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# Phosphate Transfer from Adenosine Triphosphate in a Model System[†]

Nathan Nelson[‡] and Efraim Racker[*]

ABSTRACT: In 70% dimethyl sulfoxide there is a rapid transfer of the terminal phosphate of ATP to either water or inorganic phosphate. The hydrolysis of ATP requires the simultaneous presence of $Mg^{2+}$, a dicarboxylic acid and arsenate. The transfer reaction requires only $Mg^{2+}$ but is stimulated by a dicarboxylic acid. The product of the transfer reaction appears to be exclusively inorganic pyrophosphate. These reactions may serve as model systems for phosphate transfer and ATPase activity in oxidative and photosynthetic phosphorylation.

The numerous hypothetical mechanisms and model reactions that have been devised to explain oxidative phosphorylation were reviewed by Lardy and Ferguson (1969). In most model systems an oxidation that takes place in a nonaqueous solvent is utilized for the formation of ATP or of pyrophosphate. Several of the models require formation of a phosphate ester which undergoes oxidation. The fact that in mitochondria the energy of oxidation can be preserved in the absence of phosphate (*cf.* Racker, 1965) makes these models unattractive. A more attractive model utilizes the oxidation of hemochrome with imidazole as the phosphoryl carrier (Brinigar *et al.*, 1967). Tu and Wang (1970) described a system phosphorylating imidazole in water, followed by a transfer of phosphate to AMP in dry *N,N*-dimethylacetamide. An important model reaction which takes place in aqueous solution is the formation of acid anhydrides during the oxidation of thioethers to sulfoxide (Higuchi and Gensch, 1966a,b). It is of interest to note that dicarboxylic acids facilitated the formation of the sulfoxide.

Lowenstein (1958a,b) described a phosphate transfer from ATP to inorganic phosphate catalyzed in aqueous solution at pH 9 by $Ca^{2+}$ or $Mn^{2+}$. $Mg^{2+}$ was ineffective and the reaction was comparatively slow. Hopkins and Wang (1965) described a slow transfer of the phosphate group from ATP to AMP. This reaction took place in $Me_2SO$ solution and was catalyzed by $Na^+$ or $K^+$ but not by $Ca^{2+}$ or $Mg^{2+}$.

It is the purpose of this paper to report on a phosphotransfer reaction from ATP to either water or $P_i$; this model system bears some resemblance to reactions observed in membranes catalyzing electron transport linked phosphorylation.

## Experimental Procedures

*Materials.* Dimethyl sulfoxide, ATP, ADP, Dowex-1, Tricine, Tris, and maleic acid were obtained from Sigma. Other reagents were of analytical grade. [$\gamma$-$^{32}$P]ATP was prepared as previously described (Nelson *et al.*, 1972). Crystallized yeast inorganic pyrophosphatase was a gift from Dr. L. Heppel (Heppel, 1955). Mitochondrial ATPase was prepared as previously described (Horstman and Racker, 1970).

*Assay of Transfer Reaction to $P_i$.* In a final volume of 0.5 ml, 5 μmol of ATP, 5 μmol of $MgCl_2$, and 2.5 μmol of $Na^{32}P_i$ were adjusted to pH 8 with NaOH and buffer as specified in the legends to the figures. The reaction was started by addition of 1 ml of dimethyl sulfoxide with vigorous stirring. After 10 min incubation at 37°, the reaction was terminated by addition of 1 ml of $H_2O$ or 0.1 ml of 30% trichloroacetic acid. Addition of 4 ml of 1.2% ammonium molybdate in 1 N HCl and 7 ml of a water-saturated solution of isobutyl alcohol–benzene–acetone (5:5:1) was followed by vigorous mixing in a Vortex shaker for 20 sec. The extraction was repeated twice with 7 ml of the solvent, and 1 ml of the water phase was dried and counted in a Nuclear Chicago flow counter.

*Assay of ATPase.* ATP hydrolysis was measured with [$\gamma$-$^{32}$P]ATP in the presence of sodium arsenate instead of $NaP_i$ under the same conditions used for the transfer reaction. A sample (1 ml) of the solvent phase of the first extraction was dried and counted.

