paper chromatography with R_{Ad} 1.92 in solvent B and R_{Ad} 0.45 in solvent C.

Recrystallization from 2.5 ml of water gave 54 mg of white needles, mp > 300° (darkens from 265°), and a second recrystallization gave the analytical sample, mp > 300° (darkens from 265°); $[\alpha]_D^{24}$ +28° (0.25% in water); $\lambda_{max}^{pH\,1}$ 256 mμ (ε, 12,600); $\lambda_{max}^{pH\,7}$ 252 mμ (ε, 14,000); $\lambda_{max}^{pH\,13}$ 256 mμ (ε, 11,600), 265 mμ (ε, 11,800).

Anal. Calcd. for $C_{10}H_{13}N_5O_5 \cdot H_2O$: C, 39.9; H, 5.02; N, 23.2. Found: C, 40.0; H, 5.02; N, 23.3.

Guanosine has $\lambda_{max}^{pH\,1}$ 256 mμ (ε, 12,200); $\lambda_{max}^{pH\,11.3}$ 258–266 mμ (ε, 11,300) (Beaven *et al.*, 1955).

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The Stability Constants of Metal-Adenine Nucleotide Complexes

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Values of 73,000 M^{-1} and 4,000 M^{-1} have been obtained for the stability constants of $MgATP^{2-}$ and $MgADP^{-}$, respectively, in 0.1 M *N*-ethylmorpholine buffer at 30°. In addition, reasonable confirmation of the value for $MgATP^{2-}$ has been obtained from *p*H titration data, using both an approximation method and a program written for an IBM 1620 computer. Values for the stability constants of $CaATP^{2-}$ by *p*H titration and spectrophotometry and $CaADP^{-}$ and $MnADP^{-}$ by spectrophotometry have been obtained. Results for $NaATP^{3-}$, $KATP^{3-}$, Mg-phosphocreatine, and Mg-phosphoarginine are also presented.

Divalent metal ions are required as activators (and also sometimes act as inhibitors) of many of the enzymic reactions involved in the metabolism of nucleotide phosphates. These ions interact non-enzymically with nucleotide phosphates to form the respective metal complexes, so that a knowledge of the stability constants of these complexes is necessary for the interpretation of enzyme kinetic and thermodynamic studies. This information may, in fact, be a prerequisite to many such investigations (Morrison *et al.*, 1961).

Values, varying widely in magnitude, have been given for the stability constants of $MgADP^{-}$ and $MgATP^{2-}$ (Table I; Bock, 1960). Reasons for these variations may not be obvious to an enzymologist wishing to decide on the value he should use.

It is necessary to distinguish between absolute and apparent values for these stability constants. The absolute values, usually obtained from *p*H titration data, are true constants, independent of *p*H, whereas an "apparent" value relates only to a particular *p*H, and for the reaction

$$M + L \rightleftharpoons ML$$

may be defined as

$$K_{app} = \frac{[ML]}{[M][L]_{T'}}$$

where $[L]_{T'}$ is the sum of the uncomplexed forms of L at that *p*H. Under certain conditions the "apparent" and absolute values may be approximately the same. However, where results are to apply to enzyme experiments at constant *p*H it is convenient to use the relevant "apparent" value (which can be obtained from the absolute value provided *p*H and pK_a values are known). The magnitude of both the absolute and the "apparent" values will vary with such experimental variables as the nature of the supporting medium, ionic strength, and temperature, and it is useful to consider briefly the effect of these factors. Some of these points are treated in more detail later in the text.

The application of classical potentiometric titration techniques to stability-constant determinations involving polyanionic molecules such as ATP can lead to mathematical expressions of such complexity that they are incapable of exact solution (see below). For this reason, workers (e.g., Martell and Schwarzenbach, 1956; Smith and Alberty, 1956a; Nanninga, 1961) have used various simplifications which appear to be of doubtful validity (see Bock, 1960).

The principal methods used for determinations of "apparent" constants have been dependent upon competition between ligands (e.g., Walaas, 1958; Burton, 1959; Nanninga, 1961), the required *p*H being maintained by a suitable buffer. Thus in making

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TABLE I
STABILITY CONSTANT (K) VALUES FOR MAGNESIUM-NUCLEOTIDE COMPLEXES, WHERE $K = \frac{[\text{Mg-nucleotide}]}{[Mg^{2+}][\text{free nucleotide}]}$

K (M^{-1})				
$MgADP^-$	$MgATP^{2-}$	Method	Conditions	Reference
1,000	3,000	pH titration	0.2 M tetra-n-propylammonium bromide, 25°	Smith and Alberty (1956a)
1,300	10,000	pH titration	0.1 M KCl, 20°	Martell and Schwarzenbach (1956)
560	2,000	Ion exchange	0.085 M NaCl plus 0.02 M Tris, pH 8.0, 23°	Nanninga (1957)
1,400	11,000	Ion exchange	0.1 M NaCl plus 0.01 M Tris, pH 8.2, 23°	Walaas (1958)
2,200	38,000	Spectral changes of 8-hydroxyquinoline	0.085 M tributylethylammonium bromide plus 0.025 M triethanolamine hydrochloride, pH 8.4, 25°	Burton (1959)
—	90,000	Estimated value which fits enzyme kinetic data	0.05 M glycine-NaOH, pH 9.0, 30°	Noda *et al.* (1960)

measurements it is necessary to consider the possible interaction of the buffer with the metal ion. The importance of the nature of the supporting medium is further emphasized by the fact that some workers (e.g., Martell and Schwarzenbach, 1956; Walaas, 1958) have carried out measurements in relatively high concentrations of Na^+ and K^+ and considered the interaction of these cations with the nucleotide to be negligible, though estimates of the stability constants of $NaATP^{3-}$ and $KATP^{3-}$ have been reported (Melchior, 1954; Smith and Alberty, 1956b; see also Results section of this paper). The effect of ionic strength and temperature has been demonstrated by Burton (1959), who has provided data for the deviation of the values for $MgATP^{2-}$ and $MgADP^-$ over a useful range of these variables.

The initiation in this laboratory of a program to study the metal activation of phosphoryl-group-transferring enzymes (Morrison *et al.*, 1961) led to the determination of the stability constants of metal-nucleotide complexes under conditions approximating to those used for the enzymic experiments. In a previous note (O'Sullivan and Perrin, 1961) estimates of the values for $MgATP^{2-}$ as obtained by pH titration and the spectrophotometric method of Burton (1959) were reported. The stability constant values obtained by the two methods were in good agreement, but were higher than those reported previously. This communication describes in detail the experimental techniques used in obtaining these values and also reports values for other metal-nucleotide complexes.

