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The Stimulation of Transphosphorylation by Alkali-Metal Ions

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A number of enzyme reactions involving the polyphosphate chain of nucleoside triphosphates show an absolute requirement for, or a stimulation by, alkali-metal ions. This requirement is usually met by potassium ions, but sodium, rubidium and ammonium ions have been reported to serve in some cases. Examples include fructokinase (Lardy, Parks & Ben-Gershom, 1957), myosin adenosine triphosphatase (Kielley, Kalckar & Bradley, 1956; Bowen & Kerwin, 1954), pyruvate kinase (Kachmar & Boyer, 1953) and glutathione synthetase (Snook, Yanari & Bloch, 1953). However, the majority of the enzyme-catalysed reactions which involve nucleoside triphosphates have not been tested for an alkali-metal requirement. Unless it is specifically tested for, such a requirement may easily be missed because many reagents, including adenosine triphosphate (ATP), are commonly used in the form of their sodium or potassium salts. It is likely therefore that many more reactions utilizing adenosine triphosphate possess a requirement for alkali-metal ions than are recognized to do so at present.

In a previous paper (Lowenstein, 1958) a non-enzymic transphosphorylation was described which involves the transfer of the terminal phosphate group of adenosine triphosphate to orthophosphate. The products of the reaction are inorganic pyrophosphate and adenosine diphosphate (ADP). The

reaction shows an absolute dependence on bivalent-metal ions (M^{2+}):

Adenosine triphosphate + orthophosphate

Of those tested, Ca^{2+} , Mn^{2+} and Cd^{2+} ions were the most effective.

The work presented here had two objects: the first was to provide a chemical basis for the alkali-metal requirement of enzyme-catalysed transphosphorylations. The second was to study variations in the amount of pyrophosphate formed in the non-enzymic reaction. These were observed to occur with reaction mixtures identical in every respect, except that different bases (such as potassium hydroxide, sodium hydroxide and diethanolamine) were used to neutralize the solutions of adenosine triphosphate employed in the experiments. A preliminary account of this work has been given (Lowenstein, 1959).

EXPERIMENTAL

Materials and reagents

Diethanolamine salt of adenosine triphosphate. The barium salt of adenosine triphosphate (ATP) was obtained from the Sigma Chemical Co., St. Louis, Mo., U.S.A., and from the Nutritional Biochemical Corp., Cleveland, Ohio,

U.S.A. Diethanolamine was obtained from the Matheson Co. Inc., Norwood, Ohio, U.S.A., and was purified by distillation under reduced pressure. Dowex-50 resin (8% cross-linked, 200–400 mesh, H^+ form) was obtained from The Dow Chemical Co., Midland, Mich., U.S.A. The resin was cycled through the sodium and acid forms, and was washed thoroughly with water before use.

Barium ATP 2 g. was suspended in 6 ml. of water in a 25 ml. beaker. A graduated centrifuge tube was filled with 14 ml. of a 50% (v/v) suspension of Dowex-50 resin (H^+ form) and the suspension was centrifuged. The volume of the resin was measured and adjusted to 7 ml. by the addition or subtraction of small amounts of resin and repeated centrifuging. When the desired volume of packed resin had been obtained the water was decanted. The following operations were performed on ice or in a cold room. The resin (7 ml.) was added slowly to the suspension of barium ATP with thorough stirring. The ATP dissolved very slowly at first, but, after a certain amount of resin had been added, a point was reached where the characteristic white slurry of barium ATP suddenly disappeared. This was usually accompanied by the conversion of the well-dispersed resin into a sticky mass. The remainder of the resin was added slowly with thorough stirring. After the addition of the resin was complete, stirring was continued for 5 min. The resin was filtered off on a sintered funnel and resuspended in 3 ml. of water. The suspension was stirred thoroughly and filtered on a sintered funnel. The washing of the resin was repeated twice more. The combined filtrates were neutralized to pH 3–5 by the addition of diethanolamine. This was most conveniently done by using the liquid base (m.p. 23°). A check of the total amount of diethanolamine added was made by weighing out an excess of the base and weighing the remainder at the end of the preparation. The pH was raised at this point to minimize the hydrolysis of ATP in the acid solution obtained from the resin treatment. Residual barium was then precipitated from the solution by the dropwise addition of $2N-H_2SO_4$. If the solution became too cloudy to judge the completeness of precipitation it was centrifuged, and the addition of H_2SO_4 was continued until no further precipitate formed. This point is harder to judge than is the case in, say, the precipitation of barium from solutions of its chloride. It was best to add a drop of H_2SO_4 to the clear solution and to wait a few minutes to see whether the solution remained clear, before deciding that precipitation of barium was complete. The clear, barium-free solution was neutralized to pH 9.0 by the addition of diethanolamine, the pH being checked first on indicator papers, and near the desired pH on a meter. The concentration of ATP was determined spectrophotometrically ($\epsilon_{260\text{ m}\mu}$ 15.4×10^3), and was adjusted to the desired value, usually 0.1M, with water. In a few preparations the concentration of ATP at the end of the preparation was less than 0.1M. In these cases the cold solution of ATP was placed in a round-bottomed flask and some water was removed from the cold solution in a rotary drier. No appreciable breakdown of ATP occurred in the course of this treatment. The concentration of ATP was redetermined and adjusted to the desired value.

