Biochem. J. (1976) **159**, 1–5
Printed in Great Britain

Concentration of $MgATP^{2-}$ and Other Ions in Solution

CALCULATION OF THE TRUE CONCENTRATIONS OF SPECIES PRESENT IN MIXTURES OF ASSOCIATING IONS

By ANDREW C. STORER* and ATHEL CORNISH-BOWDEN
Department of Biochemistry, University of Birmingham, P.O. Box 363, *Birmingham B*15 2*TT, U.K.*

(*Received* 25 *February* 1976)

1. A simple method is described for calculating the free concentrations of all species in a mixture of several ionic components that associate at equilibrium to any extent and with any stoicheiometry. 2. It can readily be adapted to take account of species such as protons for which the free rather than the total concentrations are controlled. 3. It was applied to mixtures of adenine nucleotides, Mg^{2+} and other ions relevant to the study of glucokinase (EC 2.7.1.2), but the qualitative conclusions are not peculiar to this system. 4. ATP exists in a high and nearly constant proportion (about 80%) as $MgATP^{2-}$ in solutions in which the total $MgCl_2$ concentration exceeds the total ATP concentration by 1–10mM. 5. By contrast, the proportion of ATP present as $MgATP^{2-}$ varies greatly if the total $MgCl_2$ and total ATP concentrations are varied in constant proportion.

A major problem in the study of enzymes that require nucleoside phosphates is the multifarious composition of mixtures of such phosphates with metal ions. For example, an equimolar mixture of ATP and $MgCl_2$ at pH7 contains appreciable proportions of $MgATP^{2-}$, ATP^{4-}, $HATP^{3-}$, Mg^{2+} and Cl^-, as well as traces of $MgHATP^-$, Mg_2ATP and $MgCl^+$. Until fairly recently it was common practice to oversimplify this problem, by assuming, for example, that if the concentrations of metal ion and nucleotide were varied in constant ratio the nucleotide would exist largely as a 1:1 complex with the metal ion. The need to place stricter controls on the way in which cation and nucleotide concentrations are varied has led to several methods for calculating the concentrations of the various species in such mixtures (e.g. Melchior & Melchior, 1958; McQuate & Utter, 1959; McGilvery, 1965; Botts *et al.*, 1966; Blair, 1970; MacFarlane & Ainsworth, 1972; De Weer & Lowe, 1973). As a necessary concomitant of these methods considerable effort has been devoted to the measurement of the stability constants of the complexes between adenine nucleotides and Mg^{2+} (McQuate & Utter, 1959; O'Sullivan & Perrin, 1961, 1964; Phillips *et al.*, 1963, 1966; Watanabe *et al.*, 1963; De Weer & Lowe, 1973).

Most current methods for calculating the composition of a given system require a set of system-specific equations, which are complicated if there are more than two components and require iterative methods for their solution. This general approach has two drawbacks: not only must a new set of equations be derived for each system studied, but when a new species is included in a system a complete set of new equations is required. In practice such methods are inflexible and difficult to apply to systems of more than two components.

In this paper we describe a simpler and more adaptable method, which is similar in principle to one used by Perrin (1965) and by Perrin & Sayce (1967) for the analysis of mixtures of inorganic complex ions. It is readily applicable to any aggregating system of several components, provided the relevant equilibrium constants are known, and thus has considerable potential for the study of systems of biological importance.

Our interest in this work orginated with the need to vary $MgATP^{2-}$ concentrations systematically in kinetic studies of rat liver glucokinase (EC 2.7.1.2). A few simple rules proved sufficient to control the concentrations of $MgATP^{2-}$ and other key species, such as Mg^{2+} and ATP^{4-}, within narrow limits, and exact calculation of concentrations was not usually necessary except as a refinement. Since these rules proved of great value in planning experiments (see Storer & Cornish-Bowden, 1976), they are described in this paper, together with suggestions about how they may be adapted for use with other enzymes.