THEORY

The Determination of Stability Constants Using the Spectral Changes of 8-Hydroxyquinoline.—The theory of this method has been fully elaborated by Burton (1959) and only the salient features are reproduced. Concerning ATP, for example, it can be shown (Burton, 1959) that the stability constant, K, of $MgATP^{2-}$ is given by $K = 1/[Mg^{2+}]_{1/2}$ where $[Mg^{2+}]_{1/2}$ is the free magnesium concentration when $[MgATP^{2-}] = \frac{1}{2}[ATP]_T$.

However, $[Mg^{2+}]_{1/2}$ was found by Burton to depend on the concentration of $[ATP]_T$. Thus K is represented by

$$K = \frac{1}{[Mg^{2+}]_{1/2}} + k_1[ATP]_T$$

where k_1 is a factor introduced by Burton to allow for this dependence, attributed by him to the formation of a ternary nucleotide-metal-8-hydroxyquinoline complex. Here K is found by plotting values of $[Mg^{2+}]_{1/2}$ for various values of $[ATP]_T$ and extrapolating to $[ATP]_T = 0$.

An estimate of K can also be obtained from the initial slope of the titration curve for ATP. Again, the value of K was found to depend on the $[ATP]_T$ concentration, so that Burton found it necessary to introduce another constant, k_2, to allow for this. He derived the expression

$$K = \frac{[MgATP^{2-}]}{[Mg^{2+}][ATP]_T} + k_2[ATP]_T$$

for $[Mg^{2+}] \to 0$, to describe the slope of the initial portion of the curve.

The Determination of Stability Constants Using pH Titration.—For the titration of the disodium salt of ATP in the presence of Mg^{2+}, the following equilibria must be considered:

$$\begin{array}{ccccc} H_2ATP^{2-} & \overset{pK_{a_1}}{\rightleftharpoons} & HATP^{3-} & \overset{pK_{a_2}}{\rightleftharpoons} & ATP^{4-} \\ \Big\| K_1 & & \Big\| K_2 & & \Big\| K_3 \\ MgH_2ATP & \overset{pK_{m_1}}{\rightleftharpoons} & MgHATP^- & \overset{pK_{m_2}}{\rightleftharpoons} & MgATP^{2-} \end{array}$$

where K_1, K_2, and K_3 are the stability constants of the appropriate magnesium complexes, with respect to dissociation into metal ion and ligand, i.e.,

$$K_1 = \frac{[MgH_2ATP]}{[Mg^{2+}][H_2ATP^{2-}]}; \qquad K_2 = \frac{[MgHATP^-]}{[Mg^{2+}][HATP^{3-}]};$$

$$K_3 = \frac{[MgATP^{2-}]}{[Mg^{2+}][ATP^{4-}]}$$

and pK_{a_1}, pK_{a_2}, pK_{m_1}, and pK_{m_2} are the dissociation constants for the species H_2ATP^{2-}, $HATP^{3-}$, Mg H_2ATP, and Mg $HATP^-$, respectively.

Linear equations describing the equilibria established during the titration may be set up in terms of total ATP, total Mg, and Σ (positive charges) = Σ (negative charges).

$$[MHL^-] + [ML^{2-}] + [H_2L^{2-}] + [HL^{3-}] + [L^{4-}] = [L]_T \quad (1)$$

$$[M^{2+}] + [MHL^-] + [ML^{2-}] = [M]_T \quad (2)$$

$$[K^+] + [H^+] + 2[L]_T + 2[M^{2+}] = [MHL^-] + 2[ML^{2-}] + 2[H_2L^{2-}] + 3[HL^{3-}] + 4[L^{4-}] + [OH^-] + 2[M]_T \quad (3)$$

(N.B. The $2[L]_T$ term arises because ATP is added as the disodium salt, the $2[M]_T$ term because magnesium is added as the chloride. For convenience, the designations L = ATP and M = Mg have been used.)

These equations contain two more unknowns than there are measurable quantities and thus are incapable of exact solution at any given titration point. Two approaches to overcome this difficulty have been made; viz., an approximation method and the use of a 1620 IBM computer, to solve the equations using pairs of selected points. The details of the latter are described in the Appendix. For the approximation method, MgH_2ATP is neglected and only the species Mg $HATP^-$ and Mg ATP^{2-}, besides the free forms of ATP, are considered. This would be reasonably valid above *p*H 5.

First Approximation.—Using equation (2) and the value for K_{m_2}, estimates of $[ML^{2-}]$ and $[MHL^-]$ can be obtained in terms of $[M^{2+}]$. Equations (1) and (2) give an expression for $[M^{2+}]$ in terms of $[M]_T$, $[L]_T$, and the different forms of L. Thus by successive substitution for $[ML^{2-}]$, $[MHL^-]$, and $[M^{2+}]$ in equation (3), an expression is obtained in which the only unknown is $[L^{4-}]$. The value obtained for $[L^{4-}]$ can be used to calculate $[M^{2+}]$ and $[ML^{2-}]$ and thus K_3. Similarly, calculation of $[MHL^-]$ gives an estimate of K_2. Once a reasonable estimate of these two constants is obtained, K_2 is utilized in a more exact theory. This makes no assumptions about pK_{m_2} but presumes that K_2 is known.

Second Approximation.—The same basic equations are used. By successive substitution in equation (3) for $[ML^{2-}]$ and $[M^{2+}]$ the expression

$$[K^+] + [H^+] - [OH^-] = 2[L]_T - [MHL^-] - 2[H_2L^{2-}] - [HL^{3-}] \quad (4)$$

is obtained, for the particular case where $[M]_T = [L]_T$.

