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## The Effect of Bivalent Metal Ions on the Hydrolysis of Adenosine Di- and Triphosphate\*

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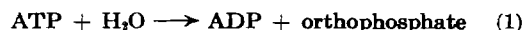
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Many bivalent metal ions catalyze the hydrolysis of adenosine triphosphate (ATP) at pH 5 and 9. In the presence of  $\text{Cu}^{2+}$  and  $\text{Zn}^{2+}$  ions, the rate of hydrolysis of ATP passes through an optimum at pH 5. These ions increase the rate of hydrolysis of ATP about 60-fold and 12-fold, respectively, at pH 5, compared to the rate of hydrolysis in the absence of bivalent metal ions. The rate of hydrolysis of adenosine diphosphate (ADP) is scarcely affected by many bivalent metal ions at pH 5 but is accelerated by most bivalent metal ions at pH 9.  $\text{Cu}^{2+}$  and  $\text{Zn}^{2+}$  ions increase the rate of hydrolysis of ADP about 9-fold and 5-fold, respectively, at pH 9. The effect of  $\text{Mn}^{2+}$  ions on both ATP and ADP hydrolysis is markedly different from that of  $\text{Cu}^{2+}$  and  $\text{Zn}^{2+}$  ions. In the hydrolysis of ATP, the amount of inorganic pyrophosphate formed in the presence of bivalent metal ions is negligible at pH 5. It is of minor importance at pH 9, except in the presence of  $\text{Ca}^{2+}$  ions, in which case about one third of the orthophosphate plus pyrophosphate formed is pyrophosphate. It is proposed that the metal ion specificities of ADP and ATP hydrolysis are the consequence of different types of chelates which are formed with different bivalent metal ions and of different reaction mechanisms which operate with different bivalent metal ions.

Bivalent metals catalyze the nonenzymatic transfer of phosphate from adenosine triphosphate (ATP) to various acceptors such as orthophosphate, acetate, glycine, and  $\beta$ -alanine (Lowenstein, 1958a,b; Lowenstein and Schatz, 1961). When orthophosphate is the phosphate acceptor, the product of the reaction is pyrophosphate and the most effective bivalent metal ions are Ca, Sr, and Ba, followed in decreasing order of effectiveness by Mn, Cd, and Zn. The pH optimum of the reaction is 9.0 (Lowenstein, 1958a, 1960). When acetate is used as acceptor in the presence of hydroxylamine, the product of the reaction is aceto-hydroxamate and the most effective bivalent metal ions are, in decreasing order of effectiveness, Be, Ni, Co, Zn, and Mn. The pH optimum of the reaction is 5.2 (Lowenstein and Schatz, 1961). Similarly, with glycine, the product of the reaction is glycine hydroxamate and the most effective bivalent metal ions are, in decreasing order of effectiveness, Be, Zn, Mn, Ca, and Cd. The pH optimum of this reaction is 5.0. Various studies of the effect of Mg, Ca, and Ba ions on the hydrolysis of ATP and adenosine diphosphate (ADP) have been reported (Lohmann, 1932; Spicer, 1952; Hock and Huber, 1956; Nanninga, 1957; Liébecq and Jacquemotte-Louis, 1958a,b; Liébecq, 1959; Blum and Felauer, 1959; Lipkin *et al.*, 1959). The effect of ten different bivalent metal ions at pH 8.5 was compared by Liébecq and Jacquemotte-Louis (1958a). Of particular interest was a report which dealt with the complex degradation of ATP in saturated barium

hydroxide. It was shown that one of the many products of this degradation is adenosine-3':5'-cyclic monophosphate (Lipkin *et al.*, 1959). Lastly, the hydrolysis of phosphate esters, including ATP, is also catalyzed by alkaline gels of  $\text{Ce}^{3+}$  and other trivalent ions (Bamann *et al.*, 1954; Bamann and Trapmann, 1959).

The present paper deals with a study of the bivalent metal ion specificity of the hydrolysis of the phosphoric anhydride linkages of ATP and ADP. The hydrolysis of ATP leads to ADP and AMP, ADP being formed by reaction (1) and AMP by reactions (2) and (3). The hydrolysis of ADP leads to AMP by reaction (3). De-



tailed analyses of the reaction products are presented, and it is shown that some bivalent metal ions exert an optimum effect at pH 5. At this pH the hydrolysis of ATP in the presence of copper ions is fifty-seven times faster than the hydrolysis of ATP in the absence of bivalent metal ions.

### METHODS

**Measurement of ADP and AMP Formed by Hydrolysis.**—The reaction times used were such that the rates of hydrolysis were proportional to time. The reaction was stopped by adding 9 ml of ice-cold water containing 40  $\mu$ moles disodium ethylenediamine tetraacetic acid (EDTA) to the reaction mixture. The resulting solution was adjusted to a pH of 8–9 by the addition of 2 N NaOH and was then poured on an ion-exchange column which contained the chloride salt of Dowex-1 resin, 200–400 mesh, 8% cross-linked, with a resin bed 1 cm in diameter and 16 cm in height. The adenine nucleo-

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tides in the mixture were separated by eluting the columns as follows (modified from Cohn, 1954). After the diluted reaction mixture had soaked into the resin, the column was washed with 15 ml of water. The combined eluates formed fraction 1 (adenine and adenosine eluted). The column was then eluted with 25-ml fractions of the following composition: fraction 2, water; fractions 3-15, 0.003 N HCl (adenosine monophosphates, including AM-3':5'-cyclic P, eluted); fractions 16-29, 0.01 M NaCl-0.01 N HCl (ADP eluted); fractions 30-39, 0.1 M NaCl-0.01 N HCl (ATP eluted). The amounts of ADP, AMP, adenosine, and adenine formed were calculated from the absorbance at 260 m $\mu$  of each fraction containing these substances. The amounts of adenine and adenosine formed during the reaction were negligible in all experiments reported here. The amounts of ATP that disappeared were essentially equal to the amounts of AMP plus ADP formed.

**Orthophosphate.**—This was measured by the method of Fiske and Subbarow (1925), except that monomethylparaaminophenol sulfate ("Elon," Eastman Kodak Co.) was used in place of aminonaphthol sulfonic acid.