Principle

Consider a mixture of m different complexes formed by association of n different components. The total concentration A_i of the ith component is the sum of its free concentration a_i and the concentrations x_j of all the m complexes, each of the latter being given a

* Present address: Department of Chemistry, Cornell University, Ithaca, NY 14850, U.S.A.

weight α_{ij} equal to the number of molecules or ions of the ith component contained in one molecule or ion of complex:

$$A_i = a_i + \sum_{j=1}^{m} \alpha_{ij} x_j \quad (1)$$

For example, consider a system of three components, R, S and T, which associate to give four different complexes, RR, RS, ST and RST. In this case eqn. (1) can be expanded for the three components as follows:

$$A_R = a_R + 2[RR] + 1[RS] + 0[ST] + 1[RST] \quad (2)$$

$$A_S = a_S + 0[RR] + 1[RS] + 1[ST] + 1[RST] \quad (3)$$

$$A_T = a_T + 0[RR] + 0[RS] + 1[ST] + 1[RST] \quad (4)$$

The concentration x_j of the jth complex can be expressed in terms of the free concentrations of the components and the association constant K_j for its formation from the free components:

$$x_j = K_j \prod_{k=1}^{n} a_k^{\alpha_{kj}} \quad (5)$$

Substituting eqn. (5) into eqn. (1) gives

$$A_i = a_i + \sum_{j=1}^{m} \left(\alpha_{ij} K_j \prod_{k=1}^{n} a_k^{\alpha_{kj}} \right) \quad (6)$$

which can be rearranged to express any of the free concentrations a_i in terms of the total concentration A_i:

$$a_i = A_i a_i \Big/ \left[a_i + \sum_{j=1}^{m} \left(\alpha_{ij} K_j \prod_{k=1}^{n} a_k^{\alpha_{kj}} \right) \right] \quad (7)$$

In general there are n equations of this type. When all of the free concentrations a_i are known they are simply truisms, containing no information. But when the a_i concentrations are estimates of the true values, they can be replaced with better estimates by successive applications of eqn. (7). Each free concentration a_i is initially set to the corresponding total concentration A_i. The resulting estimates are then inserted in the right-hand side of eqn. (7), with $i = 1$, to provide an improved estimate of a_1. This improved estimate is then used with the remaining original estimates to calculate an improved estimate of a_2. In this way improved estimates of all the a_i up to a_n are obtained. These are likely to be very poor approximations to the true values, and the entire cycle must be repeated several times until the results are self-consistent, i.e. until the left- and right-hand sides of eqn. (7) agree to within 0.01% for all n components. Provided that the procedure converges to a solution that satisfies eqn. (6) for all i components simultaneously, the solution must describe the equilibrium state of the system, because a given set of equilibrium constants defines a unique state. In our experience the procedure always converges smoothly. Once the free concentrations are known with sufficient accuracy the concentration of each complex may be calculated directly by means of eqn. (5).

It is sometimes necessary to calculate the composition of a mixture in which the free concentration of one of the components is fixed in advance. For example, if protonated and unprotonated forms of a species are distinguished the proton must be considered as one of the components. But if the solution is buffered the free proton concentration must be treated as a constant. This may be achieved by using eqn. (6) in each cycle for the fixed component instead of eqn. (7), and still using eqn. (7) for the others. This ensures that the free concentration of the fixed component remains constant during the iterative procedure, whereas the total concentration is allowed to vary.

Implementation

The iterative procedure described has been incorporated into a FORTRAN IV program, which has been used extensively in the study of glucokinase (Storer & Cornish-Bowden, 1976). Slightly different versions have been written for use with an International Computers Ltd. 1906A computer and with a Digital Equipment Corporation PDP11 computer. A listing is available on request, together with instructions for use and sample output. Table 1 illustrates the progress of the calculations in a simple case.

Applications

The most obvious application of the program described is for calculating the compositions of mixtures of interacting components. But it has also proved very useful as an aid to experimental design, and examples of this application will now be given, with some results of general importance.