Substituting for $[MHL^-] = K_2[M^{2+}][HL^{3-}]$ and subsequently again for $[M^{2+}]$ and expressing all L terms in terms of $[L^{4-}]$ gives the quadratic equation

$$[L^{4-}] = \frac{-B + \sqrt{B^2 + 4AC}}{2A} \quad (5)$$

where

$$A = K_2 \frac{[H^+]}{K_{a_2}} \left\{1 + \frac{[H^+]}{K_{a_2}} + \frac{[H^+]^2}{K_{a_1}K_{a_2}}\right\}$$

$$B = \frac{[H^+]}{K_{a_2}} \left\{1 + \frac{2[H^+]}{K_{a_1}}\right\}$$

$$C = 2[L]_T - [K^+] - [H^+] + [OH^-]$$

The ratio of bound to total metal is given by

$$\bar{n} = \frac{[MHL^-] + [ML^{2-}]}{[M^{2+}] + [MHL^-] + [ML^{2-}]} = \frac{[L]_T - [L^{4-}]\left\{1 + \frac{[H^+]}{K_{a_2}} + \frac{[H^+]^2}{K_{a_1}K_{a_2}}\right\}}{[M]_T} \quad (6)$$

Equation (6) may also be expressed in the form

$$\bar{n} = \frac{K_2[HL^{3-}] + K_3[L^{4-}]}{1 + K_2[HL^{3-}] + K_3[L^{4-}]}$$

Rearranging

$$K_3 = \frac{\bar{n}}{(1 - \bar{n})} \cdot \frac{1}{[L^{4-}]} - K_2 \frac{[HL^{3-}]}{[L^{4-}]} \quad (7)$$

Materials

Nucleotides.—Commercial samples of the sodium salts of both ADP and ATP as obtained from Sigma Chemical Co. were found to contain varying amounts of other nucleotide and inorganic phosphate impurities and were therefore subjected to further purification.

ATP was twice recrystallized from 50% ethanol (Berger, 1956), dried in air at room temperature, and stored at −10°. The resultant salt was characterized as $Na_2ATP \cdot 4\ H_2O$ by elementary analysis and by determination of the molecular extinction at 259 mμ using the extinction coefficient reported by Bock *et al.* (1956).

Anal. Calcd. for $Na_2ATP \cdot 4H_2O$: P, 14.9; N, 11.24; H_2O, 11.5%. Found: P, 14.9; N, 11.27; H_2O, 10.9.

No ultraviolet-light-absorbing impurities were observed after chromatography in isobutyric acid–NH_3 (sp gr, 0.88)–water (66:1:33, v/v) (Krebs and Hems, 1953).

ADP was purified as described previously (Morrison *et al.*, 1961). Following the determination of the ADP concentration by measurement at 259 mμ (Bock *et al.*, 1956), the solution was diluted to a concentration of 10^{-2} M and stored at −10°. In agreement with Kuby *et al.* (1962), conditions could not be found for the crystallization of NaADP.

Phosphocreatine.—Phosphocreatine was prepared by the method of Ennor and Stocken (1948). The crystalline product was dissolved in water, passed through a column of Dowex-50 (Na^+), and recrystallized from the effluent by the addition of absolute ethanol to a concentration of 80%. Elementary analysis indicated that the product was pure and contained 6 moles of water of crystallization.

Phosphoarginine.—This material was isolated as the sodium salt from crayfish muscle according to the method of Marcus and Morrison (unpublished data).

Metal Salts.—Aqueous solutions of the chlorides of Mg, Ca, and Mn (all British Drug House AnalaR) were standardized by passing measured amounts through an Amberlite IR-120 (H^+) column and titrating the effluent acid with standard alkali.

Carbonate-free Alkali.—Carbonate ion was precipitated from a stock solution of KOH (approximately 1 N prepared from British Drug House AnalaR reagent) with barium hydroxide solution and the excess barium was removed by passing the alkali through a column of Amberlite IR-120 (K^+ form). The alkali was collected under nitrogen, standardized, and diluted as required with carbonate-free water.

Buffers.—Commercial triethanolamine was purified, following the method of Germann and Knight (1933); the fraction distilled at 167–168° under 0.5 mm Hg was collected. Nitrogen analysis indicated that the purified preparation was 98–99% pure.

Tris(hydroxymethyl)aminomethane (Tris) was a Fisher Certified Reagent issued as 99.98% pure. *N*-Ethylmorpholine, as obtained from Eastman Kodak, was distilled three times under reduced pressure at 30°.

The buffers were prepared at 0.5 M concentration, adjusted to the required *p*H by the addition of 5 N HCl, and diluted as required. The *p*H values of the diluted buffer solutions were checked on a Vibron Electrometer Model 33B (Electronic Instruments, Ltd.) *p*H meter reading to 0.001 *p*H unit.

Other Reagents.—Tetraethylammonium bromide, obtained as British Drug House Laboratory Reagent, was twice recrystallized from alcohol-ether. Catechol-3,5-disulphonic acid (disodium salt) was obtained from the Aldrich Chemical Co., Inc., and 8-hydroxyquinoline was a British Drug House AnalaR product.

Methods

The Determination of Stability Constants Using the Spectral Changes of 8-Hydroxyquinoline.—The experimental procedure was similar to that described by Burton (1959), measurements at 360 mμ being made on a Shimadzu (QR-50) spectrophotometer. $MgCl_2$ (0.1 M) was added from an Agla micrometer syringe to the solution of 8-hydroxyquinoline in 0.1 M buffer maintained at 30°. The *p*H of the solution was checked on the Vibron Electrometer *p*H meter.

The stability constant of $MgATP^{2-}$ was determined by carrying out spectral measurements in 10.0 ml of solution contained in a cell of 4 cm light path, with 8-hydroxyquinoline concentrations of 0.3, 0.4, or 0.75 mM.

The stability constant of $MgADP^-$ was determined in 3.0 ml of solution in a cell of 1 cm light path, with 8-hydroxyquinoline at a concentration of 0.4 mM. The molar extinction coefficient of Mg-8-hydroxyquinoline was determined to be 1.8×10^3.

The Determination of the Stability Constants of Metal-ATP Complexes by pH Titration.—The two terminal acid dissociation constants of ATP were determined by titrating a 5.25×10^{-4} M solution of the disodium salt of ATP at 30° with KOH (0.105 N). The titration was carried out in 50 ml of solution in a 200-ml lipless Pyrex beaker closed by a rubber stopper through which passed the electrodes, a thermometer, a bubbler, and a fine-bore polyethylene tube, which was connected to an Agla micrometer syringe containing KOH. The solution was stirred by passing scrubbed nitrogen through the bubbler. The *p*H measurements were made using the Vibron Electrometer fitted with an internally shielded glass electrode. The output of the *p*H meter was recorded on a Rectiriter recording milliammeter (Texas Instruments Inc.) to facilitate assessment of attainment of equilibrium. Calibration was based on the standards, potassium hydrogen phthalate (0.05 M), $pH_{30°} = 4.011$ and sodium tetraborate (0.05 M), $pH_{30°} = 9.142$ (Perrin, 1960).

