5. The specific radioactivity of [^{14}C]amino acid in the perfusion fluid was measured at various times. The specific radioactivity of the intracellular free amino acids (L-valine or total amino acids), after perfusing for 5 hr., was about 60% of that of the corresponding amino acids in the medium at that time. The specific activity of the amino acids in the intracellular antibody was only one-sixth of that of the free amino acids on the intracellular pool. The interpretation of these findings is discussed.

6. Lungs from hyperimmune rabbits can liberate diffusible iodide from ^{131}I-labelled globulin.

We wish to thank Mr W. E. Brocklehurst for advice in the design of the perfusion apparatus, Mr R. C. Holloway for performing the iodinations and Mr G. Dickinson and Mr G. Savage for the gas counting.

It is a pleasure to acknowledge the excellent technical assistance of Miss Marion T. Smith and Miss Rosemary Willson.

REFERENCES

Askonas, B. A. (1958). *Rec. Trav. chim. Pays-Bas.* (In the Press.)

Askonas, B. A. & Humphrey, J. H. (1958). *Biochem. J.* **68**, 252.

Bradley, J. E. S., Holloway, R. C. & McFarlane, A. S. (1954). *Biochem. J.* **57**, 192.

Cohen, S., Holloway, R. C., Matthews, C. M. & McFarlane, A. S. (1956). *Biochem. J.* **62**, 143.

Coons, A. H., Leduc, E. H. & Connolly, J. M. (1955). *J. exp. Med.* **102**, 49.

Gordon, A. H. (1957). *Biochem. J.* **66**, 255.

Hanks, J. H. (1948). *J. cell. comp. Physiol.* **31**, 235.

Heidelberger, M., Kendall, F. E. & Scherp, H. W. (1936). *J. exp. Med.* **64**, 559.

Humphrey, J. H. & Sulitzeanu, B. D. (1958). *Biochem. J.* **68**, 146.

Jacobs, S. (1956). *Analyst*, **81**, 502.

Loftfield, R. B. & Harris, A. (1956). *J. biol. Chem.* **219**, 151.

McFarlane, A. S. (1956). *Biochem. J.* **62**, 135.

Miller, L. L., Bly, C. G., Watson, M. L. & Bale, W. F. (1951). *J. exp. Med.* **94**, 431.

Peters, T. (1957). *Fed. Proc.* **16**, 369.

Spector, W. S. Ed. (1956). *Handbook of Biological Data*, p. 71. London: W. B. Saunders Co. Ltd.

Steiner, D. & Anker, H. (1956). *Proc. nat. Acad. Sci., Wash.*, **42**, 580.

Van Slyke, D. D., McFadyen, D. A. & Hamilton, P. (1941). *J. biol. Chem.* **141**, 671.

Transphosphorylations Catalysed by Bivalent Metal Ions

By J. M. LOWENSTEIN*
Department of Biochemistry, University of Oxford

(*Received* 7 *March* 1958)

In all enzyme reactions involving the breakdown or the formation of the polyphosphate chain of nucleoside triphosphates there is a requirement for bivalent metal ions. This requirement is absolute in most, if not all, cases. It is most commonly met by Mg^{2+} ions, but these can frequently be replaced by Mn^{2+} ions. A few enzyme reactions which show a requirement for a bivalent metal ion also show an additional requirement for a univalent metal ion. In the examples which have come to light so far, this additional requirement is met by potassium (Hers, 1952; Parks, Ben-Gershom & Lardy, 1957; Webster & Varner, 1954*a*). Of several reviews the one by Lardy (1951) is the most relevant to the present paper. It also gives references to earlier reviews.

The work presented here arose from a study of enzyme-catalysed exchange reactions. In the course of an experiment designed to test for an enzyme-catalysed exchange between radioactive orthophosphate and adenosine triphosphate, the disappearance of radioactive orthophosphate was observed in controls which contained no enzyme.

* Beit Memorial Fellow.

The radioactive compound formed from orthophosphate was identified as pyrophosphate (Lowenstein, 1957*a*). The reaction was shown to depend on the presence of bivalent metal ions and adenosine triphosphate (ATP), and was formulated to proceed according to equation (1) (ADP represents adenosine diphosphate).

$$\text{ATP} + [^{32}\text{P}]\text{orthophosphate} \xrightarrow{Mn^{2+}} \text{ADP} + [^{32}\text{P}]\text{pyrophosphate} \quad (1)$$

This paper presents a detailed account and an extension of these observations.

A test of acceptors other than phosphates has shown that acylphosphate formation from nucleoside triphosphate and carboxylic acids also occurs in the absence of enzymes, and that it is catalysed by bivalent metal ions (Lowenstein, 1958).

EXPERIMENTAL

Materials and reagents

Nucleotides. The sodium salts of ATP and ADP were products of the Sigma Chemical Co., St Louis, Mo., U.S.A. The free acid of adenosine monophosphate was obtained

from the Pabst Laboratories, Milwaukee, Wisc., U.S.A. The substances were dissolved in water, and adjusted to the required pH with KOH. The concentrations of the solutions were determined by measuring the light-absorption at 260 mμ, and the solutions were diluted to the required concentrations.

Radioactive phosphate. Carrier-free [^{32}P]orthophosphate was obtained from the Radiochemical Centre, Amersham, Bucks.