Many $MgATP^{2-}$-dependent enzymes are affected by variation in the concentrations of Mg^{2+} and ATP^{4-}, and possibly of minor species as well. Consequently, in studying the dependence of the rate on the $MgATP^{2-}$ concentration, it is desirable not simply to know the concentrations of Mg^{2+}, ATP^{4-} and other relevant species, but also to maintain them at values as nearly constant as possible or at values that vary in a simple way with the concentration of principal interest, i.e. that of $MgATP^{2-}$. These extraneous concentrations must also be maintained at values where they do not mask or seriously interfere with the effects of the species under study.

The program described is capable of determining the exact amounts of the components required to give any concentration of a particular species in a mixture, but it is inconvenient to calculate and prepare exact amounts of all components for each assay and a more general approach is desirable. The program also

Table 1. *Calculation of true concentrations for a three-component system with four complexes*

The total concentrations of three components, R, S and T, were as follows: $A_R = 10.0$mM; $A_S = 1.0$mM; $A_T = 2.0$mM. Association constants were as follows: RR, 0.05mM^{-1}; RS, 50.0mM^{-1}; ST, 0.5mM^{-1}; RST, 25.0mM^{-2}.

(a) Iterative determination of free concentrations

Iteration	a_R (mM)	a_S (mM)	a_T (mM)	Number of values within 0.01% of previous line
0	10.000000	1.000000	2.000000	0
1	0.098039	0.084718	1.600000	0
2	1.158159	0.009431	1.565217	0
3	5.111437	0.002186	1.561905	0
4	5.862215	0.001909	1.561587	0
5	5.694078	0.001965	1.561556	1
6	5.732588	0.001952	1.561553	1
7	5.723792	0.001955	1.561553	1
8	5.725802	0.001955	1.561553	1
9	5.725343	0.001955	1.561553	3

(b) Final concentrations (to 3 significant figures)

Species	Concentration
R	5.73mM
S	1.96μM
T	1.56mM
RR	1.64mM
RS	0.560mM
ST	1.53μM
RST	0.437mM

allows the formulation of general rules for controlling concentrations in any situation. Although the examples to be given apply particularly to glucokinase kinetics, the method is general and the results can be used as guidelines for the formulation of rules for other systems.

Mixtures of ATP, $MgCl_2$ and KCl at various pH values

The first aim was to determine the pH most suitable for the study of glucokinase kinetics, and a mixture containing 5mM-ATP, 6mM-$MgCl_2$ and 100mM-KCl (total concentrations) was analysed as a function of pH. The relevant equilibria are shown in Table 2, with some others required later in this paper. At pH values below 8 the composition varies in a highly complicated way and about eight different species need to be considered (Fig. 1), but at pH values above 8 the composition not only becomes pH-independent but the species of prime interest, $MgATP^{2-}$, reaches its maximum concentration and the concentrations of the inhibitors $HATP^{3-}$ and $MgHATP^{-}$ become negligible. Similar results were obtained for other compositions of the mixture, and so the subsequent experimental work was done at pH8.0 instead of pH7.5, which had been used in previous studies of glucokinase (Parry & Walker, 1966).

The effect of varying the ionic strength was investigated similarly. The concentrations of all the species of interest exhibit a stationary point (minimum or maximum) near an ionic strength of 0.15M and vary only slightly in the ionic-strength range 0.1–0.2mol/litre. Accordingly, all experiments were done at an ionic strength of about 0.15mol/litre and this value will be assumed in the remainder of this paper.