The *p*H readings were taken after the addition of each 0.01 ml of alkali and 0.5 ml was required to complete the titration. The initial *p*H value for ATP by itself lay in the range 3.74–3.77. This value was lowered with increasing concentrations of added divalent metal ion. The titration was continued to a *p*H of approximately 8.8. The ionic strength was maintained at 0.1 by the addition of tetraethylammonium bromide, so that the activity coefficients would be constant throughout.

Results

Measurements of the apparent stability constant (K) of $MgATP^{2-}$ at *p*H 8.0 were carried out in three buffers, tris(hydroxymethyl)aminomethane (Tris), triethanolamine, and *N*-ethylmorpholine. The results are shown in Table II, together with an "absolute" value, K^*. The results obtained using 8-hydroxyquinoline at concentrations of 0.3, 0.4, and 0.75 mM were similar.

The nature of the plot from which the value of K was determined is illustrated in Figure 1, where results for $[Mg^{2+}]_{1/2}$ and for the initial slopes are shown. Greater weight has been placed on the $[Mg^{2+}]_{1/2}$ results, as it was possible to determine these with greater precision.

The variation in the value of $[Mg^{2+}]_{1/2}$ with the concentration of ATP, as determined from four different experiments, is illustrated in Figure 2 and the best line has been drawn using the average value for each concentration of ATP. Little variation in the $[Mg^{2+}]_{1/2}$ values was obtained with concentrations of $[ATP]_T$ up to 3 mM. Above this concentration the $[Mg^{2+}]_{1/2}$ values were not consistent, sometimes tending towards the x-axis. This effect could be due to the formation of the complex, $Mg(ATP)_2^{6-}$.

The effect on the apparent stability constant of $MgATP^{2-}$, produced by lowering the buffer concentration, was studied by carrying out an estimation in 0.02 M *N*-ethylmorpholine, the ionic strength being maintained at 0.1 M by the addition of tetraethylammonium bromide. The value obtained was 80,000 M^{-1}, corresponding to a K^* of 88,000 M^{-1}. This is greater than

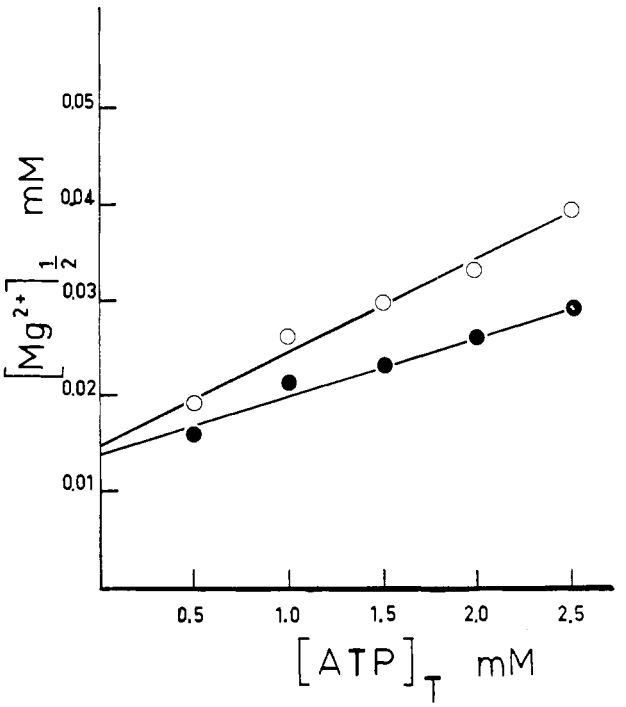

Fig. 1. Determination of the stability constant of $MgATP^{2-}$ in triethanolamine buffer (0.1 M, *p*H 8.0) at 30°. ●—●, $[Mg^{2+}]_{1/2}$ values; O—O, initial slope values. The $[Mg^{2+}]_{1/2}$ values extrapolate to 0.014 mM so that K = 71,400.

Table II
Values Obtained for the Stability Constant of $MgATP^{2-}$[a]

Method	*p*H	K (M^{-1})	K^* (M^{-1})	No. of Experiments
Spectral changes of 8-hydroxyquinoline:				
Tris buffer	7.99	18,000 ±2,000	20,000	3
Triethanolamine buffer	8.02	70,000 ±6,000	77,000	4
N-ethylmorpholine buffer	8.01	73,000 ±7,000	80,000	5
*p*H titration				
Approximation			90,000 M^{-1}	
Value obtained from computer			75,000 M^{-1}	

[a] K refers to the experimental stability constant; K^* to an "absolute" stability constant, calculated from K using the value of 7.0 determined for the pK_a of the terminal phosphate group of ATP. All buffers at 0.1 M concentration, at 30°.

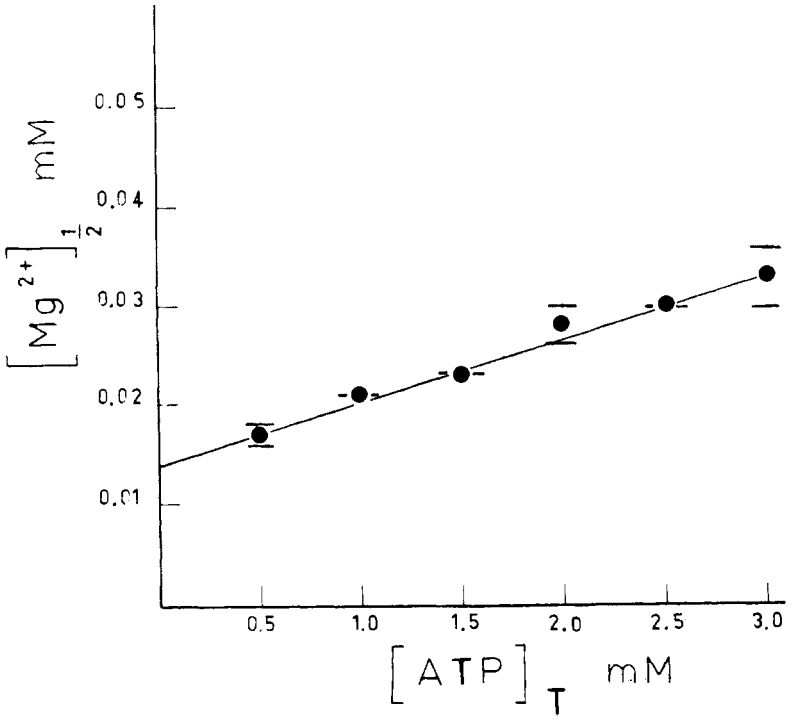

FIG. 2.—Determination of the stability constant of $MgATP^{2-}$. Variation in the values of $[Mg^{2+}]_{1/2}$ in triethanolamine buffer (0.1 M, *p*H 8.0) at 30°. The line extrapolates to $[Mg^{2+}]_{1/2}$ = 0.014 mM so that K = 71,400.