Other bases, such as 2-amino-2-hydroxymethylpropane-1,3-diol, can be used in place of diethanolamine in the above method, and a variety of metal-free base salts of ATP can thus be prepared.

Alkali-metal and ammonium salts. Rubidium chloride and CsCl were of a purified grade obtained from E. H. Sargent and Co., Chicago, Ill., U.S.A. Tetramethylammonium chloride was obtained from Eastman Organic Chemicals, Rochester, New York. Other salts were of reagent grade.

Radioactive phosphate. Carrier-free [^{32}P]orthophosphate was obtained from the Atomic Energy Commission, Oak Ridge, Tennessee, U.S.A. It was purified by paper chromatography with solvent system 2 (Lowenstein, 1957); the orthophosphate spot was detected by radioautography and was eluted with water.

Analytical methods

'Non-extractable phosphorus'. The analyses for [^{32}P]orthophosphate incorporated into the reaction product were described by Lowenstein (1958). Caesium and tetra-alkylammonium salts were found to interfere with the extraction by causing the formation of a yellow precipitate. This tended to cling to the sides of the extraction tubes and to be dispersed in the 2-methylpropan-1-ol (isobutanol) layer, making its removal difficult. The extraction procedure was modified for samples containing caesium or tetra-alkylammonium salts. The reaction was stopped by placing the reaction mixture on a column of Dowex-50 (H^+ form, 200–400 mesh, 8% cross-linked) with a resin bed 1 cm. in diameter and 5 cm. high, and not by acidifying the reaction mixture with H_2SO_4 . Immediately after the mixture had soaked into the resin the column was washed with 2×10 ml. of water. This treatment resulted in the removal of the interfering substances. Measured portions of the combined eluates were then assayed for the amount of [^{32}P]orthophosphate incorporated into the reaction product.

RESULTS

In order to facilitate comparisons, all reaction rates are expressed as the amount of pyrophosphate formed per millilitre of reaction mixture, irrespective of the actual volume used. All reaction rates quoted approximate closely to being initial rates since the reactions were stopped when they had proceeded 4% or less. The course of the reaction with time was demonstrated previously (Lowenstein, 1958).

Stimulation of transphosphorylation by potassium ions. The effect of 0.1M-KCl on the rate of transphosphorylation is shown in Table 1. The results show that the stimulating effect can be considerable (100%), and that it is not an analytical artifact. An ATP/ Mn^{2+} ion ratio of 1.11 was used in this experiment. The importance of the ATP/bivalent metal ratio in relation to the magnitude of the stimulation by alkali-metal ions is indicated later.

Effect of the concentration of potassium ions on transphosphorylation. The influence of the KCl concentration on the rate of transphosphorylation is shown in Fig. 1. At an ATP/ Mn^{2+} ion ratio of 1.11, this rate is increased 28% in the presence of 0.01M-KCl, doubled in the presence of 0.1M-KCl, and trebled in the presence of 0.4M-KCl. The possibility

of an inflexion of the curve at high KCl concentrations suggests that there might be more than one type of stimulating effect.

Specificity of univalent-metal-ion stimulation. The effect of 0.1 M-alkali-metal and ammonium salts is shown in Table 2. At the ATP/Mn²⁺ ion ratio chosen for this experiment (1.0) all substances tested show a stimulating effect on the reaction. The effect of KCl is, however, much greater than that of the other cations. Lithium chloride, NaCl, CsCl and NH₄Cl show a stimulating effect of between 24 and 29%. Rubidium chloride forms an apparent exception to this group, and shows a stimulation of 41%, but this is still considerably less than that of KCl, namely 73%. A comparison of KCl with K₂SO₄ on an equivalent basis shows KCl to be somewhat more effective than K₂SO₄. This is possibly indicative of an ionic-strength effect.