Analytical methods

'Non-extractable phosphorus'. The main analytical procedure was based on the method of orthophosphate determination of Berenblum & Chain (1938), and involves the extraction of acid phosphomolybdate with *iso*butanol. The reaction was stopped by the addition of 5 ml. of 1·5N-H_2SO_4. The solution was transferred to a 20 ml. tube and 2·5 ml. of 5% (w/v) ammonium molybdate and 5 ml. of *iso*butanol were added. The tube was stoppered and shaken rapidly for about 30 sec. After the two layers had separated, the upper *iso*butanol layer was withdrawn by means of a capillary tube which led into a filter flask attached to a water pump. A further 5 ml. of *iso*butanol was added, and the extraction was repeated four times. The capillary suction tube was rinsed with 95% ethanol from a wash-bottle after each use. After the final extraction the aqueous layer was made up to 10 ml. with 95% ethanol, and the radioactivity of ^{32}P-labelled compounds remaining in the aqueous layer was measured in a liquid counter (Veall, 1948). Only orthophosphate is extracted into the *iso*butanol under these conditions, whereas phosphate esters and pyro- and poly-phosphates remain in the aqueous phase.

Orthophosphate. This was determined by the method of Berenblum & Chain (1938). The *iso*butanol extraction procedure was modified by using stoppered tubes instead of separating funnels. The unwanted aqueous layers were removed with the capillary suction tube already referred to above.

Pyrophosphate. Portions of ion-exchange fractions containing pyrophosphate were diluted to 7·0 ml. with N-H_2SO_4. The resulting solutions were heated in a boiling-water bath for 15 min. and cooled, and the orthophosphate liberated was determined.

RESULTS

Formation and identification of product of the reaction between adenosine triphosphate and orthophosphate. The incubation of ATP and [^{32}P]orthophosphate in the presence of Mn^{2+} ions and a buffer leads to the formation of a radioactive compound not extracted by *iso*butanol in the presence of acid molybdate. As is shown in Table 1, when ATP or the bivalent metal ions are omitted, the reaction is effectively abolished. When the buffer is omitted, the amount of product formed is reduced by an amount which is not easily controlled. This is presumably due to pH changes during the reaction in the absence of the buffer. As is shown below, the reaction has a sharp pH optimum.

The tentative identification of the reaction product by ion-exchange chromatography is shown in Fig. 1. A radioactive peak in the position of elution of pyrophosphate was observed in the chromatographic elution pattern after the reaction mixture had been incubated for 6 hr., but not at zero time. Moreover, the peak corresponding to the radioactive reaction product did not absorb light at 260 mμ and thus did not contain adenine. On the basis of this evidence the reaction product was presumed to consist of pyrophosphate. Radioactivity in the position of elution of ADP and ATP was negligible.

The identity of the radioactive reaction product was confirmed by adding authentic, unlabelled pyrophosphate to a portion of the reaction mixture and subjecting the whole to the chromatographic procedure shown in Fig. 1. The eluted fractions were assayed for total phosphorus and for radioactivity. The specific activities of the fractions representing the pyrophosphate peak were constant within the limits of the experimental error (Table 2). This establishes that the reaction product is pyrophosphate.

The specific activity of the pyrophosphate peak in the absence of added carrier pyrophosphate was found to be one-half to one-third that of the starting material.

Time course of the reaction. The influence of reaction time on the formation of pyrophosphate is shown in Fig. 2. The rate of formation of pyrophosphate is approximately constant during the first 8 hr. The slight decrease in the rate observed during this period and subsequently can be partially accounted for in terms of the pH, which dropped by about one unit in 24 hr.

Specificity of bivalent-metal-ion requirement. The effect on the transphosphorylation reaction of a number of different bivalent metal ions is shown in Table 3. An ATP/metal ion ratio of 1·11 was chosen in these experiments because some of the metal salts tested form insoluble precipitates with

Table 1. *Requirements for the formation of the reaction product*

The complete reaction mixture contained 0·10 ml. of 0·05 M-[^{32}P]orthophosphate ($1·16 \times 10^5$ counts/min.), 0·5 ml. of 0·10 M-ATP, 0·10 ml. of 0·5 M-diethanolamine–diethanolamine hydrochloride buffer, pH 9·0, and 0·10 ml. of 0·45 M-$MnCl_2$. The final volume was 1·0 ml., temp. 37°, and time of incubation 5 hr.

Reaction mixture	Non-extractable radioactivity (μmole)
Complete	0·372
Buffer omitted*	0·232
ATP omitted	0·034
$MnCl_2$ omitted	0·012
Zero time	0·038

* pH adjusted to 9·0 with N-KOH.

ATP at slightly lower ratios. The results show that the effectiveness of Ca^{2+} and Cd^{2+} ions is of the same order of magnitude as that of Mn^{2+} ions, whereas the effectiveness of the other metals tested is about one order of magnitude less. Lead acetate was also tested under the conditions set out in Table 3, but no reliable results were obtained as to its effectiveness in the transphosphorylation reaction because the metal interfered with the analytical procedure. The effects of Ca^{2+} and Cd^{2+} ions were confirmed in a further experiment (Table 4). Lower concentrations of reactants were used in order to avoid the formation of precipitates

