2. Swine pyloric juice also exhibited two proteolytic pH maxima, at pH values 1·6 and 2·6, which are lower than those of swine or human fundic extracts or of human gastric juice.

3. Crystalline swine pepsin can be separated into two components, one of which digested proteins with pH maxima close to those found with swine fundic mucosa and the other with maxima close to those found with pyloric mucosa.

4. It is concluded that in man and the pig there are two main pepsins, one of which predominates in the pyloric mucosa and the other in the mucosa of the fundus and body of the stomach.

I wish to thank Mr Brian Collett and Miss Barbara Hunt for their skilful assistance in part of this work; Dr J. V. O. Reid and Mr A. M. N. Gardner for carrying out early necropsies; Professor H. W. Florey, F.R.S., and Dr N. G. Heatley for providing pig pyloric juice; and Dr R. B. Fisher and Mr J. R. P. O'Brien who read and criticized the D.M. Thesis from which the material of this paper is abridged.

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Formation Constants for the Complexes of Adenosine Di- or Tri-phosphate with Magnesium or Calcium Ions

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The formation of complexes between adenosine phosphates and bivalent metal ions has been quantitatively studied by the effects of the metals on the acid–base titration curves (Smith & Alberty, 1956*a*; Martell & Schwarzenbach, 1956) and by the use of ion-exchange resins (DiStefano & Neuman, 1953; Nanninga, 1957; Walaas, 1957, 1958). These methods have been extensively used to study many chelation complexes but there is poor agreement between the several values that have been reported for the formation constants of the complexes of the adenosine phosphates. Further study of these complexes therefore seemed to be desirable and this paper describes measurements by an independent method in which the spectral changes of 8-hydroxyquinoline are used to determine the amount of free bivalent metal ion present in solutions containing adenosine di- or tri-phosphate and magnesium or calcium ions. For most of the measurements, tributylethylammonium bromide was added to obtain a convenient ionic strength (usually 0·11). The concentration of sodium or potassium ions was usually kept low because these ions form weak complexes with the nucleotides (Melchior, 1954; Smith & Alberty, 1956*b*).

MATERIALS AND METHODS

Nucleotides. The sodium salts of adenosine triphosphate (ATP; Schwarz Laboratories Inc., Mount Vernon, N.Y., U.S.A.) and adenosine diphosphate (ADP; Sigma Chemical Co., St Louis, Mo., U.S.A.) were dissolved in water and

adjusted to the pH required in each experiment by the addition of NaOH. Approximately 2 equivalents were needed. The solutions were standardized spectrophotometrically by the difference between the absorption at 260 mμ and at 290 mμ by using the extinction coefficients of Bock, Ling, Morell & Lipton (1956). No ultraviolet-light absorbing impurities were detected after examination by paper chromatography in the *iso*butyric acid–aq. NH_3 soln. solvent of Krebs & Hems (1953).

8-Hydroxyquinoline. Analytical reagent-grade material was used.

Triethanolamine buffers. Commercial triethanolamine was redistilled under reduced pressure and a stock aqueous solution prepared by weight. Buffers were prepared by adding HCl.

Tributylethylammonium bromide. This was prepared by refluxing tributylamine with a 50 % excess of ethyl bromide for 40 hr. The solid which formed on cooling was filtered off, washed with ether, twice recrystallized from a mixture of *iso*amyl alcohol and ethyl ether and dried to constant weight *in vacuo*.

Magnesium and calcium chlorides. Aqueous solutions of $MgCl_2,6H_2O$ and $CaCl_2$ (analytical-reagent grade) were standardized by titration with $AgNO_3$. Small volumes of the $MgCl_2$ and $CaCl_2$ solutions were measured by an Agla micrometer syringe (Burroughs Wellcome Ltd.).

Spectrophotometry. If the concentration of 8-hydroxyquinoline was less than 0·5 mM, the measurements were made by a Cary recording spectrophotometer with quartz cells of 4·9 cm. light path and containing about 20 ml. of solution. To obtain the highest accuracy, the extinctions were obtained directly from the scale (instead of the recorder chart) and the slit control was set at 0. For higher concentrations of 8-hydroxyquinoline, a Beckman model DU spectrophotometer was used with 1 cm. cells of about 3 ml. capacity. The instrument was fitted with a temperature-controlled cell-holder.

Measurements of pH. The pH values of the reaction mixtures were measured at 20–25° with a glass electrode and calomel half-cell, calibrated according to B.S. 1647:1950.