**Pyrophosphate.**—The reaction was stopped by adding 5 ml of ice-cold 0.03 N HCl. The solution was then treated with 300 mg Nuchar charcoal (Crane and Lipmann, 1953), and allowed to stand on ice with occasional stirring for 10 minutes. After the solution was filtered its absorbance was measured at 260 m $\mu$ . If the absorbance was less than 0.060, the solution was considered to be sufficiently free of adenine nucleotides for pyrophosphate and orthophosphate measurements. If the optical density was higher than this value, which rarely was the case, the charcoal treatment was repeated. Controls showed that orthophosphate and pyrophosphate were not lost by this procedure. Aliquots of the filtrate were analyzed for orthophosphate before and after hydrolysis with 2.5 N H<sub>2</sub>SO<sub>4</sub> at 100° for 20 minutes. The difference between the values obtained before and after hydrolysis divided by two yielded the amount of pyrophosphate formed.

**pH Measurements.**—The values quoted were measured at 80° before the incubation. In representative experiments measurements were made both before and after the incubation. The pH changes observed were negligible below pH 7 but were appreciable ( $\Delta$  pH = -0.2 to -0.3) in the case of Cu<sup>2+</sup> and Zn<sup>2+</sup> and ATP in the range pH 7 to 9. In these cases average pH values are quoted.

**Paper Chromatography of Adenosine Phosphates.**—The system *n*-propanol-25% ammonia-water (6:3:1 by volume) was used (Hock and Huber, 1956). The distances traveled by various substances relative to AM-5'-P were: ATP, 0.23; ADP, 0.50; AM-3':5'-cyclic P, 3.1.

**Materials.**—Nucleotides were purchased from the Sigma Chemical Co., St. Louis, Mo.

## RESULTS

The present study was confined to a range of hydrogen ion concentrations between about pH 3 and 9, which covers the values which are of physiological interest. Unless otherwise indicated, experiments were performed in homogeneous solution.

**The Bivalent Metal Ion Specificity of ATP Hydrolysis at pH 5.**—With the exception of Ba<sup>2+</sup> and Mg<sup>2+</sup>, the bivalent metal ions tested accelerate the rate of hydrolysis of ATP as compared to the control containing no bivalent metal ions (Table I). The order of effectiveness is Cu<sup>2+</sup> > Zn<sup>2+</sup> > Cd<sup>2+</sup> > Mn<sup>2+</sup> > Be<sup>2+</sup>. There

TABLE I

### BIVALENT METAL ION SPECIFICITY OF ATP HYDROLYSIS AT pH 5

Each tube contained 20 mM ATP (sodium salt), 20 mM bivalent metal chloride, and 100 mM acetic acid-sodium acetate buffer, pH 5.07. In the case of the tube containing Cu<sup>2+</sup>, the pH was 5.3. Final volume 1.0 ml, temperature 80°. Time of incubation: no asterisk, 1 hour, one asterisk, 15 minutes, and two asterisks, 5 minutes. The ATP used contained 1.27% ADP and 0.028% AMP. These respective amounts have been subtracted from the amounts of ADP and AMP found after hydrolysis.

Metal	AMP Formed	ADP Formed ( $\mu$ moles/hour)	AMP + ADP Formed
Ba	0.24	2.04	2.28
Mg	0.24	2.22	2.46
None	0.25	2.49	2.74
Hg	0.21	2.68	2.89
Sr*	0.24	3.76	4.00
Co	0.32	4.7	5.02
Ca	0.22	5.0	5.22
Ni	0.49	4.9	5.39
Be	0.62	6.3	6.92
Mn	0.49	7.92	8.41
Cd*	0.26	13.64	13.90
Zn*	2.10	30.90	33.0
Cu**	17.4	138.6	156.0

is no obvious relationship between the effectiveness of a metal and its ionic radius. The ratio (ADP formed/AMP formed) for the four most effective ions is 8.0, 14.8, 52.5, and 16.2, respectively. For the other metal ions this ratio ranges between 8.5 and 23, and in the absence of bivalent metal ions the ratio is 10. These ratios show that different metal ions are responsible for large differences not only in the rates of hydrolysis but also in the relative amounts of products formed. As is shown subsequently, very little pyrophosphate is formed at pH 5. The differences in the ratio (ADP formed/AMP formed) which are observed with different metal ions are therefore primarily due to differences in the rates of hydrolysis of ADP as compared to ATP. The ratio (ADP formed/AMP formed) is exceptionally high in the case of Cd<sup>2+</sup> ions (52.5), which implies that these ions are comparatively much less effective with ADP than with ATP. This is borne out in experiments with ADP which are reported later (Table IV).

**The Bivalent Metal Ion Specificity of ATP Hydrolysis at pH 9.**—At this pH all of the bivalent metal ions tested accelerate the rate of hydrolysis of ATP as compared to a control containing no bivalent metal ions (Table II). The order of effectiveness is Ca<sup>2+</sup> > Mn<sup>2+</sup> > Cu<sup>2+</sup> > Cd<sup>2+</sup> > Zn<sup>2+</sup> > Co<sup>2+</sup>. The maximum rate of hydrolysis which was obtained in the presence of Ca<sup>2+</sup> ions is only one twenty-eighth of the maximum at pH 5 in the presence of Cu<sup>2+</sup> ions. This fact, as well as a more detailed comparison of the results shown in Tables I and II, contradicts the view that in general bivalent metal ions accelerate the rate of hydrolysis of ATP in the alkaline pH range and diminish the rate in the acidic pH range. The ratio (ADP formed/AMP formed) is highest (39) for the control containing no bivalent metal ions. A comparison of Tables II and IV shows that some of the metal ions tested at pH 9 are less effective with ATP than with ADP, although the differences are not large. Under the conditions described in Table II, some metal ions gave light precipitates toward the end of the incubation. The order of effectiveness reported by Liébecq and Jacquemotte-Louis (1958a) in the

TABLE II

## BIVALENT METAL ION SPECIFICITY OF ATP HYDROLYSIS AT pH 9

Each tube contained 20 mM ATP (diethanolamine salt), 20 mM bivalent metal ion chloride, and 100 mM diethanolamine-HCl buffer, pH 8.95. Final volume 1.0 ml, temperature 80°, time of incubation 2 hours. The results show the averages of two experiments. The ATP used contained 3.8% ADP and 2.0% AMP, except in the case of results marked by an asterisk, when the ATP used contained 2.13% ADP and 0.17% AMP. These respective amounts were subtracted from the amounts of ADP and AMP found after hydrolysis.