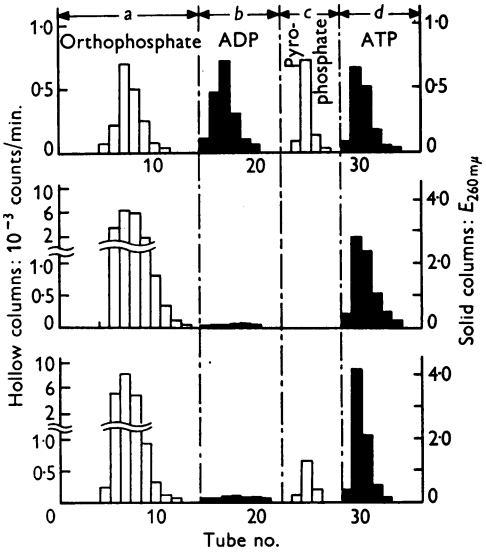

Fig. 1. Incorporation of radioactive orthophosphate into non-extractable compound. The reaction mixture contained 0·10 ml. of 0·05 M-[^{32}P]orthophosphate ($2·06 \times 10^5$ counts/min.), 0·50 ml. of 0·10 M-ATP, 0·10 ml. of 0·50 M-$MnCl_2$, 0·20 ml. of M-2-amino-2-hydroxymethylpropane-1:3-diol neutralized with HCl, pH 8·0 and 0·1 ml. of water; temperature 25°. Samples were withdrawn for analyses at 0 and 6 hr. One pair of samples was used for ion-exchange chromatography on columns of the formate salt of Dowex-2 resin, 10% cross-linked, 200–400 mesh, with a resin bed 2·5 cm. high and 1 cm. in diameter. The eluents employed are indicated by small letters: *a*, 0·15 M-ammonium formate; *b*, 3·0 N-formic acid; *c*, 0·50 M-ammonium formate; and *d*, M-ammonium formate. Fractions of 10 ml. were collected. The figure shows: top, elution pattern of a model mixture (for comparison); middle, reaction mixture at zero time; and bottom, reaction mixture after 6 hr. A second pair of samples was used for analyses of non-extractable radioactivity. The reaction was stopped by acidification with 5 ml. of 2·5 N-H_2SO_4, and orthophosphate was extracted as described under Analytical methods. The radioactivity not extracted by *iso*butanol rose from 60 counts/min./ml. at zero time to 9880 counts/min./ml. after 6 hr.

Table 2. *Identification of radioactive reaction product by co-chromatography*

The reaction mixture was similar to that described in Fig. 1. A sample of this solution, which contained about 1350 counts/min. of the reaction product, was co-chromatographed with approximately 4 μmoles of authentic pyrophosphate by the procedure described in Fig. 1. Radioactivity and phosphorus analyses for fractions 26–32 (eluent 0·5 M-ammonium formate) are shown below.

Fraction no.	Radioactivity (counts/min./ fraction)	Phosphorus (μg./fraction)	Specific activity (counts/min./μg. of phosphorus) (±S.D.)
26	12·9	2·53	5·1±0·2
27	660	134·0	4·9±0·1
28	456	92·5	4·9±0·1
29	155	31·7	4·9±0·1
30	47·8	10·0	4·8±0·1
31	14·0	3·24	4·3±0·2
32	9·1	1·86	4·9±0·2

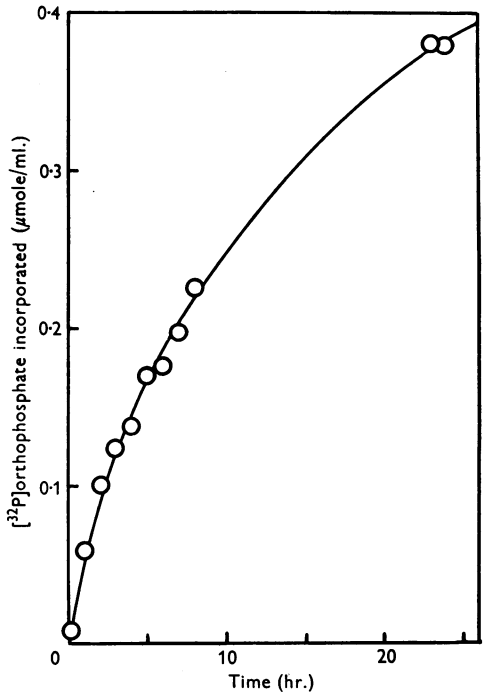

Fig. 2. Effect of time on transphosphorylation. The reaction mixture contained 2·0 ml. of 0·10 M-ATP (pH 10·0), 0·40 ml. of 0·50 M-$MnCl_2$, 0·40 ml. of 0·5 M-diethanolamine–diethanolamine hydrochloride buffer (pH 9·0), 0·40 ml. of 0·05 M-[^{32}P]orthophosphate ($7·76 \times 10^4$ counts/min.), and water to a volume of 4·0 ml. The temp. was 30°. Samples of 0·20 ml. were withdrawn for analysis at the times indicated. pH at start, 9·0; pH after 24 hr., 8·0.

Table 3. *Activation of transphosphorylation by bivalent metal ions*

The complete reaction mixture contained 0·10 ml. of 0·05 M-[^{32}P]orthophosphate ($1·16 \times 10^5$ counts/min.), 0·5 ml. of 0·10 M-ATP, 0·10 ml. of 0·5 M-diethanolamine–diethanolamine hydrochloride buffer (pH 9·0), and 0·10 ml. of 0·45 M-metal salt as indicated. The final volume was 1·0 ml., temp. 30°, and time of incubation 5 hr. Values of pyrophosphate formed are corrected for a blank containing no bivalent metal ions (0·012 μmole).

Metal salt	Pyrophosphate formed (μmole)
$MnCl_2$	0·360
$MnSO_4$	0·396
$BeSO_4$	0·022
$MgCl_2$	0·020
$CaCl_2$*	0·972
$ZnSO_4$	0·086
$CdCl_2$	0·244
$FeSO_4$	0·046
$CoCl_2$	0·062
$NiCl_2$	0·024
$CuSO_4$	0·030

* A precipitate formed in this tube.

Table 4. *Activation of transphosphorylation by bivalent metal ions*

The complete reaction mixture contained 0·05 ml. of 0·05 M-[^{32}P]orthophosphate ($8·0 \times 10^4$ counts/min.), 0·25 ml. 0·10 M-ATP, 0·10 ml. 0·5 M-diethanolamine–diethanolamine hydrochloride buffer (pH 9·0), and 0·05 ml. of 0·45 M-metal salt as indicated. The final volume was 1·0 ml. and temp. 30°.

Metal salt	Pyrophosphate formed (μm-moles)	
	Zero time	After 5 hr.
None	—	0
$MnCl_2$	0	27
$CdCl_2$	0	47
$CaCl_2$	0	294

in the case of tubes containing Ca^{2+} ions. Zero-time controls were run as an additional check on the analytical procedure. A comparison of Tables 3 and 4 shows that the relative effectiveness of Mn^{2+}, Cd^{2+} and Ca^{2+} ions varies with the reaction conditions.