THEORY

In addition to the 1:1 complex between metal ion (M) and phosphate compound (P) it is desirable to consider the possibility of the M_2P and MP_2 complexes:

$$M+P \rightleftharpoons MP,$$
$$MP+M \rightleftharpoons M_2P,$$
$$MP+P \rightleftharpoons MP_2.$$

Let a be the total concentration of metal, b the total concentration of phosphate compound, m the concentration of free metal ion and p the concentration of free phosphate compound. Let the three formation constants be

$$K_1=[MP]/mp, \quad K_2=[M_2P]/m[MP]$$

and $K_3=[MP_2]/p[MP]$ respectively. Let α be the ratio of total phosphate to bound metal, i.e. $\alpha=b/(a-m)$. Then

$$a-m=[MP]+2[M_2P]+[MP_2]$$
$$=K_1mp(1+2K_2m+K_3p). \quad (1)$$

As $m \to 0$, $p \to b$ and

$$\frac{a-m}{mb} \to K_1(1+K_3b). \quad (1a)$$

Also
$$b=[P]+[MP]+[M_2P]+2[MP_2]$$
$$=p\{1+K_1m(1+K_2m+2K_3p)\}. \quad (2)$$

From (1) and (2)

$$K_1m\{(\alpha-1)+K_2m(2\alpha-1)+K_3p(\alpha-2)\}=1. \quad (3)$$

The version for $\alpha=2$ (i.e. $a-m=\frac{1}{2}b$) is of particular value since it is independent of K_3 and is thus not affected by the possible occurrence of the MP_2 complex:

$$K_1m(1+3K_2m)=1. \quad (3a)$$

If $K_2 \ll K_1$, this becomes

$$K_1m=1. \quad (3b)$$

Consideration of ternary complex between metal, 8-hydroxyquinoline and phosphate compound. Let ϵ_0, ϵ_1 and ϵ_2 be the respective molar-extinction coefficients for the free hydroxyquinoline (O), the metal–hydroxyquinoline complex (MO) and the ternary complex (MOP). Let the formation constants of the two complexes be respectively

$$K'=[MO]/[O]\,m \quad \text{and} \quad K''=[MOP]/[O]\,[MP]$$
$$=[MOP]/K_1[O]\,mp.$$

The increase in extinction (ΔE) observed on adding metal to a mixture containing O and P is

$$\Delta E=(\epsilon_1-\epsilon_0)\,[MO]+(\epsilon_2-\epsilon_0)\,[MOP]$$
$$=[O]\,m\{(\epsilon_1-\epsilon_0)\,K'+(\epsilon_2-\epsilon_0)\,K''pK_1\}. \quad (4)$$

Let m' and o' be the respective apparent concentrations of free Mg^{2+} ion and hydroxyquinoline that would have been deduced from the value of ΔE if the MOP complex were ignored, i.e.

$$\Delta E=o'm'(\epsilon_1-\epsilon_0)\,K'. \quad (5)$$

Assuming that the MOP complex represents only a small proportion of the free hydroxyquinoline, $o' \approx [O]$ and thus from (4) and (5)

$$m'-m=\theta K_1mp, \quad (6)$$

where the constant, θ, is given by

$$\theta=(\epsilon_2-\epsilon_0)\,K''/(\epsilon_1-\epsilon_0)\,K'. \quad (7)$$

From (1), (2) and (6) it may be shown that when

$$a-m'=b/\alpha',$$
$$K_1m(\alpha'-\theta\alpha'-1)+K_2m(2\alpha'-1)+K_3p(\alpha'-2)=1. \quad (8)$$

When $\alpha'=2$ (*i.e.* $a-m'=\frac{1}{2}b$):

$$K_1m\{1-2\theta+K_2m\}=1. \quad (8a)$$

If both θ and $K_2m \ll 1$:

$$K_1m=1. \quad (8b)$$

Thus from (6) and (8*b*):

$$m'=1/K_1+\theta p.$$

By substituting for p from (2), putting $K_2 \ll K_1$ and eliminating m by (8*b*),

$$m'=\frac{1}{K_1}+\frac{\theta\{\sqrt{(1+2K_3b)}-1\}}{2K_3}. \quad (9)$$

If $K_3b \ll 1$:

$$m'=1/K_1+\tfrac{1}{2}\theta b. \quad (9a)$$

When $m \to 0$:

By substituting from (6) for m in (1*a*) and re-arranging and by using the fact that $b=p$ when $m=0$:

$$\frac{m'b}{a-m'}=\frac{1/K_1+\theta b}{1-\theta+K_3b}. \quad (10)$$

If $\theta \ll 1$:

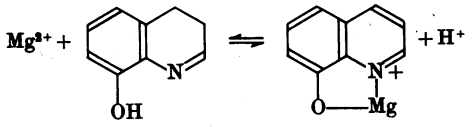
$$\frac{m'b}{a-m'} = \frac{1/K_1 + \theta b}{1 + K_3 b}. \quad (10a)$$