Metal	AMP Formed	ADP Formed ( $\mu$ moles/hour)	AMP + ADP Formed
None	0.015	0.58	0.59
Ba	0.045	0.78	0.82
Mg	0.045	1.05	1.10
Be	0.45	1.43	1.88
Hg*	0.12	1.87	1.99
Co*	0.34	2.85	3.19
Zn	0.93	2.40	3.33
Cd*	0.45	2.99	3.44
Cu*	0.95	3.05	4.00
Mn	0.78	4.35	5.13
Ca	0.55	5.03	5.58

presence of ethanolamine buffer at pH 8.5 and 80° was  $\text{Zn}^{2+} > \text{Ca}^{2+} > \text{Co}^{2+} > \text{Mn}^{2+} > \text{Fe}^{2+} > \text{Cu}^{2+} > \text{Mg}^{2+} > \text{Ba}^{2+} > \text{Be}^{2+}$ . The discrepancy between their work and ours is probably explicable as follows. Both pH 8.5 and pH 9.0 are in the region where the curve relating pH and rate of hydrolysis takes a sharp turn upward. Also, ethanolamine can be expected to bind different metal ions to different extents than diethanolamine.

In some hydrolysis experiments run at pH 9 the adenosine monophosphate fraction obtained from the ion-exchange column was neutralized, freeze-dried, and dissolved in a small amount of water. Paper chromatograms of the resulting solution revealed a single spot which possessed an  $R_f$  value identical to AM-5'-P. No spot corresponding to AM-3':5'-cyclic P could be detected.

*The Effect of Different Bivalent Metal Ions on the Amounts of Ortho- and Pyrophosphate Formed from ATP at pH 5 and 9.*—Negligible amounts of pyrophosphate are formed when ATP is incubated with various bivalent metal ions at pH 5. Control experiments in which pyrophosphate was incubated with each bivalent metal ion showed that the rate of hydrolysis of pyrophosphate at pH 5 is very slow compared to the rate of hydrolysis of ATP at pH 5. The amounts of ortho- and pyrophosphate obtained from ATP at pH 9 are shown in Table III. Pyrophosphate is formed to a relatively small extent with all bivalent metal ions tested. An exception is  $\text{Ca}^{2+}$  ions, in the presence of which 31% of the (orthophosphate + pyrophosphate) formed is pyrophosphate. Under the same conditions,  $\text{Ba}^{2+}$  ions yield little, if any, pyrophosphate. It was impractical to run control experiments to check the extent of hydrolysis of pyrophosphate in the presence of each bivalent metal ion at pH 9 because heavy precipitates were obtained on mixture of the solution of metal ion and pyrophosphate at this pH. It is possible therefore that in the presence of some bivalent metal ions the pyrophosphate which was formed initially was hydrolyzed further to orthophosphate. Considerably more variation was encountered from experiment to experiment when the ratio (orthophosphate formed/

TABLE III

## THE EFFECT OF DIFFERENT BIVALENT METAL IONS ON ORTHO- AND PYROPHOSPHATE FORMATION FROM ATP AT pH 9

The conditions were as for Table II. The results show the averages of three experiments. The ATP used contained 0.50  $\mu$ moles of orthophosphate and 0.00  $\mu$ moles of pyrophosphate. The results have been corrected for the orthophosphate present initially.

Metal	Ortho-phosphate Formed	Pyro-phosphate Formed ( $\mu$ moles/hour)	Ortho-phosphate plus Pyro-phosphate Formed
None	0.59	0.015	0.61
Ba	0.86	0.06	0.92
Ni	1.13	0.015	1.15
Hg	1.19	0.12	1.31
Mg	1.48	0.05	1.53
Cu	1.84	0.13	1.97
Co	2.01	0.07	2.08
Be	2.45	0.29	2.74
Zn	2.58	0.24	2.82
Cd	3.01	0.31	3.32
Ca	2.68	1.19	3.87
Mn	4.16	0.20	4.36

pyrophosphate formed) was measured than was observed in other experiments when the ratio (ADP formed/AMP formed) was measured.

*The Bivalent Metal Ion Specificity of ADP Hydrolysis at pH 5 and 9.*—About half of the metal ions tested exert very little effect on the hydrolysis of ADP at pH 5 (Table IV). The most effective bivalent metal ions are  $\text{Cu}^{2+}$  and  $\text{Zn}^{2+}$ , which are also the most effective with ATP at pH 5 (Table I). The least effective bivalent metal ions are  $\text{Cd}^{2+}$  and  $\text{Mn}^{2+}$ . These are the third and fourth most effective ions in the hydrolysis of ATP at pH 5.

TABLE IV

## BIVALENT METAL ION SPECIFICITY OF ADP HYDROLYSIS AT pH 5 AND 9

Each tube contained: 20 mM sodium ADP, 20 mM bivalent metal ion chloride, and 100 mM acetic acid-sodium acetate buffer, pH 5.07, or 100 mM diethanolamine-HCl buffer, pH 8.95. Final volume 1.0 ml, temperature 80°. The time of incubation for the experiment at pH 5 was one hour, or, in the case of values marked with an asterisk, 30 minutes. The results shown are the averages of two experiments. The ADP used contained 3.7% AMP. The time of incubation for the experiment at pH 9 was 2 hours. The results shown are the averages of two experiments. The ADP used contained 0.86% AMP. The results have been corrected for the AMP present initially.

Metal	AMP Formed at pH 5 ( $\mu$ moles/hour)	AMP Formed at pH 9
Mn	0.57	2.78
Cd	0.86*	2.93
Mg	1.08	0.91
Ni	1.16	2.13
Ba	1.79	2.84
Co	1.82	2.73
Ca	1.84	2.66
None	1.87	0.72
Sr	1.91	—
Hg	2.2	—
Be	3.9	2.12
Zn	11.68*	3.91
Cu	23.4*	6.23