## Results

*Hydrolysis of ATP in a Model System.* Solutions of ATP in the presence of $MgCl_2$, Tricine-maleate (pH 8), and arsenate were stable at 37° in aqueous solution for at least 4 hr. As can be seen from Figure 1, the addition of $Me_2SO$ resulted in the rapid liberation of the $\gamma$ phosphate of ATP (Figure 1) provided its concentration exceeded 50%. About 70% $Me_2SO$

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[†] From the Section of Biochemistry and Molecular Biology, Cornell University, Ithaca, New York 14850. *Received September 21, 1972.* This work was supported by National Science Foundation Grant No. GB-30850.

[‡] Present address: Department of Biology, Technion, Haifa, Israel.

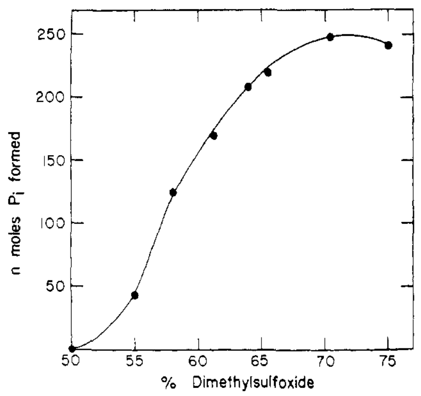

FIGURE 1: Effect of dimethyl sulfoxide concentration of ATP hydrolysis. The reaction mixture in a final volume of 0.5 ml of aqueous solution contained: 20 μmol of Tricine-maleate (pH 8), 5 μmol of ATP containing about 100,000 cpm of [γ-$^{32}$P]ATP, 2.5 μmol of sodium arsenate (pH 8), and 5 μmol of MgCl$_2$. The reaction was started by addition of different volumes of Me$_2$SO and it was terminated by 0.1 ml of 30% trichloroacetic acid after incubation for 10 min at 37°. The liberation of $^{32}$P was measured as described under Methods. The Me$_2$SO concentrations were expressed as per cent by volume.

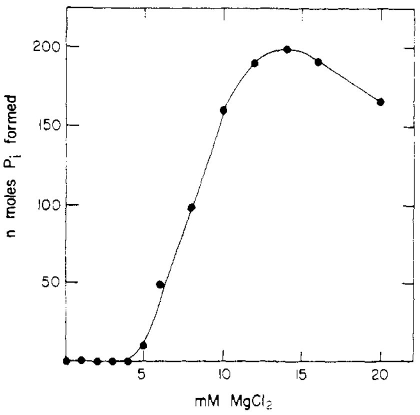

FIGURE 2: Effect of Mg$^{2+}$ concentration on the hydrolysis of ATP. The conditions were as described in Figure 1 except that the MgCl$_2$ concentration was varied and 1 ml of Me$_2$SO was used to start the reaction.

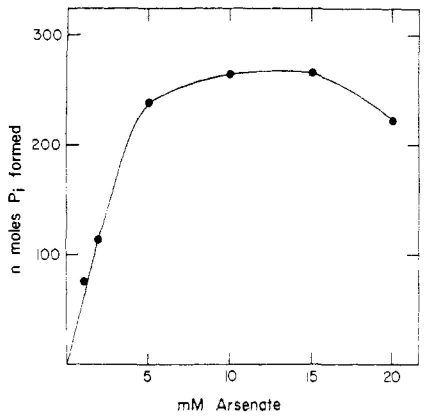

FIGURE 3: Effect of arsenate concentration of the ATP hydrolysis. The conditions were as described in Figure 1 except that the arsenate concentration was varied and 1 ml of Me$_2$SO was used to start the reaction.

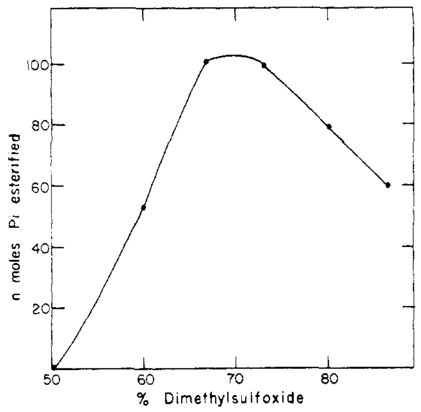

FIGURE 4: Effect of Me$_2$SO concentration on phosphate incorporation. The reaction mixture in a final volume of 0.5 ml of aqueous solution contained: 20 μmol of Tricine-NaOH (pH 8), 5 μmol of ATP, 2.5 μmol of P$_i$ containing about 500,000 cpm of $^{32}$P, and 5 μmol of MgCl$_2$. The reaction was started by addition of Me$_2$SO and was terminated after 10 min at 37° by addition of 0.1 ml of 30% trichloroacetic acid. The incorporation was measured as described under Methods.