If both θ and $K_3 b \ll 1$:

$$\frac{m'b}{a-m'} = 1/K_1 + \theta b. \quad (10b)$$

RESULTS

Reaction between 8-hydroxyquinoline and magnesium. In Fig. 1 are shown the effects of $MgCl_2$ and of $MgCl_2$ plus ATP on the absorption spectra of 8-hydroxyquinoline in buffered solution at pH 8·85. The spectrum with excess of $MgCl_2$ (20 mM) is essentially that of the 1:1 Mg–hydroxyquinoline complex (cf. Näsänen, 1952). At lower concentrations of $MgCl_2$ the spectra are consistent with their being the result of varying proportions of free hydroxyquinoline and of the 1:1 complex. The greatest change in extinction occurs at about 360 mμ, and the curves relating the increase in extinction to the amounts of $MgCl_2$ are close to those expected for the formation of the 1:1 complexes on the basis of the stability constants reported by Näsänen (1952). More of the magnesium complex is formed at higher pH values because there is an accompanying loss of H^+ ion:



Higher concentrations of free Mg^{2+} ion can therefore be more accurately measured at lower pH values. The pH can be reduced to 7·7 without appreciably affecting the binding of the metal to the adenosine phosphates because the pH values of their secondary phosphate ionizations are 6·95 or less (Smith & Alberty, 1956*b*). The upper limit of pH is set by the dissociation of the 8-hydroxyquinoline (pK 9·9; Näsänen, Lumme & Mukula, 1951) and its accompanying increase of extinction at 360 mμ. The useful range of pH is therefore 7·7–9·1.

In the solutions used, less of the Mg–hydroxyquinoline complex was formed as the temperature was increased. This may have been due mainly to the effect of temperature on the pH of the triethanolamine buffer.

The insoluble 1:2 Mg–hydroxyquinoline complex did not precipitate during the actual experiments but it did precipitate when the solutions containing $MgCl_2$ and mM-hydroxyquinoline at pH 8·4 were subsequently allowed to stand. No precipitate was formed at pH 7·9 or with 0·3 mM-hydroxyquinoline at pH 8·4.

Effects of adenosine tri- or di-phosphate. In the presence of ATP, more $MgCl_2$ has to be added to obtain the same amount of Mg–hydroxyquinoline complex. The spectrum of 8-hydroxyquinoline in the presence of 1·20 mM-$MgCl_2$ plus 0·73 mM-ATP is almost the same as that in the presence of 0·43 mM-$MgCl_2$ (Fig. 1).

In Figs. 2–4, the increase in extinction at 360 mμ has been plotted against the amounts of $MgCl_2$ in the absence and in the presence of ATP or ADP. It is convenient first to consider these curves assuming that the increase in extinction is due solely to the complex between the metal and hydroxyquinoline and that there are no complicating factors such as a ternary complex between the metal, hydroxyquinoline and ATP. The amount of metal bound to the hydroxyquinoline, which is indicated by the shaded area on the left of each graph, was calculated from the readings obtained with high concentrations of metal and 0·1–0·2 mM-8-hydroxyquinoline. For a given extinction the amount of free Mg^{2+} ion is obtained by difference from the total amount of Mg added when no ATP or ADP is present. In the presence of ATP or ADP, the extra amount of metal needed to obtain the same extinction is the amount that is bound by the nucleotide. The values are conveniently obtained directly from the graphs as described in the legend to Fig. 2. The formation constant for MgADP ($K_1 = 1640\,M^{-1}$) can thus be

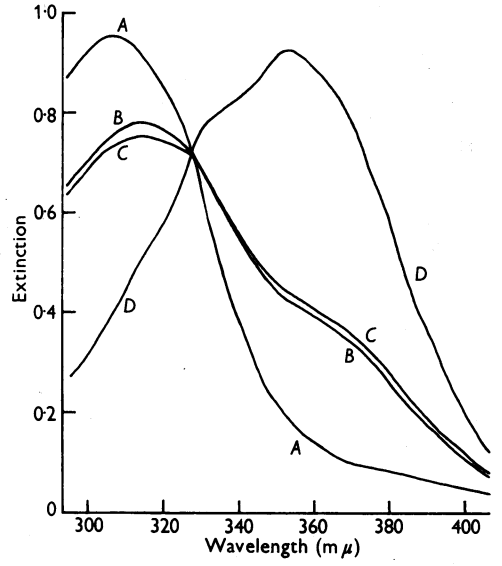


Fig. 1. Absorption spectra of 8-hydroxyquinoline. 0·37 mM-8-Hydroxyquinoline, 0·10 M-tributylethylammonium bromide, 22 mM-NH_4Cl, aq. 10 mM-NH_3 soln., pH 8·85. Cells 4·9 cm. *A*, Alone; *B*, 0·43 mM-$MgCl_2$; *C*, 1·20 mM-$MgCl_2$ plus 0·73 mM-Na_4ATP; *D*, 20 mM-$MgCl_2$.

evaluated with the use of equation 3*b*. The extinction values in Figs. 2–4 have been corrected for the small dilution of the hydroxyquinoline solution by the added $MgCl_2$. In routine measurements it is less laborious to correct for the progressive dilution by drawing the lines such as *AD* of Fig. 2 at the appropriate slight angle to the horizontal.

