affect an electrostatic repulsion dependent upon the charge density of the complexing anion. In order to conform to the observation that increasing the ionic strength of the same salt does not affect the energy of activation, we would have to postulate that these sites are fully saturated at low salt concentrations.

The following scheme is the summary of the proposed mechanism of collagen formation and its reversal

$$T' \rightleftharpoons T \rightleftharpoons T^* \rightleftharpoons \text{Collagen}$$

where T is tropocollagen, T′, the inactive form and T*, the proposed intermediate that is being formed during the lag period. T′ is formed when active sites on tropocollagen are blocked by anions, cations or by such agents as urea. Conditions which enhance hydrogen bonding favor the formation of collagen, while those which rupture hydrogen bonds reverse the equilibrium. The equilibrium rate will be influenced by *p*H, type and ionic strength of the salt present, concentration of the protein and temperature. Thus, when the collagen solution is removed from the presence of the fiber and conditions are adjusted to favor collagen formation, the system will remain poised unless conditions also favor a reasonable rate of reaction.

In writing this paper, we have avoided the use of such terms as neutral-soluble or acid-soluble collagen. The impressions gained from this work have cast considerable doubt in our minds as to whether such differences really exist. It must be realized that when HOAc solutions of collagen are adjusted to values above *p*H 7, the optimum *p*H of fiber formation is being approached. If the other variables are such as to favor coagulation, fiber formation will occur. If not, a collagen solution which is soluble in neutral or alkaline solutions will be obtained. We have found that after coagulation, the fibers can be redissolved in HOAc, the collagen reneutralized, and fibril formation repeated as before.

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[CONTRIBUTION FROM THE NATIONAL RESEARCH COUNCIL OF CANADA, PRAIRIE REGIONAL LABORATORY]

Infrared Studies on Complexes of Mg^{++} with Adenosine Phosphates[1]

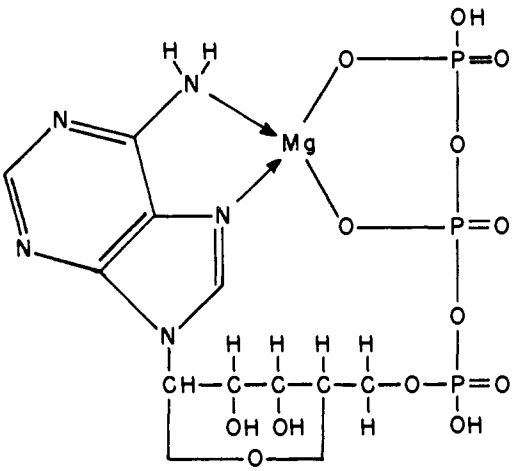
BY AGNES EPP, T. RAMASARMA[2] AND L. R. WETTER

RECEIVED AUGUST 5, 1957

Infrared spectra were obtained for the mono-, di- and triphosphate of adenosine and inosine in the presence and in the absence of Mg^{++}. The presence of the divalent cation affected the pyrophosphate group and the purine nucleus of the di- and triphosphates of adenosine. The data suggest that these divalent ions complex with the above groups but no direct evidence is obtained as to whether the complex is intramolecular or intermolecular.

Introduction

Certain inorganic cations, notably Mg^{++}, are essential for enzymic transphosphorylations involving the adenylic acid system,[3] and a complex of Mg^{++} and ATP[4] is the substrate for many kinases.[5-7] It has been suggested[8] that complexes of this type involve the cation and the pyrophosphate group. Recently, however, Szent-Györgyi[9] has postulated a quadridentate chelate between ATP and Mg^{++}, as in I, in which the bonding involves the 6-amino group, the nitrogen atom at position 7 and one oxygen atom of each of the terminal phosphate groups.



Such a chelate would be expected to give a characteristic infrared absorption spectrum distinct from that of uncomplexed ATP. Infrared spectra of nucleic acids have been recorded earlier[10-17] and

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(3) H. A. Lardy, "Phosphorus Metabolism," Vol. I, ed. by W. D. McElroy and B. Glass, The Johns Hopkins Press, Baltimore, Md., 1951, pp. 477.