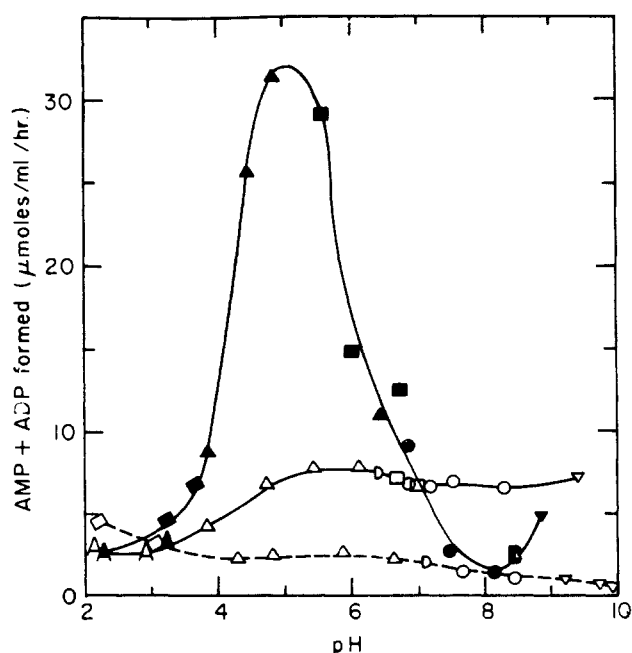


FIG. 1.—The effect of pH on the hydrolysis of ATP in the presence and absence of  $Mn^{2+}$  or  $Zn^{2+}$  ions. Each tube contained 20 mM ATP (sodium salt), 20 mM bivalent metal chloride, and 100 mM buffer, as follows:  $\nabla$ , diethanolamine; B, borate;  $\circ$ , Tris; D, imidazole;  $\square$ , dimethylglutarate;  $\triangle$ , acetate; A, alanine; and  $\diamond$ , formate. Solid symbols for  $Zn^{2+}$ , hollow symbols and solid line for  $Mn^{2+}$ , hollow symbols and broken line for absence of bivalent metal ion. Final volume 1.0 ml, temperature  $80^\circ$ , time of incubation 1 hour for points less than 6  $\mu$ moles/hour, 30 minutes for points between 6 and 15  $\mu$ moles/hour, and 10 minutes for remaining points.

All of the ions tested accelerate the hydrolysis of ADP at pH 9 (Table IV). Precipitates were formed in all cases. The order of effectiveness is  $Cu^{2+} > Zn^{2+} > Cd^{2+} > Ba^{2+} > Mn^{2+}$ . Thus  $Cu^{2+}$  and  $Zn^{2+}$  ions are the most effective ions in the case of ADP and ATP hydrolysis at pH 5 and of ADP, but not of ATP, hydrolysis at pH 9.

**The Effect of pH on the Rate of Hydrolysis of ATP.**—In the absence of bivalent metal ions the rate of ATP hydrolysis decreases with increase in pH between pH 2.2 and pH 9.6. The curve (Fig. 1) shows a shoulder near pH 6. A similar curve has been reported by Liébecq and Jacquemotte-Louis (1958a). Compared to this control, the presence of  $Mn^{2+}$  ions retards the rate of hydrolysis of ATP between pH 2.2 and pH 3.3 (Fig. 1). There is a broad maximum at about pH 5.5–6. Above pH 6 the rate drops off slightly and then remains approximately level to pH 9.5. Compared to the control, the presence of  $Zn^{2+}$  ions retards the rate of hydrolysis between pH 2.2 and 3. There is a rapid increase in the rate of hydrolysis above pH 3, and there is a pH optimum at pH 5.0 (Fig. 1). In the presence of  $Cu^{2+}$  ions there is an extremely sharp pH optimum at pH 5.4 (Fig. 2). The pH profiles obtained with  $Zn^{2+}$  or  $Cu^{2+}$  ions show that the rates of hydrolysis at pH 8 are less than those at pH 3.5, in marked contrast to the behavior observed with  $Mn^{2+}$  ions.

**The Effect of Bivalent Metal Ion Concentration on the Hydrolysis of ATP at pH 5 and 9.**—The influence of the concentration of  $Cu^{2+}$  and  $Mn^{2+}$  ions on the rate of hydrolysis of ATP at pH 5 is shown in Figure 3. An optimum concentration of either bivalent metal ion was not reached before precipitation occurred. Nevertheless the figure shows that the maximum rate

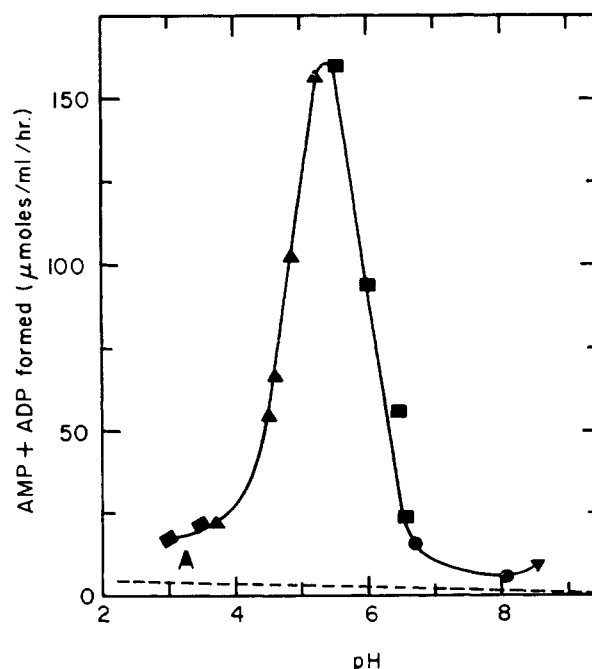


FIG. 2.—The effect of pH on the hydrolysis of ATP in the presence of  $Cu^{2+}$  ions. Conditions were as for Figure 1, except that the time of incubation was 5 minutes for points above 50  $\mu$ moles/hour. The broken line, which is reproduced from Figure 1, shows the hydrolysis of ATP in the absence of bivalent metal ions.

of hydrolysis is attained when the ATP/metal ion ratio is less than 1. Similar considerations apply to the influence of the concentration of  $Ca^{2+}$  and  $Mn^{2+}$  ions on the rate of hydrolysis of ATP at pH 9 (Fig. 4). With increasing metal ion concentration a point is reached where very light precipitates form during the second hour of incubation. At higher metal ion concentrations precipitates form sooner and become heavier. The precipitates presumably consist of metal phosphates, hydroxides, and carbonates.