K_1 can also be evaluated from the initial slopes of the curves of Fig. 2 with the use of equation 1*a* and assuming K_3 to be zero. The value obtained is $1700\,M^{-1}$.

The formation constants obtained by this procedure and by that shown in Fig. 2 were subsequently found to decrease with increasing concentration of nucleotide (Fig. 3). As shown in the Theory section, this can be accounted for by assuming that ternary complexes (MOP) are formed between the metal, the hydroxyquinoline and the nucleotide. The equations obtained (9 and 10*a*) involve the assumptions that the amount of the MOP complex is much less than the free hydroxyquinoline and that the constant θ (defined by equation 7) is much less than 1. Equations 9 and 10*a* can be further simplified if $bK_3 \ll 1$ (9*a*, 10*b*). It will be seen that in these four equations, *m*, the concentration of free metal ion, has been replaced by *m'*, which is the apparent concentration deduced (as in Fig. 2) on the assumption that the MOP complex is not formed. The experimental values of *m'* for $a-m'=\frac{1}{2}b$ and those of $m'b/(a-m')$ for $m \to 0$ have been plotted against *b*, the concentration of the nucleotide, and the results for ATP (Fig. 5) were found to be consistent with a pair of straight lines according to equations 9*a* and 10*b* with $K_1 = 38\,000\,M^{-1}$ and $\theta = 0{\cdot}03$. If the bK_3 term were appreciable, the values would have fallen on curved lines according to equations 9 and 10*a*. However, because of the scatter of the experimental values it is not clear whether they fit a curve better than a straight line and it is therefore not possible to be precise about the magnitude of K_3. A tentative maximum value for this constant $([Mg(ATP)_2^{6-}]/[MgATP^{2-}]\,[ATP^{4-}])$ is of the order of $250\,M^{-1}$. Since the value of K_1 is obtained after extrapolating to zero *b*, it is not affected by the value assumed for K_3.

The effect of nucleotide concentration is smaller for ADP (Fig. 6) than it is for ATP. The values of *m'* for $a-m'=\frac{1}{2}b$ were extrapolated to zero concentration of ADP and K_1 was found to be $2200\,M^{-1}$.

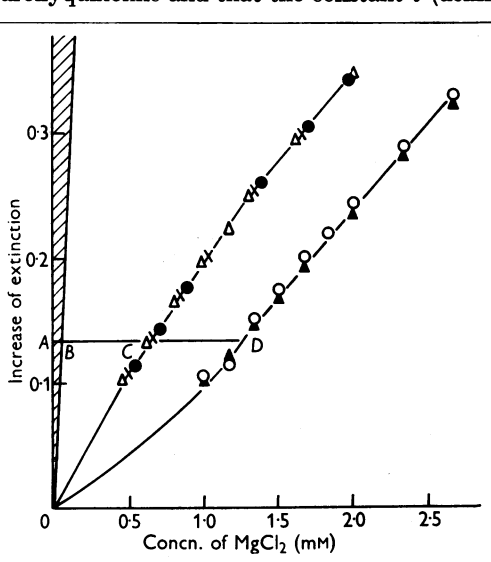


Fig. 2. Formation of MgADP complex. 0·91 mM-8-Hydroxyquinoline, 85 mM-tributylethylammonium bromide, 25 mM-triethanolamine hydrochloride, 17 mM-triethanolamine. Temp. 25°; pH 7·9. Cells 1 cm. △, ×, ●, No ADP (three separate experiments); ○, ▲, 1·23 mM-Na_3ADP (two experiments). The shaded area on the left indicates the amount of Mg–oxine complex. The curves were obtained by adding $0{\cdot}102\,M$-$MgCl_2$ from a micrometer syringe to 3 ml. of solution in a spectrophotometer cell. Extinction values were corrected for the slight progressive dilution by the $MgCl_2$. Points for extinction values less than 0·1 have been omitted. For the same extinction, and ignoring possibilities of a ternary complex of Mg, ADP and oxine, *AB* = Mg bound to the oxine (0·052 mM); *BC* = free Mg^{2+} ion ($m = 0{\cdot}61$ mM) and *CD* = Mg bound to ADP ($a - m = 0{\cdot}61$ mM). By equation 3*b*, $K_1 = 1/m = 1640\,M^{-1}$.