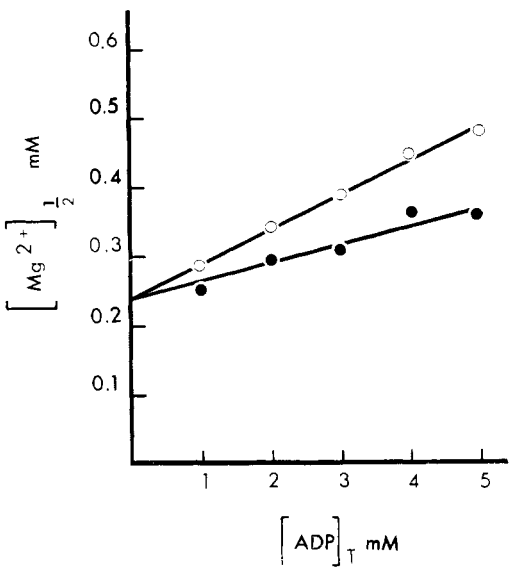

FIG. 3.—Determination of the stability constant of $MgADP^-$ in *N*-ethylmorpholine buffer (0.1 M, *p*H 8.0) at 30°. ●—●, $[Mg^{2+}]_{1/2}$ values; O—O, initial slope values. Lines extrapolate to 0.24 mM so that K = 4,150 M^{-1}.

the value obtained in 0.1 M *N*-ethylmorpholine, but the variation is within the experimental error.

Determination of the Stability Constant of $MgADP^-$.—Plots of $[Mg^{2+}]_{1/2}$ and values of the initial slope versus $[ADP]_T$ are shown in Figure 3 and from these a value of 4,150 M^{-1} was calculated for the stability constant of $MgADP^-$. A second experiment gave a value of 4,000 M^{-1}.

The *p*H optimum for the reverse reaction catalyzed by ATP–creatine phosphotransferase is at *p*H 7.0, so that in conjunction with kinetic studies on this enzyme (Morrison *et al.*, 1961) it was desirable to have an estimate of the apparent stability constant of $MgADP^-$ at *p*H 7.0. It was found that the amount of complex formation between Mg^{2+} and 8-hydroxyquinoline at *p*H 7.0 was too small for measurements to be made. However, by increasing the *p*H to 7.4 it was possible to obtain a value of 2,400 M^{-1}. Then, as the terminal phosphate group of ADP has pK_a at 6.65,[1] so that 70% and 85% of the total ADP is present as ADP^{3-} at *p*H 7.0 and *p*H 7.4, respectively, it was possible to calculate a value for the apparent stability constant of $MgADP^-$ at *p*H 7.0. The figure obtained was 2,000 M^{-1}.

The Determination of the Stability Constants of Magnesium-ATP Complexes by pH Titration.—Titration curves for the disodium salt of ATP in the absence of Mg^{2+} and in the presence of an equal concentration and of a 20-fold concentration of magnesium chloride, are shown in Figure 4. The pK_a values for ATP, calculated from the complete Henderson-Hasselbalch equation, were pK_{a_1} = 3.93 ± 0.02 and pK_{a_2} = 6.97 ± 0.02. An estimate of pK_{m_2} as 4.88 was obtained from the titration with a 20-fold excess of magnesium. (Martell and Schwarzenbach, 1956), claimed that titration of ATP with a 10-fold excess of Mg^{2+} was represented by

$$MgHATP^- \longrightarrow MgATP^{2-} + H^+$$

which would give a direct value of pK_{m_2}. In the work presented in this paper it appeared that even with a 20-fold excess of Mg^{2+} an exact estimate of pK_{m_2} was not obtained. It is possible that potassium ion at a concentration of 0.1 M was responsible for the observation by Martell and Schwarzenbach, because this cation interacts with ATP.)

In applying the approximation techniques to a titration curve, six to eight points were selected between *p*H 5.0 and *p*H 6.5. This *p*H range was chosen because below 5.0 the concentration of MgH_2ATP became increasingly important, while above *p*H 6.5 the

[1] Determined at 30° in 0.1 M tetraethylammonium bromide; W. J. O'Sullivan, unpublished results.

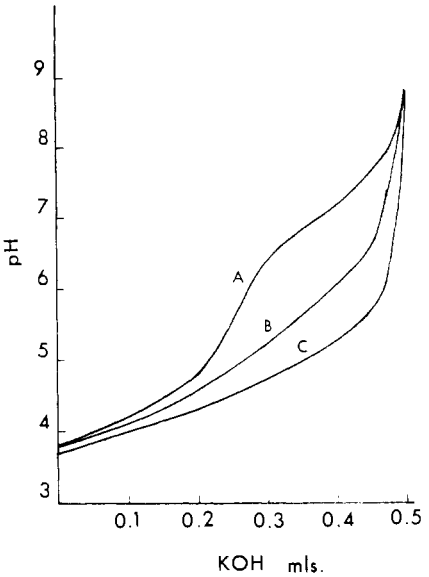

FIG. 4.—Titration curves of ATP. All curves, $[ATP]_T = 5.25 \times 10^{-4}$ M. Curve A, $[Mg]_T = 0$; curve B, $[Mg]_T = 5.25 \times 10^{-4}$ M ($[Mg]_T = [ATP]_T$); curve C, $[Mg]_T = 1.05 \times 10^{-2}$M ($[Mg]_T = 20[ATP]_T$). Titrations carried out in 50 ml of solution at 30°; tetraethylammonium bromide added to give an ionic strength of 0.1 M.