was optimal. The reaction could therefore be conveniently stopped by the addition of water. There was an absolute dependence of the ATP hydrolysis on the presence of Mg$^{2+}$ in the reaction mixture (Figure 2). In the absence of Mg$^{2+}$ the reaction mixture was turbid. It became clear on addition of 2 mM MgCl$_2$, yet, as shown in Figure 2, no ATPase activity was detected. The optimal MgCl$_2$ concentration was 10–15 mM. An important factor was the proportion of the Mg$^{2+}$ to the ATP concentrations with a ratio of 1:1.5 being optimal. Figure 3 shows the dependence of the reaction on the presence of arsenate with 5 mM being the optimal concentration.

Chloride, sulfate, or P$_i$ did not substitute for arsenate in the catalysis of ATP hydrolysis. Moreover, P$_i$ inhibited the hydrolysis in the presence of arsenate. The reaction was completely dependent on the presence of maleate. However, in the presence of high concentrations of arsenate (10 mM), this dependency was not complete.

*Phosphotransfer from ATP in a Model System.* When ATP was used in the presence of $^{32}$P$_i$ under the same conditions except for the omission of arsenate, radioactivity appeared at a rapid rate in the water phase after solvent extraction. The reaction was completely dependent on the presence of dimethyl sulfoxide as shown in Figure 4. At concentrations below 50% no activity could be detected. The time course of the reaction is shown in Figure 5 and compared to that of the arsenate-catalyzed ATP hydrolysis. Up to 10 min the rate of both reactions was linear but, especially for the transfer reaction, decreased with time, perhaps due to the formation of ADP. The transfer reaction required Mg$^{2+}$ with 8–10 mM being optimal (Figure 6). Ca$^{2+}$ or Mn$^{2+}$ could be used in place of Mg$^{2+}$. As shown in Figure 7, the reaction was accelerated several-fold by maleate; however, in contrast to the ATP hydrolysis a substantial rate was observed in the absence of maleate. Other dicarboxylic acids such as succinate and malate as well as bicarbonate were also effective. Tricine and sodium acetate also accelerated the reaction but less effectively. The reaction took place over a wide range of pH but increased at more alkaline pH. It was completely inhibited at pH values below 2 and

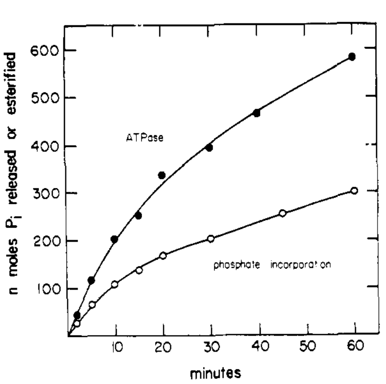

FIGURE 5: Time course for ATP hydrolysis and phosphate incorporation reactions. The reaction mixture for ATP hydrolysis was as described in Figure 1, but with 1 ml of $Me_2SO$. The reaction mixture for phosphate incorporation was identical with the ATPase except that cold ATP was used, the arsenate was omitted, and 2.5 $\mu$mol of phosphate containing $10^6$ cpm of $^{32}P$ was included. The reactions were assayed as described under Methods.

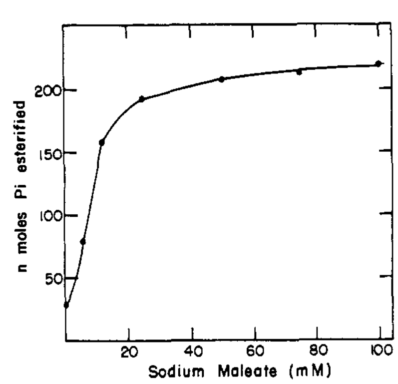

FIGURE 7: Effect of maleate concentration on phosphate incorporation. The reaction mixture in a final volume of 0.5 ml of aqueous solution contained: 5 $\mu$mol of ATP (pH 8), 2.5 $\mu$mol of $P_i$ (pH 8) containing $10^6$ cpm of $^{32}P$, 5 $\mu$mol of $MgCl_2$, and the specified concentration of sodium maleate (pH 8). The reaction was started by addition of 1 ml of $Me_2SO$. The mixture was incubated at 37° for 10 min and the reaction stopped by addition of 0.1 ml of 30% trichloroacetic acid.