It was next necessary to formulate a simple way of preparing solutions of ATP such that the nucleotide would exist largely as $MgATP^{2-}$ with small and preferably constant concentrations of other species. The proportion of ATP existing as $MgATP^{2-}$ was calculated for various mixtures of ATP and $MgCl_2$ under assay conditions, i.e. in 100mM-KCl, 50mM-tetramethylammonium glycylglycinate buffer, at pH8.0. The results (Table 3) show that if there is a constant excess of 1–10mM total $MgCl_2$ over total ATP the proportion of ATP present as $MgATP^{2-}$ is both large (84–90%) and nearly constant. If constancy of this proportion were the only consideration, an excess of 5mM would be ideal, as it gives a variation of only 89.3–90.1% over the range 0.001–100mM in the total ATP concentration. However, Mg^{2+} is an inhibitor of glucokinase, and is present at a much lower concentration if the excess is 1mM rather than 5mM. Accordingly, a 1mM excess of $MgCl_2$ over ATP is appropriate for studying glucokinase, and in the range of total ATP concentrations required, 0.1–10mM, it gives an acceptably constant proportion (84–88%) of $MgATP^{2-}$. The adequacy of this experi-

Table 2. *Complexes formed by components of the assay mixture for glucokinase*

Reaction	$K_{ass.}$ (M^{-1})	Reference
(1) $HATP^{3-}+H^+ \rightleftarrows H_2ATP^{2-}$	8.5×10^3	O'Sullivan & Perrin (1964)
(2) $ATP^{4-}+H^+ \rightleftarrows HATP^{3-}$	1.09×10^7	Phillips *et al.* (1966)*
(3) $ATP^{4-}+K^+ \rightleftarrows KATP^{3-}$	1.4×10	O'Sullivan & Perrin (1964)
(4) $H_2ATP^{2-}+Mg^{2+} \rightleftarrows MgH_2ATP$	2.0×10	O'Sullivan & Perrin (1964)
(5) $HATP^{3-}+Mg^{2+} \rightleftarrows MgHATP^-$	5.42×10^2	Phillips *et al.* (1966)*
(6) $ATP^{4-}+Mg^{2+} \rightleftarrows MgATP^{2-}$	3.48×10^4	Phillips *et al.* (1966)*
(7) $MgATP^{2-}+Mg^{2+} \rightleftarrows Mg_2ATP$	4.0×10	Noat *et al.* (1970)
(8) $Cl^-+Mg^{2+} \rightleftarrows MgCl^+$	3.4	Blair (1970)
(9) $GlyGly^-$†$+H^+ \rightleftarrows HGlyGly$	1.32×10^8	Sillén & Martell (1964)
(10) $GlyGly^-+Mg^{2+} \rightleftarrows MgGlyGly^+$	2.16×10	Sillén & Martell (1964)

* Phillips *et al.* (1966) give more general expressions for the association constants, from which the values shown in this table were calculated by assuming an ionic strength of 0.15mol/litre.

† Abbreviation: $GlyGly^-$, glycylglycinate.

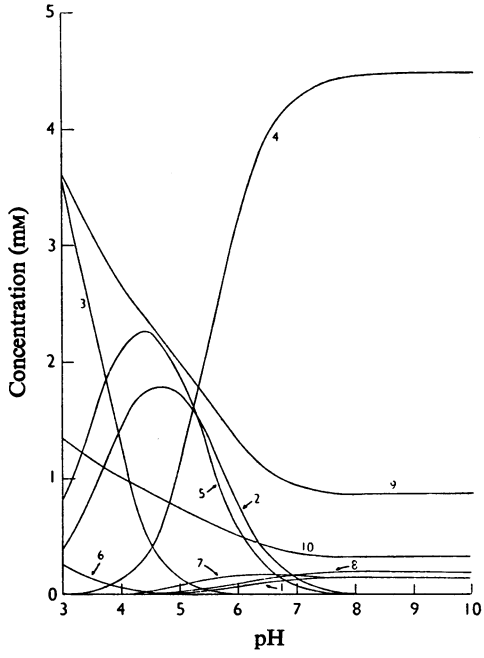

Fig. 1. *Species present in mixtures of ATP, $MgCl_2$ and KCl*

The concentrations were calculated for mixtures containing 5mM-ATP, 6mM-$MgCl_2$ and 100mM-KCl, at an ionic strength of 0.15mol/litre and variable pH as indicated. The results vary only slightly with ionic strength in the range 0.1–0.2mol/litre. Key: 1, ATP^{4-}; 2, $HATP^{3-}$; 3, H_2ATP^{2-}; 4, $MgATP^{2-}$; 5, $MgHATP^-$; 6, MgH_2ATP; 7, Mg_2ATP; 8, $KATP^{3-}$; 9, Mg^{2+}; 10, $MgCl^+$.

mental design may be judged from Fig. 2, which shows the variation of all significant species with the total ATP concentration when the total $MgCl_2$ is maintained in 1mM excess.