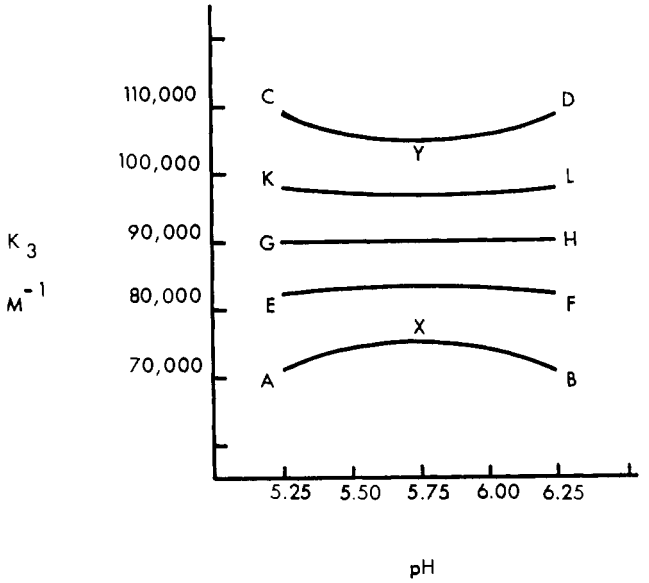

FIG. 5.—Graphical representation of the approximation method used to determine the stability constant of $MgATP^{2-}$. Curves AB and CD correspond to the values obtained by the first approximation for two independent titration curves, X and Y being taken as the best values, respectively. EF and KL correspond to the application of the second approximation, leading to the "best" value of K_3 represented by GH.

titration curve changed too rapidly with increasing concentration of alkali for accurate determinations to be made. The first approximation, utilizing the value of pK_{m2}, was used to give a series of values for K_2 and K_3, which were pH dependent. These values, plotted against pH, gave a curve which might face up or down as illustrated for the determination of K_3 in Figure 5. The values of K_2 and K_3, obtained from the maximum or minimum of such parabolas, were taken as representing the first approximation. In this way, different titration curves gave values for K_2 and K_3 from 500 to 850 M^{-1} and from 70,000 to 107,000 M^{-1}, respectively.

The value of K_2 and values slightly above and below K_2 were then used in the second approximation to calculate K_3. The effect was either to flatten out the curve (e.g., EF in Fig. 5) or to increase its curvature. The "best" estimates of K_3, and of K_2, were taken as

TABLE III
STABILITY CONSTANTS OF $MgHATP^-$ (K_2) AND $MgATP^{2-}$ (K_3)[a]

	K_2 (M^{-1})	K_3 (M^{-1})
	680	83,000
	720	97,000
	700	91,000
	750	93,000
	750	80,000
	550	75,000
	800	104,000
Average:	700	88,000

[a] Obtained by the approximation methods described in the text. Each value represents a separate experiment.

TABLE IV
STABILITY CONSTANTS OF METAL-NUCLEOTIDE COMPLEXES

Complex	Method Used to Determine Value	Conditions	K (M^{-1})
$CaATP^{2-}$	pH titration (calculated on IBM 1620 computer)	0.1 M $(C_2H_5)_4$ NBr, 30°	32,000
	Spectral changes of 8-hydroxyquinoline	0.1 M N-ethylmorpholine (pH 8.0), 30°	31,000
$CaADP^-$	Spectral changes of 8-hydroxyquinoline	0.1 M N-ethylmorpholine (pH 8.0), 30°	2,200
$MnADP^-$	Spectral changes of catechol-3,5-disulphonic acid at 325 mμ	0.1 M N-ethylmorpholine (pH 8.0), 30°	25,000
	Electron paramagnetic resonance, 27°		30,000
$NaATP^{3-}$	Effect on apparent stability constant of $MgATP^{2-}$ and $CaATP^{2-}$ using spectral changes of 8-hydroxyquinoline	0.1 M N-ethylmorpholine (pH 8.0), 30°	15
$KATP^{3-}$	Effect on apparent stability constant of $MgATP^{2-}$ using spectral changes of 8-hydroxyquinoline	0.1 M N-ethylmorpholine (pH 8.0), 30°	14

being those values which most closely approximated a straight line (GH in Fig. 5). The values, obtained in this way from separate experiments, are shown in Table III. The average values for K_2 and K_3 may be compared with the values of 800 M^{-1} and 105,000 M^{-1}, respectively, previously reported by O'Sullivan and Perrin (1961). The earlier estimates contained a small systematic error, introduced by using an erroneous value for the activity coefficient of the hydrogen ion in solution.

The program for the IBM 1620 computer was applied to pairs of points at intervals along the titration curve for total ATP equal to total magnesium. The best estimates obtained for the stability constants of MgH_2ATP, $MgHATP^-$, and $MgATP^{2-}$, were 20, 500, and 75,000 M^{-1}, respectively. Further details are presented in the Appendix.

Stability Constants of Other Metal-Nucleotide Complexes.—Determinations of the stability constants of $CaATP^{2-}$, $CaADP^-$, $MnADP^-$, $NaATP^{3-}$, and $KATP^{3-}$ were carried out and the values obtained are collected in Table IV. The analysis of the titration curve for ATP in the presence of an equimolar concentration of $CaCl_2$, by the computer program, gave the values of 20, 400, and 32,000 M^{-1} for the stability constants of CaH_2ATP, $CaHATP^-$, and $CaATP^{2-}$, respectively. Application of the approximation techniques to this data had indicated that the stability constant of $CaATP^{2-}$ was 40,000 ± 5,000 M^{-1}.

Because at *p*H 8.0 Mn^{2+} formed a precipitate with 8-hydroxyquinoline, Tiron (catechol-3,5-disulphonic acid) was used as the competing ligand. A complication was introduced by the fact that Mn^{2+} catalyzed the oxidation of the catechol, which led to an increase in the extinction at 325 mμ. However, it was possible to prevent any gross inaccuracies by carrying out the relevant measurements within 1 minute of the first addition of Mn^{2+}.[2]

It was pointed out in the introduction that some previous determinations of the stability constants of metal-adenine nucleotide complexes had been carried out in the presence of relatively high concentrations of sodium and potassium ion. For this reason, the effects of these cations on the stability constants were investigated. Determinations of the stability constants of $MgATP^{2-}$ and $CaATP^{2-}$ in the presence of 0.08 M NaCl and 0.02 M *N*-ethylmorpholine gave the apparent values of 34,000 and 13,300 M^{-1}, respectively, as against 73,000 and 31,000 M^{-1} in 0.1 M *N*-ethylmorpholine. The relationship

$$K = K_{app} \{1 + K_{NaATP^{3-}} [Na^+]\}$$

made it possible to obtain an estimate of 15 ± 2 M^{-1} for the stability constant of $NaATP^{3-}$ in 0.1 M *N*-ethylmorpholine at 30°. Similarly, a value of 14 M^{-1} was obtained for $KATP^{3-}$. (These values obtained with the monovalent ions, Na^+ and K^+, can be compared with those of Melchior (1954), who obtained approximately 10 M^{-1} in 0.2 M $(CH_3)_4N^+$ at 25° for both complexes with ATP, and with Smith and Alberty (1956b), who obtained 11 and 8.5 M^{-1} for $NaATP^{3-}$ and $KATP^{3-}$, respectively, in 0.2 M $(C_2H_5)_4N^+$ at 25°.)