Effect of tetramethylammonium chloride. The possibility that the univalent-metal-ion stimulation of transphosphorylation is solely an ionic-strength

Fig. 1. Effect of potassium chloride concentration on transphosphorylation. The reaction mixture contained: 50 μ l. of 0.05 M-[³²P]orthophosphate (6.76×10^5 counts/min.); 250 μ l. of 0.1 M-diethanolamine ATP, pH 9.0; 50 μ l. of 0.45 M-MnCl₂; KCl at the concentrations indicated. Final volume was 500 μ l.; temp., 38°; time of incubation, 3 hr.

Table 1. Stimulation of transphosphorylation by potassium ions

The complete reaction mixture contained: 50 μ l. of 0.05 M-[³²P]orthophosphate (6.76×10^5 counts/min.); 250 μ l. of 0.1 M-diethanolamine ATP, pH 9.0; 50 μ l. of 0.45 M-MnCl₂; 50 μ l. of M-KCl. Final volume was 500 μ l.; temp., 38°; time of incubation, 3 hr.

Reaction mixture	Pyrophosphate formed (μ m-mole/ml.)
Complete	172
KCl omitted	87
KCl omitted but added 5 sec. before end of incubation	90
MnCl ₂ omitted	0
Zero time	0

Table 2. Stimulation of transphosphorylation by alkali-metal and ammonium ions

The complete reaction mixture contained: 50 μ l. of 0.05 M-[³²P]orthophosphate (3.98×10^5 counts/min.); 250 μ l. of 0.1 M-diethanolamine ATP, pH 9.0; 50 μ l. of 0.45 M-MnCl₂, and, except for a control, 50 μ l. of M-chloride or of 0.5 M-sulphate as indicated. Final volume was 500 μ l.; temp., 38°; time of incubation, 3 hr. The amount of pyrophosphate formed in the control was 82.6 μ m-moles/ml.

Alkali-metal or ammonium salt	Stimulation of transphosphorylation (%)
LiCl	24
NaCl	28
KCl	73
RbCl	41
CsCl	29
K ₂ SO ₄	64
NH ₄ Cl	27
Tetramethylammonium chloride	0

Fig. 2. Effect of tetramethylammonium chloride. The reaction mixture contained 50 μ l. of 0.05 M-[³²P]orthophosphate (1.0×10^4 counts/min.); 250 μ l. of 0.1 M-diethanolamine ATP, pH 9.0; 50 μ l. of 0.4 M-MnCl₂; KCl and tetramethylammonium chloride at the concentrations indicated. Final volume was 500 μ l.; temp., 38°; time of incubation, 3 hr. $\circ$, K⁺ ion alone; $\bullet$, tetramethylammonium ion alone; Δ , tetramethylammonium ion as indicated plus 0.021 M-K⁺ ion; $\square$, tetramethylammonium ion as indicated plus 0.1 M-K⁺ ion. All analyses in this experiment involved the Dowex-50-column step described in the Experimental section.

effect is ruled out by the experiment shown in Fig. 2. Tetramethylammonium chloride has virtually no effect on the alkali-metal-free reaction mixture over the concentration range 0–0.2 M. Potassium chloride shows the usual stimulating effect. The results obtained with mixtures of the two salts indicate that tetramethylammonium chloride may shift the effect of KCl slightly.

Alkali-metal stimulation in relation to the adenosine triphosphate/Mn²⁺ ion ratio. Fig. 3 demonstrates the effect of varying the concentration of Mn²⁺ ion in the absence of alkali-metal ions, and in the presence of KCl and LiCl. In each case the

Fig. 3. Effect of K⁺ and Li⁺ ions in relation to the ratio ATP/Mn²⁺ ion. The reaction mixture contained: 50 μ l. of 0.05 M-[³²P]orthophosphate (3.94×10^5 counts/min.); 250 μ l. of 0.1 M-diethanolamine ATP, pH 9.0; MnCl₂ as indicated on the abscissa; where indicated, 50 μ l. of M-KCl or M-LiCl. Final volume was 500 μ l.; temp., 38°; time of incubation, 3 hr. The arrow indicates an ATP/Mn²⁺ ion ratio of 1.0.