**The Effect of pH on the Rate of Hydrolysis of ADP.**—In the absence of bivalent metal ions the rate of ADP hydrolysis decreases with increase in pH between pH 3.6 and pH 9.4. Compared to this control, the presence of  $Mn^{2+}$  ions retards the rate of hydrolysis between pH 3.7 and pH 7.3. There is a rapid increase in the rate of hydrolysis between pH 8 and pH 9.5 (Fig. 5). The maximum in the rate of hydrolysis of ATP plus  $Mn^{2+}$  ions, which occurs at about pH 6, is entirely absent in the case of the hydrolysis of ADP plus  $Mn^{2+}$  ions. The rates of hydrolysis of ADP in the presence of  $Zn^{2+}$  or  $Cu^{2+}$  ions show an optimum at pH 6 (Figs. 5 and 6). In the case of  $Zn^{2+}$  ions, results between pH 5.5 and 7.5 were difficult to reproduce owing to the formation of precipitates. The position of the descending portion of the curve on the alkaline side of the pH optimum is therefore uncertain. In the case of  $Cu^{2+}$  ions the results show that Tris buffer is inhibitory compared to dimethyl glutarate and diethylglutarate buffers and that the inhibition increases with increasing pH.

**The Effect of Concentration of Bivalent Metal Ions on the Hydrolysis of ADP.**—The influence of the concentration of bivalent metal ions on the rate of hydrolysis of ADP at pH 9 was similar to that found for the hydrolysis of ATP at pH 9 (Fig. 4). Again, optimum concentrations of bivalent metal ion were not reached before precipitation occurred.

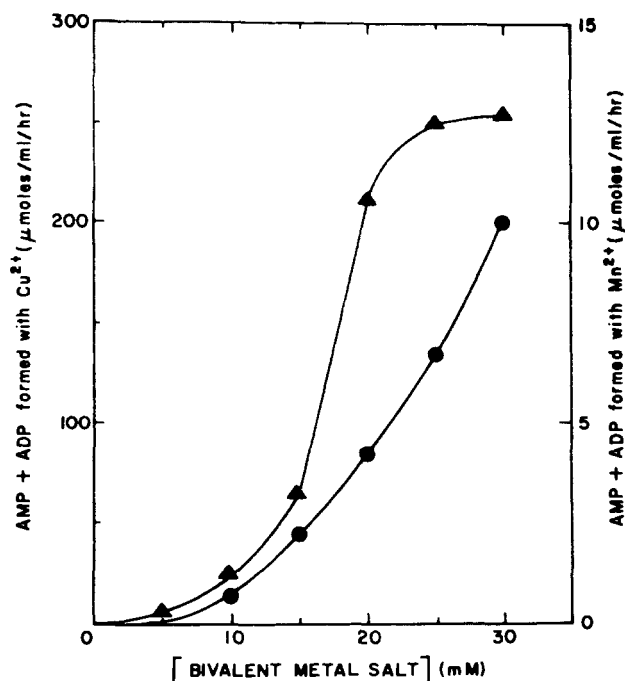


FIG. 3.—Effect of concentration of  $\text{Mn}^{2+}$  and  $\text{Cu}^{2+}$  ions on the hydrolysis of ATP at pH 5.3. Each tube contained 20 mM ATP (sodium salt), 100 mM acetic acid-sodium acetate buffer, pH 5.3, and  $\text{MnSO}_4$  or  $\text{CuSO}_4$  as indicated. Final volume 1.0 ml, temperature  $80^\circ$ , time of incubation 30 minutes for Mn points, 10 minutes for lower Cu points, and 3 minutes for upper Cu points. A control containing no bivalent metal has been subtracted from the values shown for each tube. This amounted to 2.7  $\mu\text{moles}$ . This value consists of the AMP and ADP originally present in the ATP and of the AMP and ADP formed by hydrolysis in the absence of bivalent metal. ●, manganese; ▲, copper.

### DISCUSSION

The effect of some bivalent metal ions on the hydrolysis of ATP can, under suitable conditions, be very large. What do the metal ions that are most effective in catalyzing the hydrolysis of ATP and ADP have in common? The highly effective ions of Cu, Zn, Mn, and Ca are all members of the 4th period of the periodic table. The highly effective ions of Zn and Cd are in group IIB. The ions of Be and Ca, which are highly effective under some conditions and relatively ineffective under others, are in group IIA.  $\text{Mg}^{2+}$  ions, which are conspicuous by their ineffectiveness under all conditions reported here, are also in group IIA. They are of particular interest since they are the ions which are most commonly required in the enzymatic reactions of ATP. The order in which the metals occur in group IIA is Be, Mg, Ca, Sr, Ba. This is also the order of the stability constants of the ATP-metal ion chelates ( $\text{Mg} > \text{Ca} > \text{Sr} > \text{Ba}$ ) (Nanninga, 1961; Taqui Khan and Martell, 1962). However, it is *not* the same as the order of effectiveness of the group IIA metal ions in catalyzing the hydrolysis of ATP and ADP. The stability sequence generally observed with chelates of the bivalent transition metals used in this paper is  $\text{Mn} < \text{Co} < \text{Ni} < \text{Cu} > \text{Zn}$  (Mellor and Maley, 1947, 1948; Irving and Williams, 1949; Williams, 1959). The stability constants of the corresponding ATP-metal ion chelates show the sequence  $\text{Co} < \text{Mn} < \text{Ni} < \text{Cu} > \text{Zn}$  (Brintzinger and Fallab, 1960; Taqui Khan