In these experiments no account has been taken of the possibility of a hydrolysis of pyrophosphate catalysed by metal ions.

pH *dependence of the* Mn^{2+}*-catalysed reaction.* The influence of pH on the formation of pyrophosphate is shown in Fig. 3. The transphosphorylation shows a sharp pH optimum, the reaction rates at pH 8·5 and 9·5 being only about one-half of the rate at pH 9·0.

Effect of the concentrations of Mn^{2+} *ions and adenosine triphosphate on transphosphorylation.*

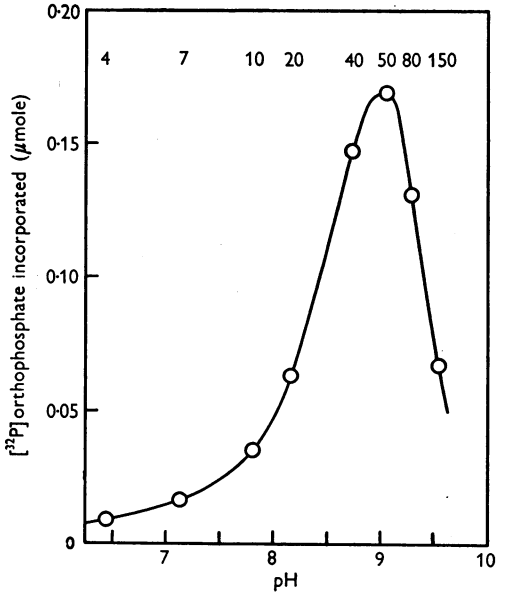

Fig. 3. Effect of pH on transphosphorylation. The complete reaction mixture contained 0·5 ml. of 0·10 M-ATP (pH 7·0), 0·1 ml. of 0·45 M-$MnCl_2$, 0·10 ml. of 0·05 M-[^{32}P]orthophosphate ($5·0 \times 10^4$ counts/min.), and 2-amino-2-hydroxymethylpropane-1:3-diol (free base) to give the pH indicated. The pH of each sample was measured at the start of the reaction by a Beckman pH meter fitted with small electrodes. Figures above the experimental points of the curve indicate the amount of base added to the tube in question (in μmoles/ml.). The final volume was 1·0 ml., temp. 38°, and time of incubation 5 hr.

Fig. 4 demonstrates the effect of varying the concentration of Mn^{2+}. An optimum concentration of Mn^{2+} ions was not reached in the experiment, but the curve is beginning to flatten out at the highest concentrations of Mn^{2+} ions used. The maximum rate of transphosphorylation is therefore attained in a region where the ATP/metal ratio is less than 1. The amount of pyrophosphate formed decreases rapidly as the ATP/metal ratio is increased from 1·0 to 2·0, and it is virtually zero for ratios greater than 2·0. Moreover, if the central part of the curve is treated as an approximately straight line, and is produced to cut the abscissa, it does so at a point equivalent to an ATP/metal ratio of 2·0.

Fig. 5 demonstrates the effect of varying the concentration of ATP. An optimum ATP concentration is approached at an ATP/metal ratio of approximately 0·6. The ATP/metal ratio could not be decreased further under the conditions of the experiments shown in Figs. 4 and 5 because of the formation of precipitates. At higher ratios the reaction rate drops off, being 69 % of the optimum when the ratio is 1·0, and 9 % of the optimum when

the ratio is 2·0. If the right-hand part of the curve is treated as an approximately straight line and is produced to cut the abscissa, it does so at a point which is practically double the concentration of $MnCl_2$ used in the reaction. The transphosphorylation reaction therefore again practically ceases when the ATP/metal ion ratio is about 2·0.

Addition of ethylenediaminetetra-acetate to the reaction mixture reduces the rate of transphosphorylation to a level which is similar to that obtained at a metal-ion concentration equal to the difference between the total metal-ion concentration minus the ethylenediaminetetra-acetate concentration (Fig. 4).

Effect of orthophosphate concentration. The rate of transphosphorylation/μmole of orthophosphate is approximately constant over a limited range of orthophosphate concentrations. Under the conditions shown in Fig. 6 this range extends from 0 to 20 μmoles of orthophosphate/ml. Thereafter the rate/μmole of orthophosphate diminishes, at first presumably due to competition for the metal by orthophosphate, and subsequently because of precipitation of orthophosphate–metal complexes.

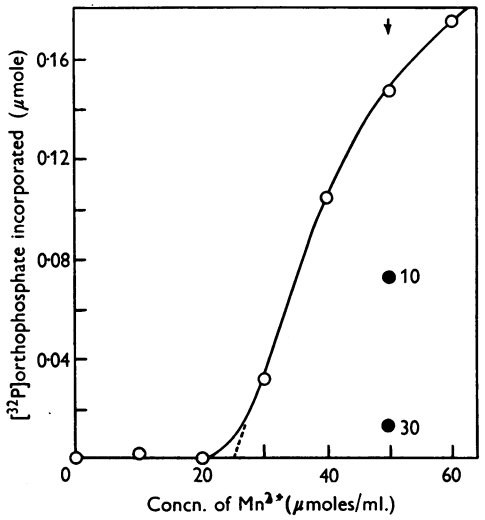

Fig. 4. Effect of concentration of Mn^{2+} ions on transphosphorylation. ○, Reaction mixtures which contained 0·5 ml. of 0·10 M-ATP (pH 9·0), 0·10 ml. of 0·05 M-[^{32}P]-orthophosphate ($3{\cdot}52 \times 10^4$ counts/min.), 0·10 ml. of 0·5 M-diethanolamine–diethanolamine hydrochloride buffer (pH 9·0), and $MnCl_2$ as indicated. ●, Reaction mixtures to which has been added ethylenediaminetetra-acetic acid, pH 9·0. The amounts added (in μmoles) are shown by the figures next to these points. The final volume was 1·0 ml., pH 9·0, temp. 36°, and time of incubation 5 hr. The arrow shows where the concentrations of ATP and $MnCl_2$ are equal.