Table 3. *Percentage of total ATP present as $MgATP^{2-}$ in solutions containing an excess of $MgCl_2$*

The values were calculated for mixtures at pH8.0 containing 50mM-glycylglycine, 100mM-KCl, total ATP concentrations as indicated, total $MgCl_2$ concentrations greater by the amounts indicated than the total ATP concentrations.

Excess of $MgCl_2$ (mM)	Total ATP (mM)					
	0.001	0.01	0.1	1.0	10.0	100.0
0.0	0.6	5.2	28.9	65.6	85.3	90.0
0.001	1.1	5.6	29.1	65.6	85.3	90.0
0.01	5.9	9.8	31.1	65.9	85.3	90.0
0.1	36.5	37.8	46.6	69.2	85.7	90.0
1.0	83.9	83.9	84.0	85.1	88.3	90.1
2.0	89.2	89.2	89.2	89.2	89.9	90.1
5.0	89.3	89.3	89.3	89.3	89.6	90.1
10.0	84.0	84.0	84.0	84.1	85.3	89.2
100.0	30.7	30.7	30.7	30.9	32.5	49.9

Effect of varying ATP and $MgCl_2$ in constant ratio

An alternative design that is sometimes used is to vary the total concentrations of ATP and $MgCl_2$ in constant ratio. This design gives very poor results, far worse than those obtained by keeping one component in constant excess over the other. For example, if the total ATP concentration varies from 0.1 to 10mM, the proportion existing as $MgATP^{2-}$ varies in the range 28.9–85.3% when the molar ratio of $MgCl_2$/ATP is 1, 46.6–85.3% when the molar ratio is 2, 74.5–89.3–52.9% when the molar ratio is 6, and so on. There are similarly large variations in the concentrations of other species, and there is no molar ratio that gives even barely acceptable results.

Discussion

Mixtures of several interacting cations and anions occur frequently in biochemistry. The methods of analysis discussed in this paper have wide potential,

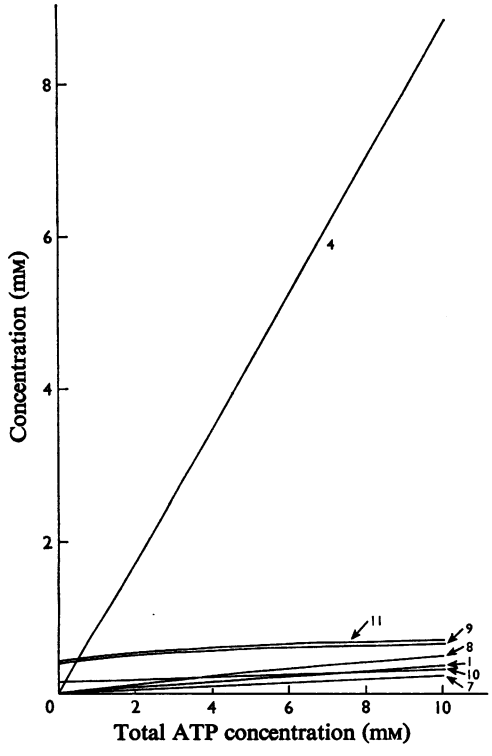

Fig. 2. *Effectiveness of maintaining $MgCl_2$ in constant excess over ATP*

The compositions were calculated of mixtures at pH 8.0 containing 100mM-KCl, 50mM-tetramethylammonium glycylglycinate, total ATP as indicated, and total $MgCl_2$ greater by 1mM than the total ATP concentrations. Key: 1, ATP^{4-}; 4, $MgATP^{2-}$; 7, Mg_2ATP; 8, $KATP^{3-}$; 9, Mg^{2+}; 10, $MgCl^+$; 11, Mg-glycylglycinate$^+$.