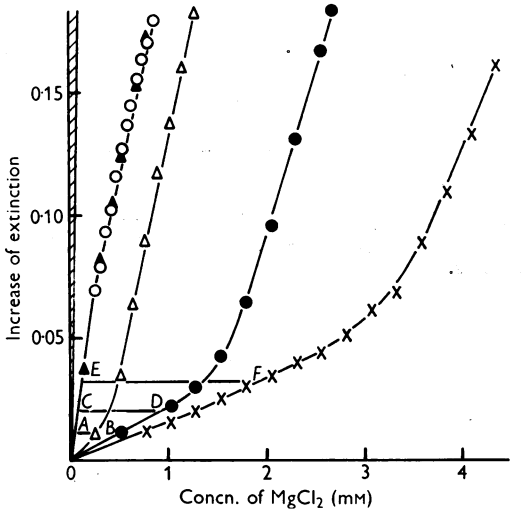


Fig. 3. Formation of MgATP complex. 0·30 mM-8-Hydroxyquinoline, 85 mM-tributylethylammonium bromide, 25 mM-triethanolamine hydrochloride; 60 mM-triethanolamine. Temp. 25°; pH 8·4. Cells 4·9 cm. ATP concentration (mM); ○, ▲, 0; △, 0·462; ●, 1·85; ×, 3·70. *AB*, *CD* and *EF* indicate the apparent binding of half an equivalent of metal at each concentration of ATP. Note the trend of the corresponding apparent concentration of free Mg^{2+} ion (*m'*) with ATP concentration (*b*) (see Fig. 5).

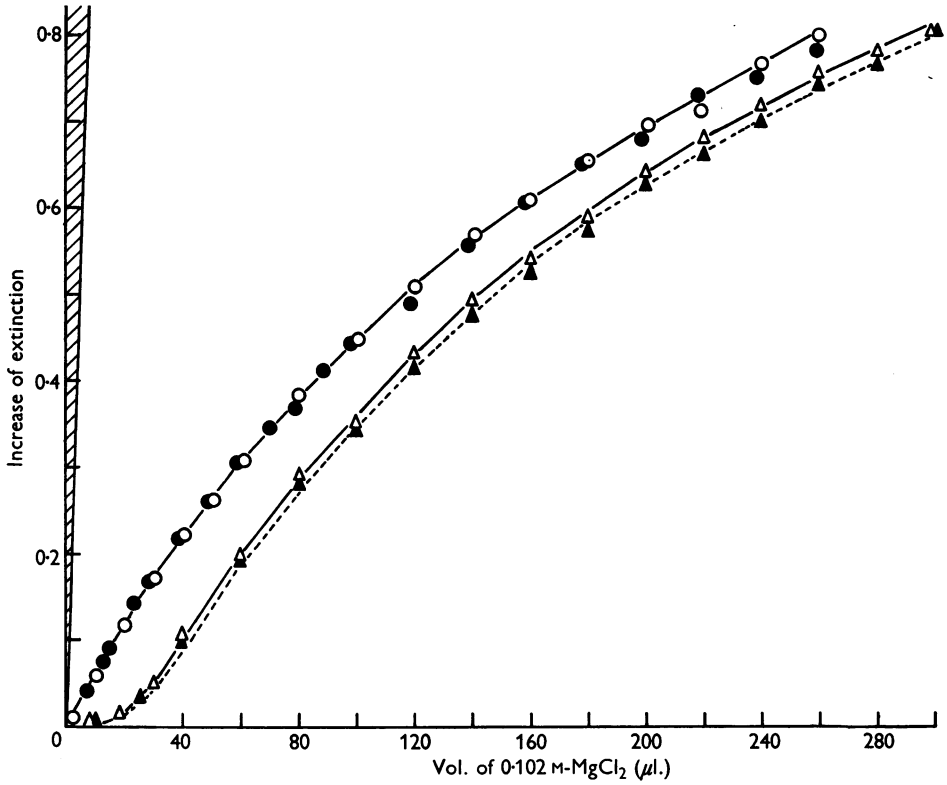


Fig. 4. Binding of Mg by ATP at high concentrations of $MgCl_2$. Conditions were as described for Fig. 2, except that temperature was 35°. pH was 7·9, measured at 25°. ○, ●, No ATP (two separate experiments); △, ▲, 0·85 mM-Na_4ATP. The continuous line is the calculated curve in the presence of ATP on the basis of the line in the absence of ATP and assuming a 1:1 complex ($K_1 = 2 \times 10^4 M^{-1}$) with no formation of a bimetallic complex (i.e. $K_2 = 0$). The broken line is the curve for the formation of a weak bimetallic complex ($K_2 = 70 M^{-1}$).