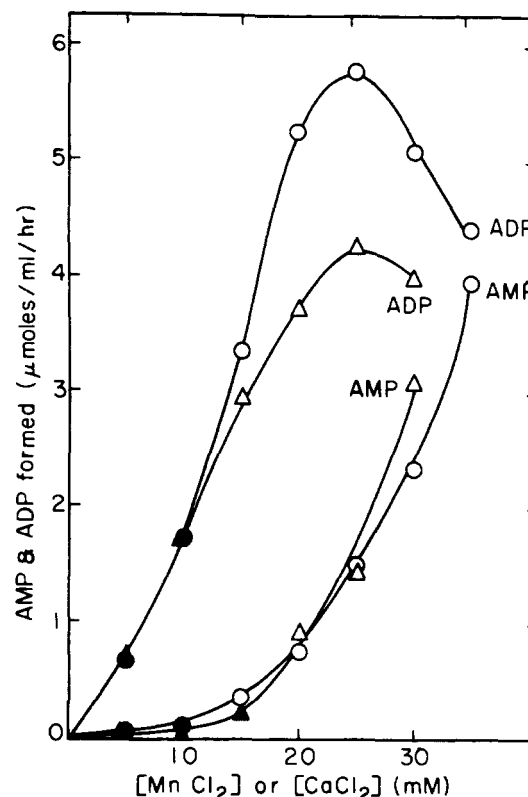


FIG. 4.—Effect of concentration of calcium and manganese ions on hydrolysis of ATP at pH 9. Each tube contained 20 mM diethanolamine salt of ATP (adjusted to pH 8.9 with diethanolamine), 100 mM diethanolamine-HCl buffer, pH 8.95, and  $\text{CaCl}_2$  or  $\text{MnCl}_2$  as indicated. Final volume 1.0 ml, temperature  $80^\circ$ , time of incubation 2 hours. Results with controls containing no bivalent metal chloride have been subtracted from each of the experimental points shown. The controls amounted to 1.21  $\mu\text{moles}$  ADP and 0.16  $\mu\text{moles}$  AMP in the experiment with  $\text{CaCl}_2$ , and 4.79  $\mu\text{moles}$  ADP and 0.61  $\mu\text{moles}$  AMP in the experiment with  $\text{MnCl}_2$ . These values consist of the ADP and AMP originally present in the ATP and of the ADP and AMP formed by hydrolysis in the absence of bivalent metal. The controls differ for the calcium and manganese experiments because different preparations of ATP were used. circles, calcium; triangle, manganese. Solid symbols represent clear solution, hollow symbols represent formation of precipitate during incubation.

and Martell, 1962). It is seen that Mn does not fit the usual sequence. The order of effectiveness in the hydrolysis of ATP and ADP is  $\text{Co} \approx \text{Ni} < \text{Cu} > \text{Zn}$ . Again Mn does not fall into the expected sequence, except in the case of ADP hydrolysis at pH 5. Figures 1 and 5 show that ATP-Mn and ADP-Mn also exhibit atypical pH optimum curves compared to the curves obtained with Cu and Zn.

Let us assume that the active chelate in the hydrolysis of ATP and ADP is that which predominates in solution, namely, the chelate containing 1 molecule of ATP and 1 metal ion (Bock, 1960; Taqui Khan and Martell, 1962). A 1:1 chelate of ATP and bivalent metal ion may have a number of different structures (Brintzinger, 1961a,b; Cohn, 1954; Liébecq and Jacquemotte-Louis, 1958a; Lowenstein, 1958a; Melchior, 1954). Measurements of stability constants which have appeared (Bock, 1960; Brintzinger and Fallab, 1960; Nanninga, 1961; Taqui Khan and Martell, 1962) give no indication of the relative extent to which such different structures occur. In most chelates of the type  $(\text{ATP-metal})^{2-}$ , the preferred

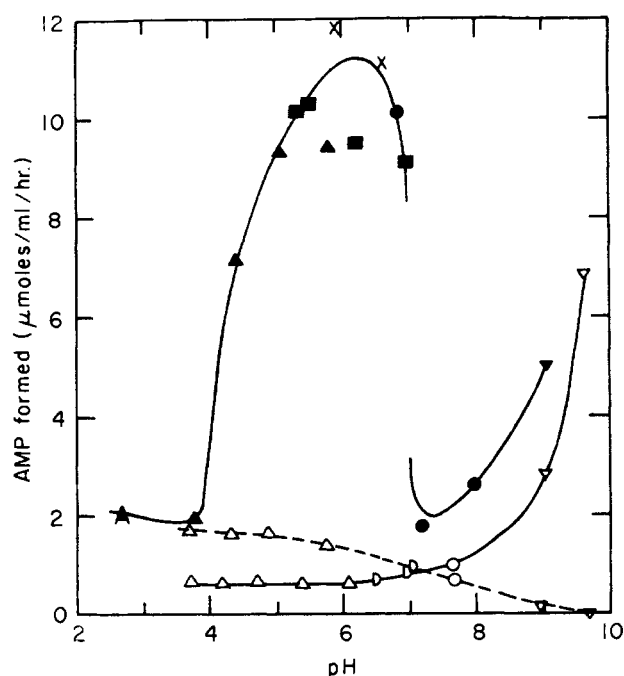


FIG. 5.—The effect of pH on the hydrolysis of ADP in the presence and absence of  $Mn^{2+}$  or  $Zn^{2+}$  ions. Each tube contained 20 mM ADP (sodium salt), 20 mM bivalent metal chloride, and 100 mM buffer. The same symbols are used as in Figure 1. In addition,  $\times$  = diethylglutarate. Final volume 1.0 ml, temperature  $80^\circ$ , time of incubation 1 hour for points less than 6  $\mu$ moles and 20 minutes for the remaining points.

structure may be A (Fig. 7), since the attraction exerted on the positively charged metal ion by the two negative charges of the  $\gamma$ -phosphate is much stronger than that exerted by the single negative charge on the  $\alpha$ -phosphate. In chelates which have one negative charge less,  $(H-ATP-metal)^-$ , the extra hydrogen is presumably on the  $\gamma$ -phosphate. Each phosphate now carries a single negative charge, and this may not favor any single chelate structure. In other words, it makes more readily possible a structure such as B (Fig. 7). Using nuclear magnetic resonance spectroscopy, Cohn and Hughes (1960, 1962) have shown that the ions of Mg, Ca, and Zn bind predominantly to the  $\beta$ - and  $\gamma$ -phosphates of ATP, Cu binds predominantly to the  $\alpha$ - and  $\beta$ -phosphates, and Mn binds to all three (but not necessarily at the same time). However, they point out that small proportions of other chelate isomers could not have been detected. Another factor that will influence the structure of the chelate is the interaction of the metal ion with the adenosine moiety of the molecule. This is much more likely in the case of the transition metals than with the group IIA metals. Cohn and Hughes (1962) also present evidence that Cu, Zn, and Mn interact with the adenine ring of ATP, whereas Mg and Ca do not. The lack of interaction of Mg with the adenine moiety of ATP had previously been demonstrated by Hammes *et al.* (1961). Lastly, atomic models<sup>1</sup> show that a structure in which the metal ion is chelated to all three phosphates of ATP simultaneously is possible without strain for metal ions with an ionic radius greater than 0.5 Å. The correlation which can be derived from these considerations is that the ions which are particularly active in catalyzing the hydrolysis of ATP interact with the

<sup>1</sup> Courtauld atomic models, obtained from The Ealing Corporation, Cambridge, Mass.