(4) The following abbreviations are used: ATP, adenosinetriphosphate; ADP, adenosinediphosphate; AMP, adenosine-5′-monophosphate; ITP, inosinetriphosphate; IDP, inosinediphosphate and IMP, inosinemonophosphate.

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some general assignments related to their structural characteristics made. The present study examines the validity of the Szent-Györgyi formula I by noting the changes induced in the infrared spectra of adenosine phosphates by Mg^{++} and related cations. A related compound, inosine phosphate, also was investigated.

Experimental

ATP was purchased from Pabst Brewing Co., Milwaukee, as the crystalline disodium salt. ADP, AMP, ITP, IDP and IMP were obtained from Nutritional Biochemical Corp., Cleveland, and were used without further purification. The compounds were dissolved in water and the *p*H of their solutions was adjusted to 7.0 with NaOH. Magnesium ion was added where indicated as an aqueous solution of $MgCl_2$. Samples for assay were freeze-dried with 5 ml. of 10% KBr solution and the powder pressed into a transparent window for infrared recordings.[18] The spectra were measured with a Perkin–Elmer, Model 21, infrared spectrophotometer equipped with a sodium chloride prism.

The major absorption bands of significance for the series of compounds investigated are given in Table I. The bands assigned to the phosphorus-containing groups are based primarily on the data of Corbridge.[19] Other group assignments are from tables prepared by Bellamy[20] and Jones and Sandorfy.[21]

Results

Spectrum of ATP.—The infrared spectrum of ATP, with and without Mg^{++}, shows three absorption bands in the region 900–1300 cm.$^{-1}$ (Fig. 1): the pyrophosphate (P–O–P) group near 900 cm.$^{-1}$, the $\overset{+}{P}\text{–}\overset{-}{O}$ and C–O (1050–1150 cm.$^{-1}$), the P=O group near 1250 cm.$^{-1}$ and a band of medium intensity at 980–990 cm.$^{-1}$ which is assigned to the terminal $PO_3^=$ group. Addition of Mg^{++}, Mn^{++} or Co^{++} causes no marked changes in this region except that the P–O–P and the $PO_3^=$ bands are shifted by 10 cm.$^{-1}$ toward higher frequencies (Table I).

In the 1500–1600 cm.$^{-1}$ region ATP exhibits a peak at 1600 cm.$^{-1}$ and a shoulder at 1575 cm.$^{-1}$ which likely are due to the C=C and C=N stretching vibrations of the purine ring. Their intensities are slightly decreased by the addition of Mg^{++} but their positions are not altered.

A strong band appears at 1640 cm.$^{-1}$ which is assigned to the NH deformation vibration of the 6-amino group. The addition of divalent cations to ATP results in the appearance in this region of a second band at 1685 cm.$^{-1}$ of medium intensity. According to Lenormant and Blout[14,15] this band can be caused by rearrangements of double bonds within the purine nucleus. Other workers[22–25] have reported that a positive charge on the nitrogen atom of the $C=\overset{+}{N}$ group gives rise to a higher

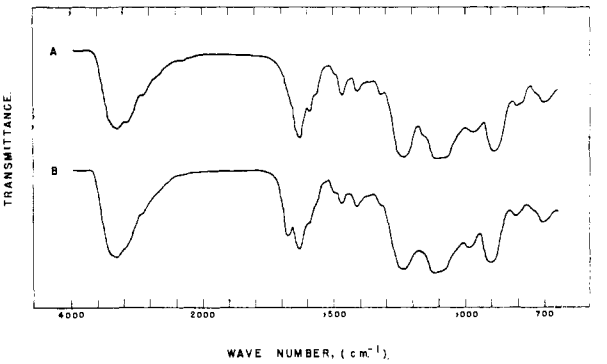

Fig. 1.—Infrared spectra of (A) ATP alone, (B) equivalent amounts of ATP and Mg^{++}.