Fig. 4. Effect of K⁺ and Li⁺ ions in relation to the ratio ATP/Mn²⁺ ion. Results of Fig. 3 have been replotted to show the net stimulation due to the addition of K⁺ or Li⁺ ions.

maximum rate of transphosphorylation is reached in a region where the ATP/Mn²⁺ ion ratio is 1 or less than 1, in agreement with earlier observations (Lowenstein, 1958). The alkali-metal stimulation is maximal in a region where the ATP/Mn²⁺ ion ratio is 1.1 in the presence of K⁺ ion and 1.4 in the presence of Li⁺ ion. This is demonstrated by replotting the data from Fig. 3 in the manner shown in Fig. 4. In the latter the control curve obtained with no added alkali metal has been subtracted from the rates obtained in the presence of KCl or LiCl. This method of plotting the results serves to make clear the quantitative differences between the stimulation obtained with K⁺ and Li⁺ ions.

The percentage stimulation of the alkali-metal ions, as distinct from the net stimulation, is maximal in a region where the ATP/Mn²⁺ ion ratio is about 2. This is demonstrated by replotting the data from Fig. 3 in the manner shown in Fig. 5. Broken lines are used at MnCl₂ concentrations of less than 30 μ moles/ml., because the relative errors in the measurement of the stimulation increase as the rate of transphosphorylation decreases. The percentage stimulation by KCl is still considerable when the ATP/Mn²⁺ ion ratio is 0.6, but stimulation by LiCl becomes zero at an ATP/Mn²⁺ ion ratio of 0.8. Figs. 3–5 therefore show the different effects of KCl and LiCl more clearly than does a comparison of these salts at a single ratio of ATP/Mn²⁺ ion.

DISCUSSION

The results reported here demonstrate that alkali-metal ions stimulate the non-enzymic-transphosphorylation reaction from ATP to orthophosphate (equation 1). The most striking aspect of this stimulation is the observation that the effect of K⁺ ions is exceptionally large compared with that

Fig. 5. Effect of K⁺ and Li⁺ ions in relation to the ratio ATP/Mn²⁺ ion. The results of Fig. 3 have been replotted to show the percentage stimulation due to the addition of K⁺ or Li⁺ ions.

of other alkali-metal ions (Table 2). The alkali-metal ions do not act simply by substituting for or satisfying the requirement for bivalent-metal ions. This is demonstrated in Table 1 and in Fig. 3, which show the bivalent-metal requirement to be absolute even in the presence of 0.1M-alkali-metal ions.

In a previous paper (Lowenstein, 1958) the catalytic action of bivalent-metal ions was accounted for as follows: two of the negative charges of the polyphosphate chain of ATP are neutralized by chelation with the bivalent-metal ion. This greatly increases the probability of the attack on ATP by anions which would otherwise tend to be repelled. Chelation of the attacking agent to the ATP-bivalent-metal complex can further facilitate the phosphate-transfer reaction. The probability of successful attack on ATP by neutral nucleophilic agents is also increased, since chelation with the bivalent metal increases the electrophilic character of the phosphorus atom. The structures of a number of ATP-bivalent-metal complexes were discussed by Liebecq & Jacquemotte-Louis (1958) and by Lowenstein (1958).

The simplest hypothesis to account for the effects described in this paper is that the alkali-metal ions exert their effect by neutralizing the negative charges on the polyphosphate chain of ATP which remain after the latter has combined with one bivalent-metal ion. According to this postulate, Li^+ ion would be expected to be the most effective ion because it possesses the smallest ionic radius of the univalent ions which were tested. Li^+ ion is indeed more effective than K^+ ion at high ATP/ Mn^{2+} ion ratios (Figs. 3-5). But this simple postulate is inadequate to account for the different stimulating effects observed with different alkali metals at ATP/ Mn^{2+} ion ratios in the region of 1 (Table 2; Figs. 3-5).

The available measurements of the stability constants of alkali metals with ATP provide no clue to the specificity of the stimulating effect of K^+ ions. Melchior (1954) found the stability constants for sodium ATP and potassium ATP to be the same (K , 10), although Alberty & Smith (1956) found slight differences (K_{K^+} , 11.5; K_{Na^+} , 14.3).

