ON THE SEPARATION OF INORGANIC PYROPHOSPHATE (PP) FROM THE PHOSPHORIC ESTERS OF ADENOSINE. SOLUBILIZING ACTION OF ATP ON THE Mn-SALTS OF PP

by

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The methods so far existing for the separation of inorganic pyrophosphate (PP) from the phosphoric esters of adenosine (ATP, ADP, AMP) and the following quantitative determination of the former, are those worked out by JONES[1] and KORNBERG[2], taking advantage of the precipitation of PP in the form of a complex with Mn, at pH 4.1 according to JONES, at pH 5 according to KORNBERG. The washed precipitate is dissolved in 0.1 *N* HCl and PP is valued by determining orthophosphate (P) after acid hydrolysis at 100° C. According to KORNBERG, a correction is given for the ATP eventually present in the precipitate, this compound being determined spectrophotometrically.

The use of both these methods is, however, restricted to a narrow range of conditions, and they lack an exact estimation indicating which are the limits and the errors in their use for quantitative purposes.

The recent investigations by NEUBERG AND MANDL[3] and MANDL, GRAVER AND NEUBERG[4], which point out the solubilizing action of ATP on various inorganic salts, have focussed our attention on the importance that the reciprocal concentrations of PP and ATP might have when one of these compounds is determined in the presence of the other.

In the present study we have, therefore, intended to make clear whether ATP has a solubilizing action on PP, and how such an action might influence the quantitative determination of PP over a wide range of pH (from pH 4.8 to pH 8.6).

The amounts and the concentrations of the substances used correspond more or less to those existing in tissue samples on which the determination of PP is usually carried out.

The solutions, containing the various substances at the molarity shown in Table I, were initially adjusted to pH 4.8 with 1 *M* acetate buffer.

After addition of 0.2 ml 0.1 *M* $MnCl_2$, the solution was left standing at room temperature for at least 30 minutes and the precipitate formed was collected by centrifugation.

The pH of the clear supernatant was then raised by 0.2 units with NaOH and in the same way it was successively raised at intervals up to pH 8.6, the precipitate formed at each pH interval being each time removed by centrifugation.

Each single precipitate removed was dissolved in 1.4 ml 0.1 *N* HCl and diluted with 1.8 ml H_2O. From this solution aliquots were removed for:

1. The determination[5] of P before and after acid hydrolysis for 15′ at 100° C. The difference between the two values thus obtained (acid-labile P) gives the amount of PP, if no other substance able to yield P during acid hydrolysis at 100° is in the precipitate.

2. The determination of AMP, ADP, ATP with the enzymic spectrophotometric technique of KALCKAR[6].

The same determinations were performed also on the clear supernatant fluid residual at pH 8.6 so as to value the amount of the various substances still dissolved at this pH.

No precipitate is formed at pH 5.2 in pure solutions of PP in which the concentration of PP is below 0.00016 M. For higher concentrations of PP, PP is precipitated by Mn^{+2} from pure solutions (Fig. 1 A) in the proportion of 95% at pH 4.8 and of the 100% at pH 5.7. At pH 8.6 a recovery of 107% is obtained. At this pH traces of PP are still found dissolved.

