pH 7 the interaction of norepinephrine with ATP shifted both $P^+—O^-$ bands to lower frequencies, whereas only one of them was affected by pH.

The P=O band from catecholamine·ATP complexes would be expected to exhibit a tendency toward increased double-bond character and an increased absorption frequency. In actuality, the band was shifted from 1240–1250 cm^{-1} to 1230–1235 cm^{-1}. One explanation for this finding is that the interaction of norepinephrine cations with the negatively charged oxygen atoms of ATP causes the hydroxyl groups of the ethanolamine moieties to form hydrogen bonds with the P=O groups. This possibility, which is extremely likely on stereochemical grounds, was also proposed by Weiner and Jardetzky (1). Such hydrogen bonding would probably serve to reduce the dissociation of the complex. In harmony with this idea, phenethylamine and dopamine did not decrease the frequency of the P=O band of ATP, whereas phenylethanolamine behaved like norepinephrine.

Observations on mixtures revealed that Mg^{++} has a greater affinity than norepinephrine for ATP. Indeed, the phosphate absorption of such ternary mixtures was

indistinguishable from that of ATP and Mg^{++} alone. Thus, in view of reports that adrenal medullary granules contain large amounts of alkaline earth metals (9, 10), it was startling to discover that the spectra of the soluble constituents of these particles were very similar to those of mixtures of ATP and norepinephrine. This finding provides the first direct evidence for catecholamine·ATP complexes in adrenal granules. At the same time it raises some questions about the role of the metals. Inasmuch as the ethanolamine moiety of the catecholamines is involved in the complexes, it is not available to chelate metals. Moreover, earlier work (11) seems to have eliminated the possibility of chelation by the catechol moiety at physiological pH values.

In connection with the disposition of the alkaline earth metals in adrenal granules, three possibilities may be considered. First, the soluble constituents of the granules may contain an unrecognized compound which surpasses ATP in its ability to bind the metals. Second, the metals may be associated with the granular membranes rather than the contents. It is important to note that the only data on this question (9, 10) were derived from analyses of whole granules. Third, the metals may be located in subcellular particles present as contaminants in the granule preparations, such as mitochondria. Although a final answer must await further work, it is worth mentioning that the granules used in the present experiments must have contained only minute amounts of Mg^{++} and Ca^{++}. This assertion is based on the observation that the addition of Mg^{++} to the supernatant solution of lysed granules converted the spectrum to that of the ATP·Mg^{++} chelate.

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