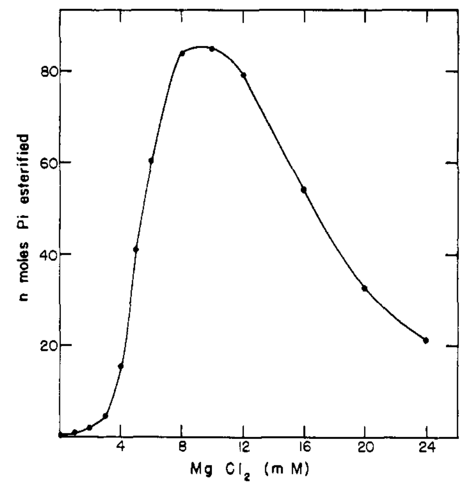

FIGURE 6: Effect of $Mg^{2+}$ concentration on phosphate incorporation. The reaction conditions were as described in Figure 5 except that $MgCl_2$ concentration was varied and 1 ml of $Me_2SO$ was used.

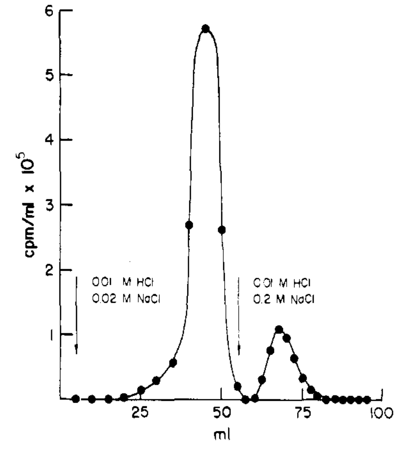

FIGURE 8: Elution pattern of Dowex-1 column. The reaction mixture in a final volume of 2.5 ml of aqueous solution contained: 250 $\mu$mol of sodium maleate (pH 8), 25 $\mu$mol of ATP, 12.5 $\mu$mol of $P_i$ containing $^{32}P$, and 25 $\mu$mol of $MgCl_2$. The reaction was started by addition of 5 ml of $Me_2SO$. After 20 min of incubation at 37° the reaction was stopped by addition of 7.5 ml of $H_2O$ and the solution was applied on a Dowex-1 (chloride form) column (1 × 4 cm). The column was washed by 20 ml of $H_2O$, 30 ml of 3 mM HCl, and then 0.01 M HCl + 0.02 M NaCl and 0.01 M HCl + 0.2 M NaCl. The elutants of the last two solutions are shown in the figure. Samples of 0.1 ml of each fraction were removed, dried, and counted for $^{32}P$.

could therefore be stopped by addition of trichloroacetic acid.

*Identification of Products of Transfer Reaction.* To separate the products of the transfer reaction, the mixture was diluted with water and applied on a Dowex-1 column (1 × 4 cm). All radioactivity was adsorbed and elution was achieved as shown in Figure 8. Samples from each fraction were extracted with ammonium molybdate and isobutyl alcohol-benzene-acetone as described under Experimental Procedures, dried, and counted. The radioactivity was recovered in two peaks; the first was extracted into the organic phase of the extraction mixture and the second into the water phase. The first peak was in the position of $P_i$, the second in the position of inorganic pyrophosphate. For positive identification of the product, samples were exposed to inorganic pyrophosphatase. This rendered all the counts extractable into the organic phase of the extraction mixture. This conversion by the enzyme was markedly inhibited by addition of inorganic pyrophosphate. With [$\gamma$-$^{32}P$]ATP and $P_i$ the product of the transfer reaction was also identified as radioactive pyrophosphate. In this experiment the mitochondrial ATPase ($F_1$)[1] was used to hydrolyze the remaining ATP. It can be seen from Table I that the counts that have disappeared from the ATP fraction were recovered in the pyrophosphate fraction as shown by the action of pyrophosphatase. The fact that $F_1$ hydrolyzed the remaining ATP suggests that the reaction conditions did not modify the ATP even after 6 hr of incubation.

Pyrophosphate formation also occurred when CTP, GTP, or ITP were used instead of ATP. With ADP only 2% of the rate with ATP was observed. Moreover, addition of ADP to a reaction mixture containing ATP resulted in an inhibition of pyrophosphate formation.

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[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: $F_1$, mitochondrial ATPase; $CF_1$, spinach chloroplast coupling factor.