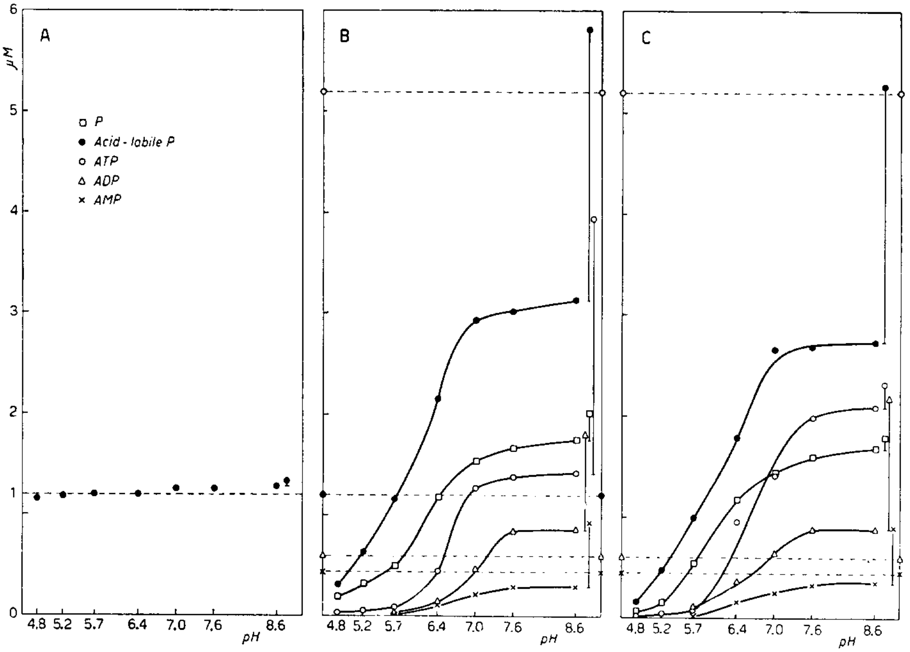

Fig. 1. Solution A contained 1.2 μM $Na_4P_2O_7$; Solution B 1.2 μM $Na_4P_2O_7$ + 5.2 μM NaATP + 0.59 μM NaADP + 0.443 μM AMP; Solution C 5.2 μM NaATP + 0.59 μM NaADP + 0.443 μM AMP. They were all brought to a final volume of 3.4 ml by addition of 0.4 ml 1 M acetate buffer pH 4.8. Results are given in μM of the substances determined. For acid-labile P they are given in μM of PP. The figures plotted at each pH are obtained by adding to the values determined on the precipitate formed at the pH concerned, the values determined on the precipitates formed at lower pH's. The vertical lines show the amount of each substance still in solution at pH 8.6 while the horizontal dotted lines show the amount of the substance initially present in the solution. All data are the average of three or more different determinations.

PP shows an identical behaviour when in solutions containing also P (Table I A), while on the contrary, P is precipitated by Mn^{+2} in amounts increasing with the pH value, reaching nearly full precipitation at pH 8.6 (Table I D).

In solutions containing both PP and ATP (Fig. 1 B), the amount of acid-labile P determined in the precipitate for pH values below pH 6 is much lower than the amount corresponding to the PP present in the solution at the beginning, while, for pH values over pH 6, the amount of acid-labile P determined sharply exceeds the PP initially present in the solution. A solubilizing action of ATP on the complex PP–Mn is therefore manifest below pH 6. Above pH 6 the finding of both ATP and ADP in the precipitate accounts for the higher values of acid-labile P obtained: in fact, during acid hydrolysis ATP and ADP yield 2 and 1 mole of P, respectively. A small percentage of the acid labile P is also due to PP formed owing to the hydrolysis of ATP (see also Table I B).

Fig. 1 C, referring to a pure solution of ATP, confirms the characteristics of Fig. 1 B: in fact it gives evidence of a moderate formation of PP due to hydrolysis of ATP in the solution, and shows that, from pH 6 up, remarkable amounts of ATP are found in the precipitate together with AMP, ADP, PP and P, that is to say all possible products of the hydrolytic breakdown of ATP that precipitate the more easily the higher the pH (see also Table I C, D, E, B).

TABLE I

	A		*B*					*C*	*D*	*E*
	1.2 μM $Na_4P_2O_7$ + *0.78 μM* K_2HPO_4		*1.2 μM* $Na_4P_2O_7$ + *0.78 μM* K_2HPO_4 + *5.2 μM NaATP + 0.59 μM NaADP + 0.443 μM adenylic acid*					*2.17 μM ADP Na-salt*	*0.78 μM* K_2HPO_4	*2.88 μM adenylic acid*
pH	*P*	*acid-labile P*	*P*	*acid-labile P*	*ATP*	*ADP*	*AMP*	*acid-labile P*	*P*	*AMP*
4.8	0.167	1.13	0.128	0.225	0.027	0	0	0	0	0
5.2	0.281	1.206	0.202	0.429	0.051	0	0	0		
5.7	0.415	1.241	0.506	1.046	0.186	0.059	0.025	0.143	0.143	0.479
6.4	0.844	1.274	1.315	2.275	0.326	0.199	0.093	0.672		
7	0.892	1.314	1.88	2.863	1.02	0.331	0.184	1.446	0.49	2.369
7.6	0.893	1.331	1.894	2.936	1.419	0.602	0.231	1.893		
8.6	0.932	1.34	1.933	2.992	1.434	0.605	0.24	2.083	0.52	2.527
total recovery including supernatant	1.095	1.342	2.462	6.154	3.874	1.845	0.873	2.115	0.74	2.956