C=N frequency. The nitrogen at position 7 and the amino group at 6 will be positively charged if they take part in complex formation with divalent cations, since electrons are being shared with the chelating metal ion. Thus the appearance of the band at 1685 cm.$^{-1}$ probably is due to the $C=N^+$ at position 7 and suggests that the purine ring is involved in the chelation.

Spectrum of ADP.—The spectrum of ADP resembles that of ATP in all respects except that the $PO_3^=$ band is absent[26] and the P=O band is slightly weaker. The addition of Mg^{++} to ADP causes a shift of 15 cm.$^{-1}$ in P–O–P band toward higher frequencies, and the $C=N^+$ band appears at 1680 cm.$^{-1}$ (Table I).

Spectrum of AMP.—The spectrum of AMP differs considerably from those of ATP and ADP. The absorption bands caused by P–O–P and P=O groups are absent. However, the band at 975 cm.$^{-1}$ assigned to the $PO_3^=$ group is strong and well resolved. The absorption band at 1075–1150 cm.$^{-1}$ caused by $\overset{+}{P}\text{–}\overset{-}{O}$ and C–O as well as the NH deformation vibration band at 1640 cm.$^{-1}$ are present. The addition of Mg^{++} does not result in the appearance of the $C=N^+$ band for AMP thus indicating that no complexing between the nitrogen and the cation has taken place. However, there is a shift of the band at 975 to 1000 cm.$^{-1}$ indicating that some interaction takes place between Mg^{++} and the phosphate group.

Spectrum of AMP in Presence of Inorganic Pyrophosphate.—The appearance of the $C=\overset{+}{N}$ band (1685 cm.$^{-1}$) in the ATP and ADP spectra in the presence of Mg^{++} and its absence in the AMP spectrum suggests that the P–O–P group plays a role in the chelation of divalent cations. The spectra of AMP with an equimolar amount of inorganic pyrophosphate and of adenosine with an equimolar amount of inorganic pyrophosphate or tripolyphosphate are equivalent to the sum of the individual compounds. The addition of Mg^{++} to these combinations does not result in the appearance of the $C=N^+$ band which suggests that chelation will not take place unless adenosine and P–O–P are in the same molecule.

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(26) The PO_3^- band was present in the spectra of ATP, AMP, ITP and IMP but not ADP or IDP. It was noted that the spectrum for sodium pyrophosphate showed a very weak PO_3^- band whereas the spectrum for sodium tripolyphosphate gave a sharp band at 970 c.m.$^{-1}$.

TABLE I

MAJOR INFRARED ABSORPTION BANDS OF ADENOSINE AND INOSINE PHOSPHATES ALONE AND IN THE PRESENCE OF DIVALENT CATIONS[a]

Compound[b]	P–O–P	PO_3^-	$P^+–O^-$ and C–O[c]	P=O	Aromatic ring	NH	C=O	$C=N^+$
ATP	900s	980m	1075–1185s	1245s	1580sh 1603m	1640s	...	
ATP + Mg^{++}	910s	990m	1085–1185s	1245s	1580sh 1603m	1640s	...	1683m
ATP + Mn^{++}	910s	990m	1100–1140s	1240s	1580sh 1603m	1640s	...	1685m
ATP + Co^{++}	905s	990m	1095–1130s	1245s	1580sh 1605m	1645s	...	1685m
ITP	900s	995w	1075–1130s	1255s	1515w 1552w 1588w	...	1685s	....
ITP + Mg^{++}	910	995sh	1080–1135s	1250s	1515w 1552w 1585w	...	1685s	?[d]
ADP	915s	....	1100s	1235s	1580sh 1605m	1643s	...	....
ADP + Mg^{++}	930s	....	1100	1220s	1580sh 1605sh	1640s	...	1680m
IDP	900s	....	1065–1120s	1235s	1512w 1550w 1585w	...	1685s	
IDP + Mg^{++}	925s 960s	....	1060–1130s	1225s	1510w 1550w 1583w	...	1685s	?[d]
AMP	..	975s	1100s	...	1580sh 1603m	1640s	...	....
AMP + Mg^{++}	..	1000s	1110s	...	1580sh 1603m	1640s	...	....
IMP	..	975s	1080s	...	1515w 1550w 1590w	...	1680s	....
IMP + Mg^{++}	..	995s	1100–1125s	...	1513w 1550w 1586w	...	1680s	....