Table 1. *Formation constants for the calcium and magnesium complexes of adenosine tri- and di-phosphate*

85 mM-Tributylethylammonium bromide; 25 mM-triethanolamine chloride and 0·9 mM-8-hydroxyquinoline. Additional triethanolamine (17mM–0·23M) was added to obtain the required pH. Values at 37° (in italics) were obtained by interpolation.

Complex	Total nucleotide concn. (b)	pH (25°)	$10^{-3} K_1$: Evaluated ignoring MOP complexes — No. of values	Mean ± range (M^{-1})	Extrapolated to $b=0$ (M^{-1})	Temperature
$MgATP^{2-}$			See Fig. 5		38	25°
					55	*37*
	0·426	8·8	8	71 ±11	98	64
	0·852	8·8	4	57 ±6		
$MgADP^-$	0·62	7·9	8	2·0 ±0·4	2·2	25
	1·23	7·9	3	1·7 ±0·1	(see Fig. 6)	
	0·62	7·9	4	2·5 ±0·1	3·0	35
	1·23	7·9	4	2·05±0·1		
					3·3	*37*
	0·62	8·8	4	5·3 ±0·3	6·9	64
	1·23	8·8	4	4·5 ±0·2		
$CaATP^{2-}$	0·84	9·0	4	28 ±10	—	25
$CaADP^-$	1·23	8·8	2	0·72, 0·82	—	25

No statement can be made about K_3 because of the large scatter in the values obtained from the initial slopes of the extinction–[$MgCl_2$] curves.

Higher concentrations of magnesium chloride. To investigate the possibility that the Mg_2ATP complex may be formed, concentrations of up to 80 mM-$MgCl_2$ have been used. As shown in Fig. 4, there is no clear indication that more than one atom of magnesium is bound per molecule of ATP. The experiment of Fig. 4 at 35° and other similar experiments at 25° indicate that, if the Mg_2ATP complex is formed, the upper limit for K_2 ([M_2P]/[M] [MP]) is about 70 M^{-1}. A similar result was obtained for ADP.

Temperature. Larger values for K_2 were found at higher temperatures (Table 1). Since the error of each value is about ±10%, ΔH for forming the complexes can be estimated only approximately. The values are about 5 kcal./mole for $MgATP^{2-}$ and about 6 kcal. for $MgADP^-$, and for both complexes ΔS is about 36 cal./degree/mole. A large increase of entropy is expected because of the dehydration of the Mg^{2+} ion.

Other conditions. Some other measurements have been made at 25° but at different ionic strengths or with KCl replacing the tributylethylammonium bromide. The following values of K_1 for ATP were obtained after extrapolating to zero concentration of nucleotide: $2{\cdot}0$–$2{\cdot}5 \times 10^4$ M^{-1} (I 0·22) and $1{\cdot}6$–$2{\cdot}0 \times 10^4$ (I 0·11; 0·10 M-KCl). For ADP, K_1 was found to be about 1700 at I 0·22.

Calcium complexes. The values obtained are included in Table 1. Accurate values for the ATP complexes were not obtained because, even at pH 9, the binding of calcium by the hydroxyquinoline is not sufficient compared with the binding by ATP. No evidence of bimetallic complexes was found at concentrations up to 80 mM-$CaCl_2$.

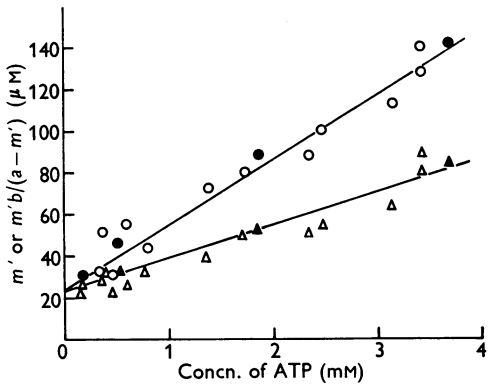


Fig. 5. Effect of ATP concentration on the apparent binding of magnesium. Values of m' for $a - m' = b/2$ (○, ●) and values of $m'b/(a - m')$ for $m \to 0$ (△, ▲) plotted against total ATP concentration (b). 8-Hydroxyquinoline concentration: 0·9 mM (○, △) or 0·3 mM (●, ▲). Other conditions were as described for Fig. 3. The straight lines fit equations 9a and 10b with $K_1 = 38\,000$ M^{-1} and $\theta = 0{\cdot}03$.

DISCUSSION

The values reported in this paper are higher than those of other authors (see Table 2) except for the value of DiStefano & Neuman (1953) for CaADP.