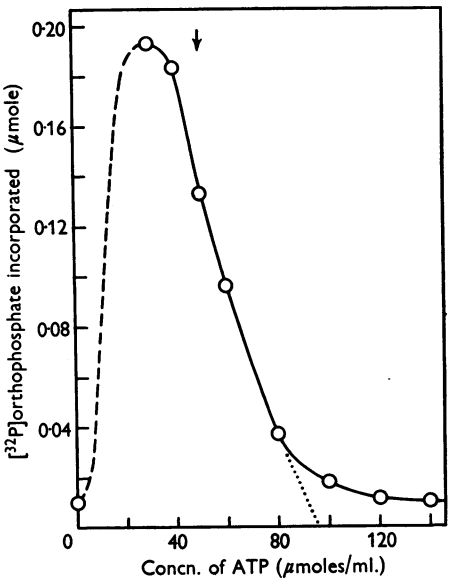

Fig. 5. Effect of concentration of ATP on transphosphorylation. The reaction mixture contained 0·10 ml. of 0·05 M-[^{32}P]orthophosphate ($1{\cdot}0 \times 10^5$ counts/min.), 0·1 ml. of 0·50 M-$MnCl_2$, 0·10 ml. of 0·5 M-diethanolamine–diethanolamine hydrochloride buffer (pH 9·0), and ATP as indicated. The final volume was 1·0 ml., pH 9·0, temp. 30°, and time of incubation 5 hr. Precipitation occurs at the ATP concentrations indicated by the broken part of the curve. The arrow shows where the concentrations of $MnCl_2$ and ATP are equal.

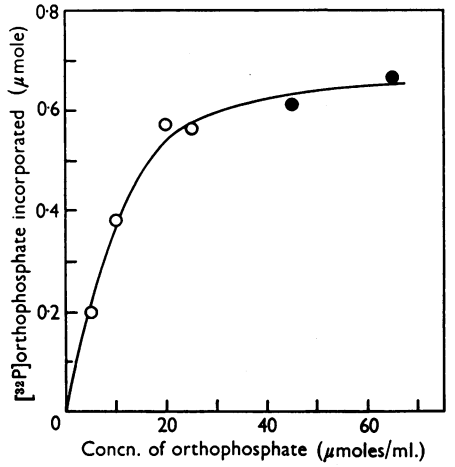

Fig. 6. Effect of orthophosphate concentration on transphosphorylation. The reaction mixture contained 0·5 ml. of 0·10 M-ATP (pH 9·0), 0·1 ml. of 0·50 M-$MnCl_2$, 0·10 ml. of 0·5 M-diethanolamine–diethanolamine hydrochloride buffer (pH 9·0), and [^{32}P]orthophosphate ($5{\cdot}26 \times 10^4$ counts/min.) at the concentrations indicated. The final volume was 1·0 ml., pH 9·0, temp. 30°, and time of incubation 5 hr. ○, Clear solution; ●, turbid solution.

DISCUSSION

The following abbreviations are used below. Nucleoside mono-, di- and tri-phosphate are represented by NP, NPP and NPPP respectively. Pyrophosphate is represented by PP. Radioactive phosphate is indicated by an asterisk, e.g. P*. Acceptor molecules are indicated by X. A chelate containing one molecule of NPPP and one metal ion will be designated ZM, a chelate containing one molecule of NPPP and two metal ions will be designated ZM_2, and a chelate containing two molecules of NPPP and one metal ion will be designated Z_2M.

The work reported here demonstrates the occurrence of a transphosphorylation reaction which is catalysed by bivalent metal ions, and which proceeds according to equation (1). The evidence for the reaction consists of the identification of pyrophosphate as the reaction product by isotope dilution (Table 1). The specific activity of the reaction product was also in accord with that expected from equation (1), and the formation of pyrophosphate was associated with an increase in the amount of ADP (Fig. 1).

The non-enzymic transphosphorylation reaction has a number of features in common with nucleoside triphosphate (NPPP) phosphotransferases. The most important of these is the bivalent-metal-ion requirement. Others include the inhibition of the reaction when the NPPP/metal ratio is greater than 1, the pH optimum (which falls within two units of that of most NPPP-phosphotransferases), the shape of the pH–activity curve and the temperature at which the reaction proceeds. A direct comparison of reaction rates is not possible since the rates of enzyme-catalysed reactions are by convention expressed in terms of enzyme concentrations. Moreover a comparison of rates with respect to the amounts of metal present in enzymic and non-enzymic transphosphorylations is not strictly justified in this case because the metal is not an integral part of the enzyme. However, one obtains the impression that the non-enzymic reaction is much slower than the enzyme-catalysed reaction.

The relative effectiveness of different bivalent-metal ions (Tables 3 and 4) varies with the reaction conditions and awaits detailed study. An interpretation of the pH-optimum curve (Fig. 3) probably involves many factors. Those which may have to be taken into account include the stability of ATP and pyrophosphate chelates at different pH values, the competitive binding of Mn^{2+} by hydroxyl ions at high pH values, pK_3 for orthophosphate and pK_4 for pyrophosphate. pK_4 for ATP can probably be ignored above pH 8·0.