Using these figures and correcting for ionic strength and temperature according to Burton (1959), it is possible to obtain the values of 15,000 and 6,600 M^{-1} for the apparent stability constants of $MgATP^{2-}$ and $CaATP^{2-}$ under approximately physiological conditions (0.165 M Na^+, *p*H 7.4, 37°).

Stability Constants of Magnesium-Phosphagen Complexes.—In studying the metal activation of phosphoryl-group-transferring enzymes it may be necessary to consider the interaction of the metal with the nonnucleotide substrate. Thus to provide information for the studies on ATP–creatine phosphotransferase (Morrison *et al.*, 1961) an estimate of the stability constant of Mg-phosphocreatine was made, using Burton's (1959) method. The value obtained was 40 ± 16 M^{-1} at 30° in 0.1 M *N*-ethylmorpholine buffer at *p*H 8.0. The standard error of this result is rather high because the weakness of the complex prevented estimation of $[Mg^{2+}]_{1/2}$ values, so that reliance had to be placed on plots of initial slope against phosphocreatine concentration. It is in reasonable agreement with the value of 20 M^{-1} reported by Smith and Alberty (1956a).

A similar procedure gave 100 M^{-1} for the stability constant of Mg-phosphoarginine under the same conditions. This higher value may arise because the larger hydrocarbon chain of this molecule makes possible the formation of a metal complex involving not only the phosphate moiety but also the carboxylate group.

Discussion

This study was initiated in an attempt to obtain reliable values for the stability constants of $MgADP^-$ and $MgATP^{2-}$ under conditions similar to those being used for kinetic studies of enzymic reactions (Morrison *et al.*, 1961). The initial phase was extended to include other metal complexes of interest. The agreement between the results obtained for $MgATP^{2-}$ (and $CaATP^{2-}$) using both *p*H titration and spectrophotometry suggests that the values are reliable. The spectrophotometric technique (Burton, 1959) seems to have more general practical application, as it can be used to obtain "apparent" constants under specified conditions.

In general, the present values are higher than any reported previously, although they are not very different from those of Burton (1959). For example, if Burton's value for the stability constant of $MgATP^{2-}$ is extrapolated to the experimental conditions reported above (using his temperature coefficient and ignoring the slight difference in ionic strength), a value of approximately 45,000 M^{-1} is obtained, which may be compared to 70,000 M^{-1} reported in this study.

Recently, two publications have given comparable estimates for $MgATP^{2-}$. Watanabe (1962) reported, "The fluorescence of Mg^{2+}-(8-hydroxy)quinoline was used to estimate the apparent binding (or stability) constants for complexes in 0.1 M triethanolamine at 25°. Such constants exceed those reported by others, except that the Mg^{2+}-ATP^{4-} value resembles that of O'Sullivan and Perrin," and Asai and Morales (1962)

[2] In conjunction with Dr. M. E. Winfield of the Division of Physical Chemistry, C.S.I.R.O., Melbourne, an attempt was made to measure the stability constant of $MnADP^-$ using an electron resonance technique. It was noted that the amplitude of the signal due to Mn^{2+} was reduced on the addition of ADP. As $MnADP^-$ would have been expected to be formed under the experimental conditions, this reduction in signal was taken as a measure of the concentration of this complex and was thus used to obtain an estimate of the stability constant. There is some uncertainty about this figure, as it appears that more than one complex is formed (M. E. Winfield, personal communication), but it is probable that the 1:1 complex predominates and that the value of 25,000 M^{-1} would be suitable for use as an apparent stability constant under the conditions of determination. When the different experimental conditions are taken into account, this value is in reasonable agreement with that of 10,000 M^{-1} reported by Cohn and Leigh (1962).

obtained results in the range 60,000–90,000 M^{-1} in 0.1 M Tris at *p*H 9.0, 23°.[3]

In applying the results reported in this paper to experiments, it is evident from the above and from Burton's (1959) work that changes in *p*H, ionic strength, and temperature, and the presence of appreciable concentrations of ions such as Na^+ and K^+ can profoundly affect the magnitude of the "apparent" stability constant. (It should be noted that the value, 16,500 M^{-1}, reported for $MgATP^{2-}$ by Taqui Khan and Martell (1962) was obtained in the presence of 0.1 M KNO_3.) The interaction between tetraethylammonium ion and ATP has been reported to be negligible (Smith and Alberty, 1956b). The agreement between the *p*H values and the values obtained for the stability constants in *N*-ethylmorpholine and triethanolamine also suggests that neither of the buffer cations interacts appreciably with either the phosphate moiety or Mg^{2+}. (There is no significant interaction between Mg^{2+} and the anions used, viz., Br^- and Cl^- [Bjerrum *et al.*, 1958].) However, it is seen that Tris does have an effect on the apparent stability constant (Table II). This would most probably be due to interaction between Tris and Mg^{2+}, though it has been claimed that the binding of Mg^{2+} and Ca^{2+} to this buffer may be neglected (Nanninga, 1957), but some interaction between Tris anion and the phosphate moiety of ATP may be possible.

The validity of the values obtained is dependent on there being no significant breakdown of the nucleotides during the measurements. Experiments in this laboratory indicated that this was the case at *p*H 8.0 and it was also estimated that any breakdown during the short period that the ATP was subject to acid condition during the titration experiments was negligible (see Tetas and Lowenstein, 1963). Also, the *pK* for the reaction

$$Mg^{2+} + OH^- \rightleftharpoons MgOH^+$$

was determined as 12.8 (in 0.1 M KCl at 30°) by Chaberek *et al.* (1952) so that this reaction would not interfere with the measurements.

Cohn and Hughes (1962) found that for $MgATP^{2-}$ and $CaATP^{2-}$ the metal ion is bound only to the phosphorus oxyanions. If this is also true for other nucleotides, then it is probable that the nature of the nitrogen base would not have a substantial effect on the magnitude of the stability constants of their Mg and Ca complexes. Thus, provided that the differences in the pK_a values for the different nucleotide triphosphates are sufficiently small, it might be expected that their Mg and Ca complexes would be similar to those for $MgATP^{2-}$ and $CaATP^{2-}$ under the same experimental conditions. This would be in accordance with the findings of Walaas (1958).