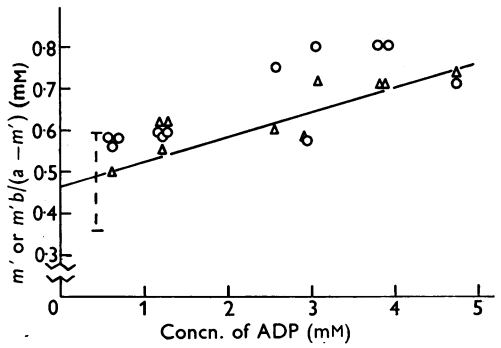


Fig. 6. Effect of ADP concentration on the apparent binding of magnesium. Conditions were as described for Fig. 5 except that pH = 7·9. The line fits equation 9a with $K_1 = 2\,200$ M^{-1} and $\theta = 0{\cdot}12$.

Table 2. *Comparison of previously published values for the formation constants*

References: (1) Smith & Alberty (1956*a*); (2) Nanninga (1957); (3) Martell & Schwarzenbach (1956); (4) Walaas (1958); (5) DiStefano & Neuman (1953); (6) Melchior & Melchior (1958 and personal communication). Methods: *A*, acid–base titration; *B*, cation-exchange resin; *C*, anion-exchange resin; *D*, effect of $MgCl_2$ on pH of nucleotide solution.

	(1)	(2)	(3)	(4)	(5)	(6)
Method	*A*	*B*	*A*	*B*	*C*	*D*
Ionic strength	0·20	0·1	0·10	0·10	0·10	0·2
Temperature	25°	23°	20°	23°	37°	25°
Alkali metal ion	Absent	0·085 M-Na^+	0·1 M-K^+	0·1 M-Na^+	0·1 M-Na^+	Absent
Values of 10^{-3} K_1 (M^{-1}):						
MgATP	2·97	2·0	10	11	—	20
MgADP	1·00	0·56	1·28	1·41	—	—
CaATP	1·97	0·75	4·0	5·9	11·5	—
CaADP	0·64	0·48	0·60	0·66	5·5	—

Because the alkali metal ions form weak complexes with the nucleotides (Melchior, 1954), the amounts of sodium or potassium ions present during most of the other measurements are expected to have reduced the observed formation constants by a factor which is of the order of two.

If the formation constants for the sodium complexes at an ionic strength of 0·2 (Smith & Alberty, 1956*b*) are assumed to apply at an ionic strength of 0·1, the values for MgATP and MgADP reported by Walaas (1958) may be converted into 26 700 and 2350 M^{-1} respectively in the absence of sodium. This value for MgATP is 30% lower than that found in the present work but there is good agreement between the values for MgADP. Part of the difference between the MgATP values is probably caused by the effects of ionic strength on the formation constant of the sodium complex. The value obtained in this work for MgATP at I 0·22 is reasonably consistent with the value reported by Melchior & Melchior (1958).

In considering the other measurements shown in Table 2, it should be mentioned that Martell & Schwarzenbach (1956) have tacitly assumed that the ionization of the adenine amino group causes the complete dissociation of the metal from complexes such as $MgHATP^-$. This probably holds for MgHADP but it does not seem to be justified for $MgHATP^-$. This would mean that Martell & Schwarzenbach's titration curves could be consistent with higher values for the formation constants of the $MgATP^{2-}$ and $CaATP^{2-}$ complexes. After allowing for the difference in temperature, the values found in this work for MgATP are about 60% higher than those given by Martell and Schwarzenbach. The results for CaATP also indicate a higher value.

Bimetallic complexes. There is as yet no direct evidence for the occurrence of the Mg_2ATP complex. In this work, no clear evidence was found at a concentration of 80 mM-free Mg^{2+} ion. The possibility of its formation at higher concentrations is suggested by the effects of magnesium on the hexokinase equilibrium (Robbins & Boyer, 1957).

Because Smith & Alberty (1956*a*) used relatively high concentrations of bivalent metal ions, the possibility has been considered that the low values obtained for the formation constants may have been due to the formation of bimetallic complexes such as Mg_2ATP. For example, within the stated experimental errors the results can fit a larger constant (2×10^4 M^{-1}) for the $MgATP^{2-}$ complex with values of 420 M^{-1} for the $MgHATP^-$ complex and 10 M^{-1} for the constant (K_2 above) of the Mg_2ATP complex. Other suggestive evidence for the Mg_2ATP complex is provided by the kinetics of the metal catalysis of certain reactions of ATP (Liebecq & Jacquemotte-Louis, 1958; Lowenstein, 1958).