The influence of the ATP/metal ratio on the rate of transphosphorylation is shown in Figs. 4 and 5. The rate increases as the ATP/metal ratio is decreased from 2·0 to 0·6. On the other hand, the rate of transphosphorylation tends towards zero as the ATP/metal ratio approaches 2·0, and it does not increase again at ratios greater than 2. These phenomena can be accounted for in terms of the existence of chelates with different ATP/metal ratios and with different net charges. A number of possible chelates and their interrelationships are shown in Fig. 7. Each of the individual chelates represented in Fig. 7 may occur in more than one structural form. For example, three possible structures of ZM^{2-} are shown in Fig. 8. Since pK_4 of ATP lies between 6·5 and 7·0 (Martell & Schwarzenbach, 1956; Smith & Alberty, 1956) the only species which need be considered at pH 9·0 are those in the lower line of Fig. 7, that is those derived from Z^{4-}. The experimental evidence shows that the chelates reactive in the transphosphorylation are either ZM^{2-} or ZM_2, or both, and that the species Z_2M^{4-} is not reactive. The decrease in the reaction rate as the ATP/metal ratio is increased from 1·0 to 2·0 is then most simply accounted for by the transition from mainly ZM^{2-}, at a ratio of 1·0, to mainly Z_2M^{4-} at a ratio of 2·0. The attainment of the maximum rate of transphosphorylation at ATP/metal ratios of less than 1·0 indicates that ZM_2 is the more reactive species.

The results also constitute evidence for the formation of the chelates Z_2M^{4-} and ZM_2. Stability constants for ZM have been measured by Smith & Alberty (1956), and by Martell & Schwarzenbach (1956), but none are available for ZM_2 and Z_2M. Martell & Schwarzenbach (1956) advance arguments that the stability constant for ZM_2 can be neglected by comparison with that for ZM in ATP–Mg and –Ca chelates. However, for manganese, Cohn (1954) mentions complexes with more than one metal atom/molecule of ATP. Although the ATP/metal ratio of the reactive chelate is thus not certain, for the following argument it is assumed to be 1·0. In such a chelate some of the negative charges of the polyphosphate chain are screened by the metal (Fig. 8). This screening greatly increases the probability of the attack on NPPP by anions which would otherwise

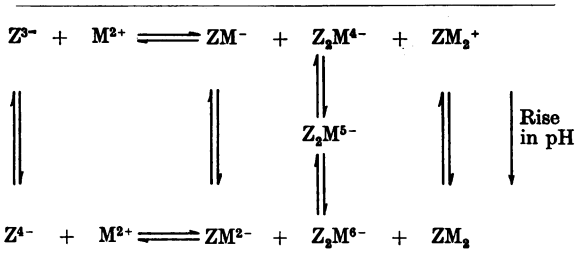

Fig. 7. Some possible chelates and their interrelationships (NPPP is denoted by Z, metal by M).

tend to be repelled, such as phosphomonoesters and carboxylic acids. For a transfer of the γ-phosphate from NPPP to an acceptor (equation 2), the chelate is probably that shown in Fig. 8*a* or *c*. In the former the metal is shown to span the β- and γ-phosphates of NPPP, and an attack on the γ-phosphate of the chelate by, for example, a phosphomonoester would take the course shown in Fig. 9*a*. The enzyme reactions may be formulated to proceed by a similar mechanism (Fig. 9*b*) except that at pH 7·4 the reactive chelate may be derived from either Z^{4-} or Z^{3-}. An attack on the α- or β-phosphates of NPPP, which occurs in enzyme reactions of the type shown in equations (3) and (4), could proceed by a similar mechanism.

$$\text{NPPP} + \text{X} \rightarrow \begin{cases} \text{XP} + \text{NPP} & (2) \\ \text{NPX} + \text{PP} & (3) \\ \text{XPP} + \text{NP} & (4) \end{cases}$$

If the reactive chelate in reactions (2), (3) and (4) is that shown in Fig. 8*c*, the enzyme may exert its specificity by influencing the charge distribution along the polyphosphate chain. On the other hand, the chelate involved in reaction (3) may be that shown in Fig. 8*b*, and that involved in reaction 4 may be either that shown in Fig. 8*a* or that shown in Fig. 8*b*.

Certain enzymic transphosphorylations from NPPP result in the phosphorylation not of anions but of neutral molecules. Such reactions can be formulated as nucleophilic attacks by the neutral molecules on a phosphorus atom of NPPP. In these cases chelation also greatly increases the electrophilic character of the phosphorus atom, and thus facilitates the attack by the neutral molecule.

It has been proposed that the mechanism of action of ATP-phosphotransferases involves the phosphorylation of the enzyme by ATP. Further, the formation of a phosphorylated enzyme was postulated to represent one general mechanism for the utilization of ATP in enzyme reactions (Webster & Varner, 1954*a*, *b*; Snoke, 1953; Jones, Lipmann, Hilz & Lynen, 1953; Lipmann, 1954). If reactions (2), (3) and (4) proceed by a mechanism involving the formation of a phosphorylated enzyme as an intermediate step, the enzymes concerned should catalyse exchange reactions between NP*P* and NPPP, P*P* and NPPP, and NP* and NPPP respectively, and the exchange should occur in the absence of acceptor X. This should be so unless it is postulated that the acceptor must be present to fulfil some purpose other than acting as acceptor. On the other hand, in the enzymic mechanism of transphosphorylation shown in Fig. 9*b* the enzyme does not act as a phosphoryl carrier, and no exchange should be observed in the absence of acceptor X. Exchange criteria of this type have been employed by Berg (1956) for the acetate-activating enzyme, and have led him to propose