The observed effects may be accounted for in terms of the formation of complexes in which an alkali-metal ion as well as the bivalent-metal ion is chelated to ATP. A diagram of such a chelate is shown in Fig. 6*a*. It is proposed that the different stimulating effects reported here can be accounted for in terms of different modifications which each type of alkali-metal ion makes to the structure of the ATP-bivalent-metal chelate. The relative specificity of potassium may be because the charge-ionic radius relationship of the K^+ ion is such as to make its chelate with (ATP-bivalent metal) $^{2-}$

more active than that of the other alkali metals. Part of the observed effects must, however, be due to a simple neutralization of the remaining charges of ATP without chelation of the univalent ion, that is by ion-pair formation. This follows firstly because the NH_4^+ ion, which has no vacant coordination points available, also has a stimulating effect. Compared with potassium this amounts to about one-third under the experimental conditions used in Table 2. Secondly, the chelate shown in Fig. 6*a* still possesses one negative charge. If this charge is also neutralized by a second alkali-metal ion this process would involve only ion-pair attraction, there being no possibility of the formation of five- or six-membered chelate rings with other oxygens of the polyphosphate chain. Fig. 3 shows that the much larger tetra-alkylammonium ion does not stimulate the reaction, and indicates that the ion-pair effect varies with the size of the cation. The magnitude of the ion-pair effect for different alkali-metal ions cannot, however, be estimated from the data presented here.

The ATP chelate shown in Fig. 6*a* is not the only structure of its type which can be written. The possibility of position isomerism clearly exists. Thus the bivalent metal may also span the α - and β -phosphates of ATP, as was discussed previously (Lowenstein, 1958), and the position(s) occupied by the alkali-metal ion(s) would then also be different. Moreover, the chelate shown in Fig. 6*a* does not have a planar structure. Fig. 6*b* shows a possible conformation of this chelate. It consists of two chairs arranged so that the legs of one chair touch the back of the other chair. The two chairs do not face in the same direction: the top chair faces towards the reader and the bottom chair faces to his left.

Possible structures of complex ions containing one molecule of ATP and one metal ion were discussed by Melchior (1954). It was suggested that

the structure of such complexes would vary with the size of the metal ion. Small metal ions would be completely surrounded by the polyphosphate chain of ATP, intermediate-sized metal ions would rest in a 'shallow cup' formed by the polyphosphate chain, and complexes with large metal ions would have an 'open' configuration. It was pointed out that such differences in the shape of the ATP molecule could profoundly affect the rate of enzyme-catalysed reactions. The non-enzymic reaction described here has an absolute requirement for a bivalent-metal ion, and shows a selective stimulation by K^+ and Rb^+ ions. This implies that in the alkali-metal-stimulated reaction the active complex contains at least two metal ions, and that its structure differs from those described by Melchior (1954). In close analogy, most or all enzyme-catalysed-transphosphorylation reactions from ATP show an absolute requirement for a bivalent metal. A number of such reactions show a selective stimulation by univalent cations, and here the active complex must also involve at least two metal ions. The structures proposed by Melchior (1954) are therefore also insufficient to account for the alkali-metal-ion stimulation of enzyme-catalysed transphosphorylations from ATP.

No complex-formation between the 6-amino group of adenine and the co-ordination points left vacant on the metal is indicated in Fig. 6. The balance of the evidence available at present (e.g. Spicer, 1952; Martell & Schwarzenbach, 1956) is against such an interaction. A similar interaction with the heterocyclic-ring system cannot be ruled out, but metals such as magnesium, calcium and potassium, which are involved in chelate formation with ATP in the cell, show a strong preference for chelating with oxygen as compared with nitrogen (Sidgwick, 1941).

SUMMARY

1. The stimulation by alkali-metal ions of a non-enzymic-phosphate transfer from adenosine tri-

phosphate is described. The alkali-metal-ion stimulation does not abolish the absolute requirement by the reaction for bivalent-metal ions (M^{2+}).

2. Potassium ions produce a greater stimulation than other alkali-metal ions at adenosine triphosphate/ M^{2+} ion ratios in the region of 1.

3. The observed effects are discussed in terms of ion-pair and of chelate formation.

4. The results provide a chemical basis for the alkali-metal-ion dependence or stimulation observed in many enzyme-catalysed-phosphate-transfer reactions involving adenosine triphosphate and similar substances.

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The Component Fatty Acids of *Sporidesmium bakeri* Lipids

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Under certain climatic conditions, such as warm autumn rains after summers of above-average temperatures, pastures in some New Zealand districts have been known to produce a virulent poison causing serious losses in sheep and cattle.

Some of the affected animals show symptoms of photosensitization described as 'facial eczema', but the disease consists essentially in severe liver damage (Cunningham, Hopkirk & Filmer, 1942). Thornton & Percival (1959) have recently re-