Finally, it should be stressed that the computer is no more able than the original approximation techniques to solve the equations set up to obtain the stability constants from the *p*H titration data. Rather, it allows the testing of a large range of estimates of K_1, K_2, and K_3 to see which values best fit the experimental results. It is interesting to note that the approximation method, in which K_1 was considered to be negligible at sufficiently high *p*H values, gave results in reasonable agreement with those obtained from the computer. The existence of complexes such as $NaATP^{3-}$ and Mg_2ATP was not considered, as to do so made the program too unwieldy. The assumption that these complexes were present at only insignificant concentrations for the titration with equimolar concentrations of ATP and Mg appears to be justified from the good agreement between the *p*H titration and spectrophotometric results.

Acknowledgment

We should like to thank Dr. J. F. Morrison for his interest in this work.

Appendix

Analysis of Data by the IBM 1620 Computer.—The same basic equations describing the equilibria between the different species of ATP and the corresponding magnesium complexes were used except that K_1 (the stability constant of MgH_2ATP) was included. Equations (1), (2), and (3) may be expressed as:

$$[L]_T = pc + pm(k_1h^2 + k_2h + k_3) \quad \text{(i)}$$

$$[M]_T = m + pm(k_1h^2 + k_2h + k_3) \quad \text{(ii)}$$

$$b + 2m = k_2hpm + 2k_3pm + ap \quad \text{(iii)}$$

where $a = 4 + \frac{3h}{K_{a_2}} + \frac{2h^2}{K_{a_1}K_{a_2}}$

$$b = [K^+] + h + 2[L]_T - [OH^-] - 2[M]_T$$

$$c = 1 + \frac{h}{K_{a_2}} + \frac{h^2}{K_{a_1}K_{a_2}}$$

and $m = [Mg^{2+}]$, $p = [L^{4-}]$, $h = [H^+]$,

$$k_1 = \frac{K_1}{K_{a_1}K_{a_2}}, \quad k_2 = \frac{K_3}{K_{a_2}}, \quad k_a = K_3$$

From equations (i) and (ii)

$$[L]_T - [M]_T = pc - m = d$$

Substituting for m in equations (ii) and (iii) and expanding gives:

$$cp^2(k_1h^2 + k_2h + k_3) + p(c - dk_1h^2 - dk_2h - dk_3) - [L]_T = 0 \quad \text{(iv)}$$

and

$$cp^2(k_2h + 2k_3) + p(a - 2c - dk_2h - 2dk_3) - (b - 2d) = 0 \quad \text{(v)}$$

Provided that $(b - 2d) \neq 0$, then equation (iv) may be multiplied by $(b - 2d)$, equation (v) by $[L]_T$, and the two equated. Rearrangement gives an expression in p, viz.,

$$p = \frac{[L]_T(a - 2c - dk_2h - 2dk_3) - (b - 2d)(c - dk_1h^2 - dk_2h - dk_3)}{c(b - 2d)(k_1h^2 + k_2h + k_3) - c[L]_T(k_2h + 2k_3)} \quad \text{(vi)}$$

This value of p may be inserted in equation (v) to give an equation in k_2 and k_3. By selecting points in pairs, two equations in k_2 and k_3 are obtained and hence solutions for k_2 and k_3. Then k_1 may be obtained from equation (iv).

A program to solve for K_1, K_2, and K_3 was written by Miss E. A. Reid for an IBM 1620 digital computer using Fortran. The logic of this program was as follows: The constants, K_1, K_2, and K_3, are assigned arbitrary values. Pairs of points, designated as the $2n$th and nth

[3] Note added in proof: S. Watanabe, T. Troper, M. Lynn, and L. Evenson (1963; *J. Biochem. (Tokyo) 54*, 17) have published details of stability-constant measurements utilizing the fluorescence of magnesium-8-hydroxyquinoline. The results are, in general, similar to those reported here. They obtained values of 6–8 × 10^4 M^{-1} and 3–5 × 10^4 M^{-1} for $MgATP^{2-}$ at 25° in 0.1 M triethanolamine and 0.1 M Tris, respectively, in the *p*H range 8.0–8.3.

points, at intervals along the titration curve were selected for solution.

Using the data and the $2n$th point, and with K_1 and K_2 zero, equation (vi) is solved for p and the values obtained substituted into equation (v) to give a value, S. If S is positive, K_3 is increased by an increment, z, and the process repeated until S just changes sign. If S is negative, K_3 is decreased by z, again until S changes sign. The value of K_3 obtained to just cause S to change sign is designated α. The process is repeated at the nth point to obtain a second estimate, γ, of K_3.

The whole procedure is repeated using a second value of K_2, this constant being incremented by y. In this way, two further estimates of K_3 are obtained; viz., β and δ, at the $2n$th and nth points, respectively. The estimates of K_3 thus obtained with different values of K_2 are compared.

If $|\beta - \delta| < |\alpha - \gamma|$, the process is repeated, with $K_2 = K_2 + 2y$.

If $|\beta - \delta| \geq |\alpha - \gamma|$, the computer prints out the values of K_2 and K_3, and of p and m at the $2n$th and nth points.

The entire process is repeated with K_1 increased by an increment, x. In this way a particular pair of points would give values of K_2 and K_3 for each selected value of K_1.

At the start of a computation, K_1 and K_2 were set equal to zero, K_3 usually at 50,000 M^{-1}. The increments y and z for K_2 and K_3, respectively, were usually set at 100 M^{-1}, and x, for K_1, at 10 M^{-1}. It was found that the magnitude of K_1 had a comparatively small effect on the value obtained for K_3 but a somewhat larger effect on K_2. In general, meaningful results were not obtained with K_1 greater than 20 M^{-1}.

A summary of some values obtained is shown in Table V.

TABLE V

SUMMARY OF SOME REPRESENTATIVE VALUES OBTAINED FROM THE IBM 1620 COMPUTER FOR THE STABILITY CONSTANTS OF THE COMPLEXES MgH_2ATP (K_1), $MgHATP^-$ (K_2), AND $MgATP^{2-}$ (K_3).

K_1[a]	K_2[a]	K_3[a]	Points Used[b]
Zero	400	73,500	25, 15
	570	74,200	30, 20
	480	78,200	36, 26
	420	75,800	41, 31
20	400	74,200	25, 15

[a] All values are expressed as M^{-1}.

[b] The position of the points refers to each addition of 0.01 ml KOH (see Methods), the titration curve being composed of 50 points.

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