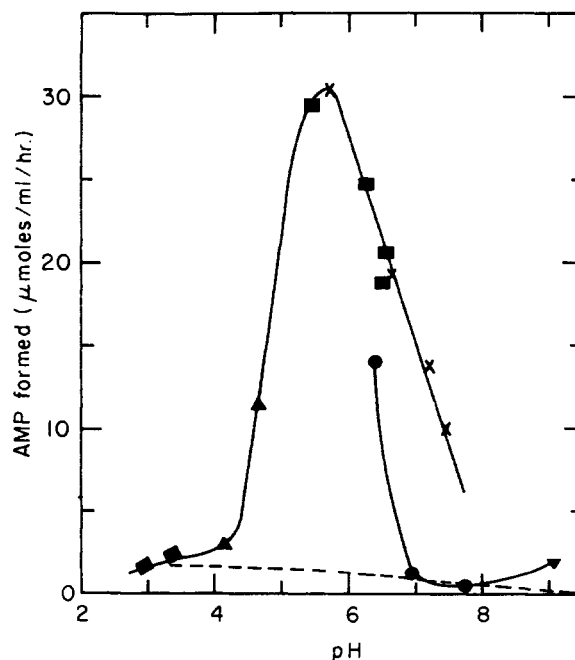


FIG. 6.—The effect of pH on the hydrolysis of ADP in the presence of  $Cu^{2+}$  ions. Conditions were as for Figure 5, except that the time of incubation was 10 minutes for points above 20  $\mu$ moles. The broken line, which is reproduced from Figure 5, shows the hydrolysis of ADP in the absence of bivalent metal ions.

adenine ring system (Cu, Zn, Mn) or do not fit into the expected order of stability constants (Mn).

Specific mechanisms will now be discussed. Nucleophilic attack by water on the  $\gamma$ -phosphate of the ATP-chelate A is very similar to attack by water on the  $\beta$ -phosphate of the ADP-chelate D (Fig. 7). However, mechanisms of hydrolysis are available to the chelate with ATP which are not available to the chelate with ADP. For example, an attack by water on the  $\gamma$ -phosphate or  $\beta$ -phosphate of ATP-chelate B (Fig. 7, B1 or B2) is different from any attack which can be formulated for the ADP-chelate D. To account for the large differences in the rates of hydrolysis of the  $Cu^{2+}$  chelates of ATP and ADP it may be postulated that ATP-Cu is hydrolyzed by mechanisms B1 or B2, which are very different from mechanism D, and that it is not hydrolyzed by mechanism A, which is very similar to mechanism D. Mechanisms B1 and B2 as formulated in Figure 7 are both bimolecular nucleophilic substitution reactions. It may be argued that of the two mechanisms B1 is the more likely, since in the chelate structure shown the  $\beta$ -phosphate is the more electropositive. An entirely different mechanism may, however, be operative. The curves relating pH and rate of hydrolysis shown in Figures 1 and 2 are very similar to some of the profiles obtained in the laboratories of Vernon and of Westheimer on the effect of pH on the rate of hydrolysis of phosphomonoesters. Among the plausible mechanisms that have been proposed to account for the shape of such curves is an elimination mechanism which leads to the transient formation of the metaphosphate anion (Butcher and Westheimer, 1955; Kumamoto and Westheimer, 1955; Vernon, 1957; Bunton *et al.*, 1958). Considerable support for this type of mechanism has recently been put forward by DiSabato and Jencks (1961). An elimination mechanism for the hydrolysis of the  $\alpha,\beta$ -chelate of ATP is shown in Figure 7E. The  $\alpha,\beta$ -chelate is assumed to be involved in the elimination, since in

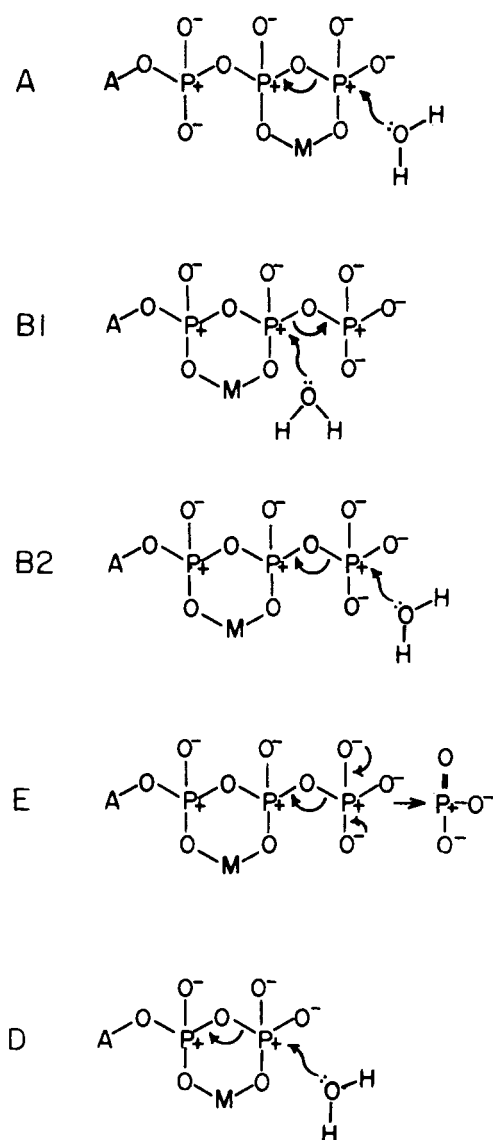


FIG. 7.—Mechanisms to be considered for the hydrolysis of ATP and ADP in the presence of bivalent metal ions.

this structure the leaving group is the  $\alpha,\beta$ -cholate of ADP. This is a much better leaving group than is ADP in the  $\beta,\gamma$ -cholate of ATP. According to this mechanism the descending limb on the acid side of the pH optimum could be attributed to the protonation of the  $\gamma$ -phosphate, which would reduce the negative charge which is the driving force for this group to leave. The  $pK$  values for the ionizations  $(H\text{-ATP-metal})^{1-} \rightarrow (ATP\text{-metal})^{2-}$  and  $(H\text{-ADP-metal})^0 \rightarrow (ADP\text{-metal})^{1-}$  are about 4.6 for the chelates formed with  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  (Martell and Schwarzenbach, 1956). The corresponding  $pK$  values in the presence of  $\text{Cu}^{2+}$ ,  $\text{Zn}^{2+}$ , or  $\text{Mn}^{2+}$  may be expected to fall in the same region.