TABLE I: Recovery of ATP and Pyrophosphate after Prolonged Incubation in 66% $Me_2SO$.[a]

| | μmol |
|---|---|
| ATP added | 5.0 |
| ATP after 6 hr of incubation | 3.98 |
| Pyrophosphate formed after 6 hr of incubation | 1.08 |

[a] The reaction mixture in a final volume of 0.5 ml of aqueous solution contained: 5 μmol of ATP containing about 100,000 cpm of [$\gamma$-$^{32}$P]ATP, 5 μmol of $MgCl_2$, 20 μmol of Tricine-maleate (pH 8), and 2.5 μmol of $P_i$ (pH 8). After addition of 1 ml of $Me_2SO$ the solution was incubated for 6 hr at 37°. Samples of 0.1 ml were removed and diluted to 1 ml by 20 mM Tricine and incubated for 10 min at 37° in the presence of Tris-maleate (pH 8) with either 5 μg of inorganic pyrophosphatase or 10 μg of $F_1$. Pyrophosphate and ATP were calculated from the counts released by the above enzymes.

The same reaction was observed with other organic solvents such as dioxane, tetrahydrofuran, formamide, *N,N*-dimethylformamide, and hexamethylphosphoramide; however, $Me_2SO$ was the most effective solvent under the experimental conditions described.

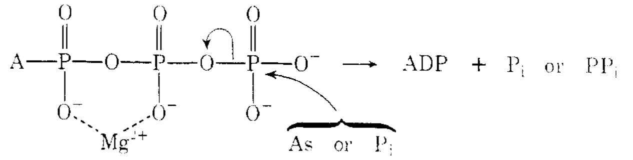
## Discussion

We have presented data on ATP hydrolysis and phosphate transfer reactions which take place in a 30% aqueous solution in the absence of enzymes. Since the reactions occur in different organic solvents of different chemical composition, it is unlikely that the solvent itself participates in the reaction by forming a chemical intermediate with one of the reagents. It is more likely that the solvent provides an appropriate hydrophobic environment for the reaction. Six possible mechanisms for phosphate transfer from ATP are mentioned by Jencks (1969).

The fact that ADP failed to replace ATP might suggest that $Mg^{2+}$ ion forms a complex with the $\alpha$ and $\beta$ phosphates of ATP, leaving the $\gamma$ phosphate available for a nucleophilic attack by arsenate or inorganic phosphate. The arsenate–phosphate bond which might be formed as a result of such attack should be unstable thus giving rise to ATP hydrolysis. When $P_i$ makes a nucleophilic attack on the $\gamma$ phosphate of ATP, pyrophosphate is formed.

The role of the dicarboxylic acid is not understood. It may be participating in a general base catalysis. The importance of general base catalysis in $Me_2SO$ solution was recently pointed out by Margolin and Long (1972). Lowenstein (1958b) noted formation of hydroxamic acid from carboxylic acid and ATP in the presence of $Mn^{2+}$ in aqueous solutions. It is possible that a similar reaction is involved in 70% $Me_2SO$, but we also consider the possibility that ATP might be surrounded by water molecules separating it from the hydrophobic phase of the solvent mixture. The organic acid might facilitate the removal of the protons formed during the reaction from the aqueous phase of the nucleotide molecule into the hydrophobic phase. This suggestion is consistent with the fact that protonated maleate is more soluble in $Me_2SO$ than the charged molecule.

The suggested mechanism can be summarized as follows·



The model system described in this paper seems attractive for the following reasons. (1) The reaction takes place in 30% aqueous solution which mimics the hydrophobic environment of the active center of $F_1$ or $CF_1$ enzymes. (2) The reaction is dependent on divalent cations which is one of the main features of the catalysis of phosphate transfer reactions in mitochondria and chloroplasts. (3) The effect of arsenate in the model system resembles the uncoupling effect of arsenate in chloroplasts and mitochondria. (4) The effect of dicarboxylic acids in the model system is remarkably similar to their effect on the $Mg^{2+}$-dependent ATPase activity of $CF_1$ (Nelson *et al.*, 1972). (5) If the organic acids are indeed involved in the removal of protons formed during the reaction, this system might serve as a model for the chemiosmotic hypothesis (Mitchell, 1966) which suggests a similar movement of protons through the hydrophobic membrane.

The model reactions we have presented here suggest the existence of phosphate or nucleotide phosphate in the active site of $CF_1$ and $F_1$, which facilitates the catalysis of their ATPase and phosphate transfer activities. Bound nucleotides were indeed reported to be present in isolated $CF_1$ and $F_1$ (Roy and Mondrianakis, 1971; Zalkin *et al.*, 1965).

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