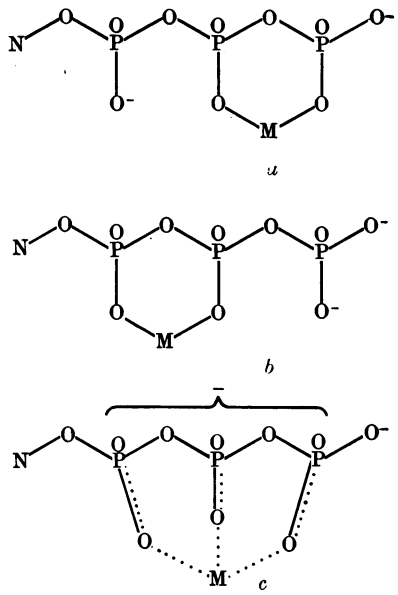

Fig. 8. Three possible structures of the chelate ZM^{2-}. In *a* the metal spans the α- and β-phosphates, in *b* the β- and γ-phosphates, and in *c* all three phosphates. In *c* one of the charges may be on any one of the three phosphates.

enzymic mechanisms for the formation of acetyl phosphate and adenyl acetate similar to those shown in Fig. 9. A similar mechanism is probably also operative in the non-enzymic activation of acetate demonstrated by Lowenstein (1958). Chemical mechanisms in enzymic transfer reactions have also been discussed by Koshland (1954) and by Boyer & Harrison (1954).

The non-enzymic reaction and the results of enzymic-exchange experiments (Berg, 1956; Lowenstein, unpublished observations) show that transphosphorylations from ATP need not proceed via phosphorylated enzymes. This conclusion does not apply to reactions which do not require bivalent metal ions or which involve the transfer of phosphate groups with a free energy of hydrolysis comparable with that of the common sugar phosphates. A case in point is phosphoglucomutase, which has been shown to be phosphorylated in the course of the reaction that it catalyses (Anderson & Jollès, 1957; Kennedy & Koshland, 1957).

Metal chelates of NPPP have been postulated to be the 'true' substrates in enzyme reactions involving nucleotides, the role of the metal being to bind the nucleotide to the enzyme (for a discussion see Calvin, 1954). The reaction demonstrated above shows that the role of the metal ion in enzymic transphosphorylations goes beyond that of a passive binding function. Instead the enzyme may be considered to make more specific the non-enzymic mechanism by the selective adsorption of

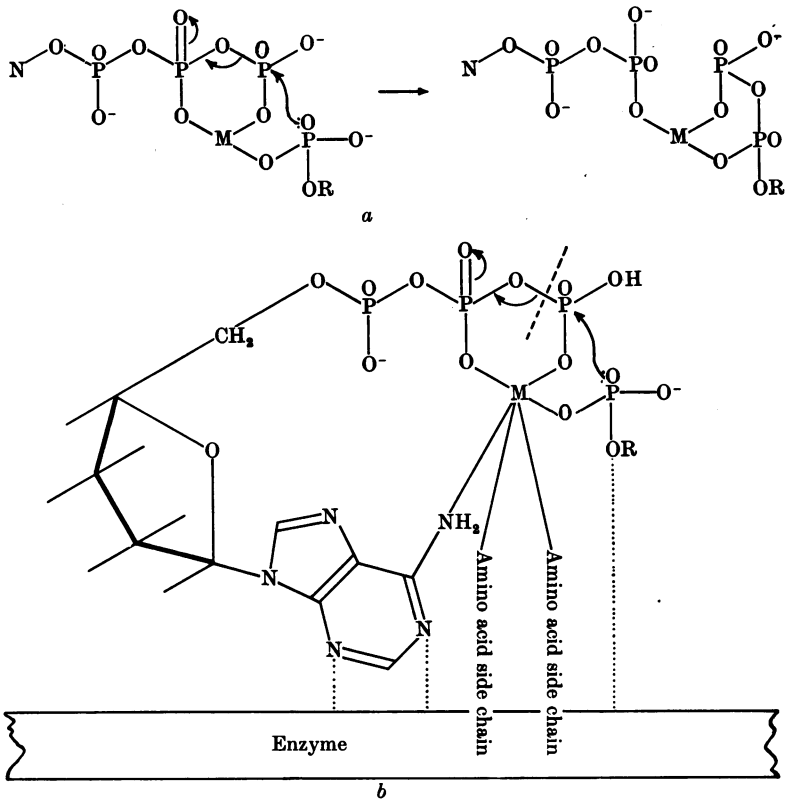

Fig. 9. *a*, Mechanism of non-enzymic transphosphorylation by the nucleophilic attack of a phosphomonoester on the γ-phosphate of NPPP. *b*, Diagrammatic representation of the same mechanism in enzymic transphosphorylation (only the initial attack is shown).

specific nucleotides and specific acceptors. It may do this by binding the NPPP–metal chelate to the enzyme surface through vacant co-ordination valencies of the metal. In addition the chelate may complex with the acceptor molecule as is suggested in Fig. 9*b*. No experimental data exist on the binding of NPPP–metal chelates to enzymes, but it has been demonstrated by Murphy & Martell (1957) that the co-ordination of metals with simple peptide linkages is too weak to account for the strength of co-ordination which has been observed with ions as basic as Mg^{2+} and Mn^{2+}. Thus binding of these metals must occur through groups, such as carboxyls and iminazoles, available on the side chains of amino acids.

The substrate concentrations commonly employed in enzymic transphosphorylations are approximately one-fiftieth of those employed in the non-enzymic reaction. These differences can be partially reconciled in that the adsorption and orientation of the substrates on the enzyme surface is, other influences apart, tantamount to a localized raising of the substrate concentrations.

Polyphosphates in the form of partially hydrated polyphosphoric acid and partially hydrated phosphoryl chloride have been employed as phosphorylating agents (for references see Seegmiller & Horecker, 1951; Lowenstein, 1957*b*). The compounds phosphorylated were generally alcohols, the reaction conditions usually involved heating, and no catalysis by bivalent metal ion was reported. The reaction conditions were thus quite different from those reported here.

SUMMARY

1. A non-enzymic transphosphorylation has been demonstrated to occur between adenosine triphosphate and orthophosphate.

2. The reaction is catalysed by bivalent metal ions. Of those tested, Mn^{2+}, Ca^{2+} and Cd^{2+} were found to be the most effective.