Effects on the free energy of hydrolysis of adenosine triphosphate. Because ATP binds magnesium more strongly than ADP does, the presence or absence of the magnesium will appear to change the free energy of hydrolysis of ATP to ADP (Burton & Krebs, 1953; Robbins & Boyer, 1957). The effect was underestimated in these earlier discussions and in the recent survey of Burton (1958) because the values of Burton & Krebs (1953) or of Smith & Alberty (1956*a*) were used for the formation constants of the metal complexes. Thus the values for the free energy of hydrolysis of ATP as summarized by Burton (1958) have tended to represent reaction (1) rather than reaction (2).

$$MgATP^{2-} + H_2O \rightarrow MgADP^- + HPO_4^{2-} + H^+ \quad (1)$$

$$ATP^{4-} + H_2O \rightarrow ADP^{3-} + HPO_4^{2-} + H^+ \quad (2)$$

According to Table 1, $\Delta G'$ for reaction (2) will be more negative than that for reaction (1) by 1·7 kcal. at 25° or 37°.

In a following paper Benzinger, Hems, Burton & Kitzinger (1959) discuss the free energy of hydrolysis of ATP, using the data for the magnesium complexes obtained in this paper.

Control of metabolism. Raaflaub (1956) has suggested that the different complexing powers of ATP and its hydrolysis products could be the basis of a mechanism to control the rate of glycolysis. It is interesting to note that the formation constants reported in this paper lend support to this possibility in that they indicate a greater difference between ATP and ADP than do the measurements that have been reported by other workers. More direct evidence is needed to determine whether this suggested mechanism is important.

SUMMARY

1. The formation of complexes between adenosine tri- or di-phosphate (ATP or ADP) and Mg^{2+} or Ca^{2+} ions has been studied in aqueous solution at pH 7·9–8·9. A spectrophotometric method was used in which 8-hydroxyquinoline acts as an indicator of free metal ion.

2. Adenosine triphosphate has a much greater affinity for both metals than has adenosine diphosphate. The formation constants (i.e. reciprocals of the dissociation constants) for the $MgATP^{2-}$ and $MgADP^{2-}$ complexes were found to be 38 000 and 2200 M^{-1} respectively at 25°, or 98 000 and 6900 M^{-1} at 64° (I 0·11; 85 mM-tributylethylammonium bromide plus 25 mM-triethanolamine hydrochloride). The interpolated values for 37° are 55 000 and 3300 M^{-1} respectively.

3. There was no clear indication that bimetallic complexes were formed at concentrations of up to 80 mM-Mg^{2+} or -Ca^{2+} ion.

4. The results also indicate the formation of a ternary complex between magnesium, adenosine triphosphate and 8-hydroxyquinoline.

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Enthalpies of Hydrolysis of Glutamine and Asparagine and of Ionization of Glutamic and Aspartic Acids

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Thermodynamic data for glutamine are of special biochemical interest because in the glutamine synthetase reaction (Elliott, 1953) the synthesis of glutamine is coupled with the hydrolysis of adenosine triphosphate. Levintow & Meister (1954) showed that this reaction is readily reversible, and found that the equilibrium constant is $1{\cdot}2 \times 10^3$. If the free energy of hydrolysis of glutamine were known, it would be possible to evaluate, in a simple manner, the free energy of hydrolysis of adenosine triphosphate.

There are no data in the literature for the free energy of hydrolysis of glutamine. The glutaminase- or acid-catalysed hydrolysis of glutamine is considered practically irreversible, and its equilibrium constant and free energy appear therefore not to be readily measurable by chemical analysis of reactants and products. For an approach from thermal data, heat of combustion and low-temperature heat-capacity data are available for glutamic acid, but not for glutamine. Levintow & Meister (1954) and M. F. Morales (appendix to Levintow & Meister, 1954) have therefore assumed that the hydrolysis of glutamine is thermodynamically analogous to that of certain dipeptides or of asparagine. The free energy of hydrolysis of these compounds can be calculated from available heat of combustion and heat-capacity data (Borsook & Huffman, 1938).

In order to examine the assumed analogy between glutamine and asparagine, we have directly measured, in two steps, the enthalpy changes associated with the transformation of glutamine and asparagine into the undissociated glutamic and aspartic acids.

EXPERIMENTAL

Method of heat measurements

Heats of reaction were measured by dissipation microcalorimetry (Benzinger & Kitzinger, 1954, 1956; Kitzinger & Benzinger, 1955), with quantities of reactants ranging from 1 to 16 μmoles. The solutions are placed in glass or Teflon vessels of approx. 16 ml. capacity. In 'bicompartmented' vessels, similar quantities of the two solutions (8 ml. each) can be kept separated by a circular partitioning wall during the initial equilibration period. In 'drop' vessels, droplets of one solution are measured with an Agla microsyringe into small dimple-shaped recesses and thus kept apart from the main bulk (16 ml.) of the other solution. The larger volumes are measured by suitable hypodermic syringes or pipettes. Solutions are placed in the vessels with polyethylene tubing attached to the syringes or pipettes.

After thermal equilibrium has been attained, the solutions are mixed by rotation of the entire calorimeter, thus