therefore, beyond the restricted context in which they have been developed. It is of general interest, for example, that maintaining a total $MgCl_2$ concentration in constant excess over the total ATP concentration ensures, for a wide range of concentrations, that the proportion of ATP present as $MgATP^{2-}$ is both high and nearly constant, and that the concentration of Mg^{2+} varies rather little. Similar results must also apply to other metal ions and other anions, because they are a direct effect of the law of mass action as applied to a binary complex at equilibrium, and do not depend on any special properties of Mg^{2+} and ATP^{4-}. Only the quantitative details need to be established for each system, and these may require only minor modification from those that apply to the phosphorylation of glucose. For example, substitution of another sugar or another non-ionic substrate for glucose is unlikely to have any drastic effects on the ionic interactions.

When modifications to the results given in this paper are appropriate they are often obvious: for example, in the study of an enzyme that is not inhibited by Mg^{2+} there is no need to keep the concentration of Mg^{2+} low and a 5mM excess of $MgCl_2$ over ATP is likely to give better results than the 1mM excess found to be suitable for glucokinase (cf. Table 3).

In this paper we have discussed in detail only one relatively simple system. However, we have applied the same methods to several more complex systems, to determine, for example, the effects of ADP and glucose 6-phosphate on the glucokinase assay mixture, or the stoicheiometry of proton release at different pH values in the glucokinase reaction. The methods described proved easily capable of dealing with these systems, and details are in Storer (1975).

We thank the Medical Research Council for financial assistance, and the University of Birmingham for a University Research Scholarship (to A. C. S.).

References

Blair, J. McD. (1970) *Eur. J. Biochem.* **13**, 384–390
Botts, J., Chashin, A. & Schmidt, L. (1966) *Biochemistry* **5**, 1360–1364
De Weer, P. & Lowe, A. G. (1973) *J. Biol. Chem.* **248**, 2829–2835
MacFarlane, N. & Ainsworth, S. (1972) *Biochem. J.* **129**, 1035–1047
McGilvery, R. W. (1965) *Biochemistry* **4**, 1924–1930
McQuate, J. T. & Utter, M. F. (1959) *J. Biol. Chem.* **234**, 2151–2157
Melchior, N. C. & Melchior, J. B. (1958) *J. Biol. Chem.* **231**, 609–623
Noat, G., Ricard, J., Borel, M. & Got, C. (1970) *Eur. J. Biochem.* **13**, 347–363
O'Sullivan, W. J. & Perrin, D. D. (1961) *Biochim. Biophys. Acta* **52**, 612–614
O'Sullivan, W. J. & Perrin, D. D. (1964) *Biochemistry* **3**, 18–26
Parry, M. J. & Walker, D. G. (1966) *Biochem. J.* **99**, 266–274
Perrin, D. D. (1965) *Nature (London)* **206**, 170–171
Perrin, D. D. & Sayce, I. G. (1967) *Talanta* **14**, 833–842
Phillips, R. C., George, P. & Rutman, R. J. (1963) *Biochemistry* **2**, 501–508
Phillips, R. C., George, P. & Rutman, R. J. (1966) *J. Am. Chem. Soc.* **88**, 2631–2640
Sillén, L. G. & Martell, A. E. (1964) *Chem. Soc. Spec. Publ.* **17**, 430–431
Storer, A. C. (1975) Ph.D. Thesis, University of Birmingham
Storer, A. C. & Cornish-Bowden, A. (1976) *Biochem. J.* **159**, 7–14
Watanabe, S., Trosper, T., Lynn, M. & Evenson, L. (1963) *J. Biochem. (Tokyo)* **54**, 17–24