The decrease in the rates of hydrolysis on the alkaline side of the pH optima for  $\text{Cu}^{2+}$  and  $\text{Zn}^{2+}$  may be due to a number of factors. For example, a shift may occur from the chelate structure that favors ATP hydrolysis to one that does not, a shift, say, from structure B to structure A. Another possibility is that, as the pH rises, water molecules bound to the metal ion of the chelate become replaced by hydroxide ions, and the resulting chelate is less active because of the reduced electron-withdrawing power which the

metal ion now exerts on the polyphosphate chain. In the presence of  $\text{Cu}^{2+}$  or  $\text{Zn}^{2+}$  the rates of hydrolysis of both ATP and ADP reach a minimum near pH 8, which is followed once more by an increase in the rates of hydrolysis above pH 8. The increase is probably a reflection of the increasing importance of nucleophilic attack by hydroxide ions. This is analogous to the nucleophilic attack on ATP by orthophosphate described earlier (Lowenstein, 1958a). The curve relating the pH to the rate of hydrolysis for ATP-Mn does not show a pronounced minimum at pH 8, and the curve for ADP-Mn does not show a maximum between pH 5 and 6. Perhaps the difference between  $\text{Cu}^{2+}$  and  $\text{Zn}^{2+}$  ions on the one hand and  $\text{Mn}^{2+}$  ions on the other may be resolved as follows. In the case of ATP hydrolysis,  $\text{Cu}^{2+}$  and  $\text{Zn}^{2+}$  ions function through an elimination mechanism involving the  $\alpha,\beta$ -cholate of ATP, while  $\text{Mn}^{2+}$  ions function through a nucleophilic attack mechanism involving the  $\beta,\gamma$ -cholate of ATP. In the case of ADP hydrolysis,  $\text{Cu}^{2+}$  and  $\text{Zn}^{2+}$  ions function through a nucleophilic attack mechanism involving the  $\alpha,\beta$ -cholate of ADP, while  $\text{Mn}^{2+}$  ions function by the same mechanism, only much less effectively than  $\text{Cu}^{2+}$  and  $\text{Zn}^{2+}$ .

The formation of pyrophosphate may involve attack by hydroxide, favored by high pH, on the  $\beta$ -phosphate of chelates A or B. The formation of AM-3',5'-cyclic-P described by Lipkin *et al.* (1959) is also favored by high pH. In the present work, the AMP fractions obtained after the hydrolysis of ATP at pH 9 (or 5) showed no detectable amounts of the cyclic phosphate. A pH higher than 9 is necessary to obtain this compound. Lipkin *et al.* (1959) favor a mechanism involving an attack by the 3'-hydroxyl group of ribose on the  $\alpha$ -phosphorus of ATP. It is, however, difficult to see why this mechanism is favored by high pH. A possible explanation is that at higher pH values, say above 12, the hydroxyls of the ribose moiety of ATP ionize. This would greatly facilitate a nucleophilic attack by the 3'-oxygen of ribose on the  $\alpha$ -phosphorus of ATP. An enzymatic counterpart of the nonenzymatic cyclization reaction has been described by Sutherland and Rall (1958).

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## Uncoupling of Oxidative Phosphorylation by Carbonyl Cyanide Phenylhydrazones.

### I. Some Characteristics of *m*-Cl-CCP Action on Mitochondria and Chloroplasts

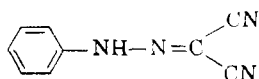
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Received August 14, 1962

Carbonyl cyanide *m*-chlorophenylhydrazone (*m*-Cl-CCP), at concentrations between  $10^{-7}$  and  $10^{-6}$  M, is an effective uncoupler of oxidative phosphorylation in plant, animal, and insect mitochondria. While in some respects its action on mitochondria resembles that of 2,4-dinitrophenol—*e.g.*, apparent ATPase of intact mitochondria is stimulated and swelling of resting mitochondria is inhibited—*m*-Cl-CCP also exhibits several unusual characteristics. It does not stimulate or inhibit purified soluble ATPase from mitochondria; its action is specifically blocked by 1,2- and 1,3-aminothiols; and it strongly inhibits photophosphorylation in chloroplasts. The *m*-Cl compound is representative of the class of CCP uncouplers, which appear to be useful tools in phosphorylation studies.

Preliminary reports from this laboratory (Heytler *et al.*, 1962; Heytler and Prichard, 1962) described a new class of uncoupling agents, ring-substituted phenylhydrazones of carbonyl cyanide.



Carbonyl Cyanide Phenylhydrazone (CCP)

Some of these derivatives are at least as effective as the best antibiotic uncouplers (*e.g.*, gramicidin [Hotchkiss, 1944] or valinomycin [McMurray and Begg, 1959]) and represent the most potent synthetic inhibitors of this type reported. Also, as we reported (*loc. cit.*), these materials block photosynthetic phosphorylation, which is rather resistant to most uncoupling agents. The CCP derivatives, therefore, are promising tools in the study of both these energy-conversion mechanisms, and their mode of action is being studied in greater detail. The present report deals with some characteristics of the *m*-chloro derivative (*m*-Cl-CCP), which has

relatively high uncoupling activity and appears to be qualitatively characteristic of the class.

#### MATERIALS AND METHODS

The carbonyl cyanide phenylhydrazones were prepared by Dr. W. W. Prichard of this laboratory; details will be published shortly. Briefly, the corresponding aniline is diazotized by conventional means, then coupled with malononitrile in mildly basic solution. The yellow product precipitates upon subsequent acidification and is recrystallized from chloroform or benzene. Buffered solutions of the Tris or sodium salt were used routinely.

Oligomycin was the generous gift of Prof. H. A. Lardy.

Other reagents were used as obtained commercially. Enzymes and most biochemicals were purchased from Sigma Chemical Company in highest routinely available grades.

Mitochondria from several sources were prepared by minor modifications of standard differential centrifugation techniques (Schneider and Hogeboom, 1950). Homogenization in 0.25 M sucrose medium was followed by centrifugation at  $1,000 \times g$  for 12 minutes to remove debris and at  $10,000 \times g$  for 20 minutes to sediment

\* Contribution No. 803.