Each solution, containing the different substances in the amounts described above, was brought to a final volume of 3.4 ml by addition of 0.4 ml 1 *M* acetate buffer pH 4.8.

The Na salt of ATP used contained impurities of ADP and AMP, as shown in column B;

Results are given in *μM* of the substance determined. For acid-labile P they are given in *μM* of PP in columns A and B (2 P = PP = ATP) and in *μM* of P in column C (ADP = P).

The figures referred for each pH are obtained by adding to the values determined on the precipitate formed at the pH concerned, the values determined on the precipitates formed at lower pH's.

Each datum is the average of three or more different determinations.

We may therefore conclude stating that a solubilizing action of ATP on the complex PP–Mn is beyond question.

A quantitative determination of PP by means of a precipitation with Mn^{+2} is possible only in samples free from the phosphoric esters of adenosine. When moderate amounts of these substances are present, the quantitative determination of PP is possible in theory—in the range from pH 5.6 to pH 5.8—giving a correction for the ATP found in the precipitate: however, where the concentration of ATP exceeds the concentration of PP, the quantitative separation of PP becomes precarious and almost impossible, owing to the solubilizing action of ATP on the complex PP–Mn, and to the fact that PP is yielded by ATP and that, above pH 6, ATP itself is found in the precipitate.

SUMMARY

The influence of the ATP concentration on the precipitation of pyrophosphate (PP) by Mn^{+2}, from solutions containing PP and phosphoric esters of adenosine, has been studied over a wide range of pH.

At the same time the modalities of the precipitation with Mn^{+2} of the hydrolytic breakdown products of ATP, namely orthophosphate, adenilic acid and ADP, have been investigated.

A solubilizing action of ATP on the PP–Mn complex can be demonstrated, and, in connection with this, the optimal conditions for the quantitative determination of PP, using the precipitation with Mn^{+2}, have been established.

RÉSUMÉ

Sur des solutions contenant pyrophosphate (PP) en présence des esthers phosphoriques de l'adénosine, on a etudié, à différents pH, l'influence de la concentration de l'ATP sur la précipitation du PP avec Mn^{+2}.

Du même temps on a examiné les modalités de la précipitation avec Mn^{+2} des produits de démolition de l'ATP, c'est à dire l'ortophosphate, l'acide adénilique et l'adénosine diphosphate.

On a pu mettre en évidence une action solubilisante de l'ATP sur le complexe PP–Mn, et, en rapport avec celle ci, on a établi les conditions optimales pour la détermination quantitative du PP en employant la précipitation avec Mn^{+2}.

ZUSAMMENFASSUNG

Es wurde der Einfluss der Konzentration der ATP auf die Ausfällung des PP mit Mn^{+2} aus Pyrophosphat enthaltenden Lösungen bei Gegenwart von phosphorsauren Estern des Adenosins bei verschiedenem pH studiert.

Zu gleicher Zeit wurden die Ausfällungsbedingungen der Abbauprodukte des ATP, d.h. des Orthophosphats, der Adeninsäure und des Adenosindiphosphats mit Mn^{+2} untersucht.

Es wurde eine lösende Wirkung des ATP auf den PP–Mn-Komplex augenscheinlich gemacht und im Hinblick darauf wurden die optimalen Bedingungen für die quantitative Bestimmung des PP durch die Ausfällung mit Mn^{++} festgesetzt.

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