[a] These various abbreviations are used in the table to give a rough indication of band intensities: s = strong, m = medium, w = weak, sh = shoulder on the side of another band. [b] Two μM of the phosphates and 2 μM of divalent cations, where mentioned, were used per run. [c] Whenever a definite maximum of the band is observed, one frequency value is given; whenever the maximum extends over a certain region its frequency limits are given. [d] The $C=N^+$ band is probably overlapped by the C=O band.

Spectra of Inosine Phosphates.—The spectra of inosine phosphates are very like those of the adenosine phosphates (Table I). The carbonyl group at position 6 in inosine gives rise to a strong absorption band at 1685 cm.$^{-1}$. The addition of Mg^{++} to the inosine phosphates shows changes similar to those observed for the adenosine phosphates in that the P–O–P bands shift. Since the C=O group absorbs strongly at 1685 cm.$^{-1}$, one obtains only a widening of this band after the addition of Mg^{++}; for this reason no information is forthcoming regarding the Mg^{++}–ITP complex.

Discussion

Investigations of the enzymatic reactions involving ATP and Mg^{++} show that a complex of equimolar Mg^{++} and ATP is an active substrate.[5-7] Mg^{++} has been shown to complex with the various ionic species of ATP and ADP, the complexes formed with the fully ionized nucleotides (ATP^{-4} and ADP^{-3}) having the greatest affinity for Mg^{++}.[27] However, Robbins and Boyer[28] showed that Mg–ATP^{-2} and Mg–ADP^{-1} are present in the highest concentration at equilibrium in the hexokinase reaction and these complexes therefore appear to be the active species.

The Mg^{++}–ATP chelate (I) of the type suggested by Szent-Györgyi[9] finds some support in the present infrared absorption data. The shift of the pyrophosphate to high frequencies together with the appearance of the $C=N^+$ band when Mg^{++} is added to ATP and ADP indicate that the pyrophosphate and very likely the purine nucleus are concerned in chelation. Further, adenosine in the presence of sodium pyrophosphate or sodium polytriphosphate does not complex with Mg^{++}, which indicates that the pyrophosphate or triphosphate group must be attached directly to the adenosine in order to chelate. It should be noted that ADP

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acts similarly to ATP in regard to chelation with Mg^{++} as observed in infrared spectra. However, Szent-Györgyi concludes that chelation with Mg^{++} is possible only with ATP and not with ADP, but the present results suggest that ATP and ADP form similar types of chelates. Since the first and second phosphate groups are common to both ATP and ADP, it suggests that these groups are involved in the complex formation. It is interesting to point out that not only does ATP require a divalent cation to transfer the terminal phosphate, but in enzymatic reactions in which ADP accepts a phosphate group[6] the divalent cation is again required for the operation.

The infrared evidence indicates the P–O–P and the purine nucleus are involved in the complex formation with Mg^{++} and that the two former groups should be within the same molecule; however, it does not clearly demonstrate whether the complexing is intramolecular or intermolecular. It is possible that the purine nucleus of one molecule can complex with the P–O–P of another through Mg^{++} and still maintain the 1:1 ratio of Mg^{++} to nucleotide. Either a dimer or a polymer could satisfy this condition and the present investigation does not clarify the point. It is hoped that future investigations will be able to determine whether the complexing is intra- or intermolecular.