3. The reaction has a sharp pH optimum. With Mn^{2+} ions the rate of transphosphorylation is optimum at adenosine triphosphate/metal ratios between 0·6 and 1·0.

4. A mechanism is proposed for the reaction, and its relation to enzymic transphosphorylation is discussed.

I am indebted to Mr Roy Cooke for invaluable technical assistance. My thanks go to Professor Sir Hans A. Krebs, F.R.S., and to Drs W. Bartley, K. Burton and R. J. Williams for reading the manuscript.

This work was supported in part by a grant from the British Empire Cancer Campaign. I am grateful to the Sigma Chemical Co., St Louis, Missouri, for a generous gift of nucleotides.

REFERENCES

Anderson, L. & Jollès, G. R. (1957). *Arch. Biochem. Biophys.* **70**, 121.
Berenblum, I. & Chain, E. (1938). *Biochem. J.* **32**, 295.
Berg, P. (1956). *J. biol. Chem.* **222**, 991.
Boyer, P. D. & Harrison, W. H. (1954). *The Mechanism of Enzyme Action*, p. 658. Ed. by McElroy, W. D. & Glass, B. Baltimore: Johns Hopkins Press.
Calvin, M. (1954). *The Mechanism of Enzyme Action*, p. 239. Ed. by McElroy, W. D. & Glass, B. Baltimore: Johns Hopkins Press.
Cohn, M. (1954). *The Mechanism of Enzyme Action*, p. 246. Ed. by McElroy, W. D. & Glass, B. Baltimore: Johns Hopkins Press.
Hers, H. G. (1952). *Biochim. biophys. Acta*, **8**, 416.
Jones, M. E., Lipmann, F., Hilz, H. & Lynen, F. (1953). *J. Amer. chem. Soc.* **75**, 3285.
Kennedy, E. P. & Koshland, D. E. (1957). *J. biol. Chem.* **228**, 419.
Koshland, D. E. (1954). *The Mechanism of Enzyme Action*, p. 608. Ed. by McElroy, W. D. & Glass, B. Baltimore: Johns Hopkins Press.
Lardy, H. (1951). *Phosphorus Metabolism*, vol. 1, p. 477. Ed. by McElroy, W. D. & Glass, B. Baltimore: Johns Hopkins Press.
Lipmann, F. (1954). *The Mechanism of Enzyme Action*, p. 599. Ed. by McElroy, W. D. & Glass, B. Baltimore: Johns Hopkins Press.
Lowenstein, J. M. (1957*a*). *Biochem. J.* **65**, 40P.
Lowenstein, J. M. (1957*b*). *Spec. Publ. Chem. Soc.* **8**, 100.
Lowenstein, J. M. (1958). *Biochim. biophys. Acta*, **28**, 206.
Martell, A. E. & Schwarzenbach, G. (1956). *Helv. chim. Acta*, **39**, 653.
Murphy, C. B. & Martell, A. E. (1957). *J. biol. Chem.* **226**, 37.
Parks, R. E., Ben-Gershom, E. & Lardy, H. A. (1957). *J. biol. Chem.* **227**, 231.
Seegmiller, J. E. & Horecker, B. L. (1951). *J. biol. Chem.* **192**, 175.
Smith, R. M. & Alberty, R. A. (1956). *J. Amer. chem. Soc.* **78**, 2376.
Snoke, J. E. (1953). *J. Amer. chem. Soc.* **75**, 4872.
Veall, N. (1948). *Brit. J. Radiol., N.S.*, **21**, 347.
Webster, G. C. & Varner, J. E. (1954*a*). *Arch. Biochem. Biophys.* **52**, 22.
Webster, G. C. & Varner, J. E. (1954*b*). *J. Amer. chem. Soc.* **76**, 633.

The Biosynthetic Preparation of [16-^{3}H]Aldosterone and [16-^{3}H]Corticosterone

By P. J. AYRES, W. H. PEARLMAN,* J. F. TAIT† and SYLVIA A. S. TAIT†
Guy's Hospital Medical School, London, S.E. 1, *and The Middlesex Hospital Medical School, London, W.* 1

(*Received* 18 *March* 1958)

For both metabolic *in vivo* and analytical *in vitro* uses of radioactive steroids it is an advantage to add amounts of the labelled compound much smaller than are already present in the animal or extract but sufficiently radioactive to be followed with ease through any process. [4-^{14}C]Cortisol of sufficiently high specific activity (about 0·004 μc/μg.) for most purposes is now available. However, [4-^{14}C]corticosterone of similar specific activity is not suitable for many applications owing to the low rate of secretion of this hormone in man. (Ayres *et al.* 1957*a*; Peterson, 1957). Radioactive aldosterone, of specific activity high enough to be useful in metabolic studies, has not previously been prepared.

The advantages in the preparation and use of tritiated steroids are the ease and cheapness of their synthesis at high specific activities and their relative radiation safety for metabolic studies in man. The disadvantages of their use are the difficulty of assay, which is overcome by the application of methods employing a flow counter (Banks, Crawhall & Smyth, 1956) or a liquid scintillator (Davidson & Feigelson, 1957), and also the possibility of unstable labelling. This latter consideration has been discussed elsewhere (Pearlman, 1957*b*) and found to be unlikely for most metabolic applications of [16-^{3}H]progesterone. The same remarks apply to the use of 16-^{3}H-labelled corticosteroids.

The preparation of [16-^{3}H]progesterone of high specific activity has been described previously (Pearlman, 1957*a*). Progesterone has been shown to be an intermediate in the biosynthesis of aldosterone and corticosterone by capsule strippings of

* Present address: Waldemar Medical Research Foundation Inc., 16 Sintsink Drive East, Port Washington, New York, U.S.A.
† External Scientific Staff, Medical Research Council, Great Britain.