SASKATOON, SASK., CANADA

[CONTRIBUTION FROM THE RESEARCH LABORATORIES, ETHYL CORPORATION]

The Synthesis of Alkyl Aryl Phosphates from Aryl Phosphorochloridates. I. The Sodium Alkoxide Route[1]

BY HAROLD D. ORLOFF, CALVIN J. WORREL AND FRANCIS X. MARKLEY

RECEIVED JULY 30, 1957

The synthesis of aryl phosphorodichloridates by the reaction of phenols with phosphoryl chloride is readily catalyzed by aluminum chloride. The yield of dichloridate is decreased by the presence of electron-withdrawing substituents such as chlorine in the aryl group. High yields of dialkyl aryl phosphates have been obtained by treating the dichloridate with sodium methoxide under controlled *p*H conditions. With excess of alkoxide, the product yield is reduced chiefly by an ester interchange reaction which yields trimethyl phosphate. The kinetics of the reactions of dialkyl aryl phosphates and trimethyl phosphates with sodium methoxide and sodium phenoxide are discussed.

Although phosphate esters have been the subject of intensive study for more than a century,[2,3] the preparation in high yield of mixed alkyl aryl phosphates presents a number of problems of interest from a synthetic, kinetic and mechanistic standpoint. Dialkyl aryl phosphates may be synthesized by the reaction of a phenol with a dialkyl phosphorochloridate, $(RO)_2POCl$, or of an alcohol with an aryl phosphorodichloridate, $ArOPOCl_2$, usually in the presence of a base or acid acceptor.

Of four methods considered[4] for the preparation of $(RO)_2POCl$, the reaction of an alcohol with phosphoryl chloride was found suitable for the formation of alkyl phosphorodichloridate, $ROPOCl_2$,[5,6] but not for the monochloridate. A more attractive route for the latter was stated to involve the reaction sequence

$$3C_2H_5OH + PCl_3 \longrightarrow (C_2H_5O)_2PHO + C_2H_5Cl + 2HCl \quad (1)$$

$$(C_2H_5O)_2PHO + Cl_2 \longrightarrow (C_2H_5O)_2POCl + HCl \quad (2)$$

wherein step 1 probably involves cleavage by hydrogen chloride of triethyl phosphite formed initially in the reaction.[7–9] A series of dialkyl aryl phosphates, hitherto unreported in the literature, except for diethyl *m*-tolyl phosphate, has been synthesized by this route in crude yields of 48–90%, based on dialkyl hydrogen phosphite (Table I). These preparations were carried out without distillation of the chloridate prior to reaction of the latter with aqueous sodium phenoxide. The low yields may be attributable in part to partial hydrolysis of the chloridate by the aqueous medium which would have the effect of removing $(RO)_2POCl$ in the form of the dialkyl hydrogen phosphate, $(RO)_2POOH$, and of liberating free phenol which was often difficult to remove from the product.

The Phosphorylation Reaction

In an attempt to achieve better utilization of phosphorus, avoid an aqueous system and eliminate the necessity for removal of free phenol from the products, the reaction series

$$ArOH + POCl_3 \longrightarrow ArOPOCl_2 + HCl \quad (3)$$

$$ArOPOCl_2 + 2ROH \longrightarrow (RO)_2(ArO)PO + 2HCl \quad (4)$$

was considered in detail. While a mixture of $ArOPOCl_2$, $(ArO)_2POCl$ and the triaryl phosphate, $(ArO)_3PO$, was expected from the phosphorylation reaction 3, the object of our study was to establish the effect of phenol structure, phosphoryl chloride to phenol ratio, reaction conditions and catalysts upon the yield of $ArOPOCl_2$.

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(8) An alternate route, $PCl_3 + 2C_2H_5OH + H_2O \rightarrow (C_2H_5O)_2PHO + 3HCl$, has been the subject of several patents, *e.g.*, H. Coates, British Patent 684,835, Dec. 24, 1952.

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