Modifying Effects of Anions on the Alkali-Cation-Activated AMP Deaminase of Human Erythrocyte

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SUMMARY

Human erythrocyte contains an AMP deaminase (AMP aminohydrolase, EC 3.5.4.6) which requires the presence of certain alkali cations. ATP modifies the selective response of this enzyme to various alkali cations. The object of this work was to determine whether anions other than ATP could have similar effects.

AMP deaminase activities in the presence of various anions (SO_4^{2-}, Cl^-, Br^-, I^-, NO_3^-, and $PO_4^- - PO_4^{2-}$) were measured. The results showed that a change in the major anion of the system modifies the enzyme activity, and that the relative effects of anions are influenced by the nature of the activating cation. The effects of the substrate concentration and the activator-cation concentration on the enzyme activity were studied under conditions of constant and maximal anion effects. The data did not conform to simple Michaelis-Menten kinetics. At lower ranges of substrate and cation concentrations the results could be described by the empirical Hill equation. Although some reaction schemes consistent with the kinetic data were suggested, no definite conclusions about the mechanisms of the cation and anion effects could be reached. The studies did prove, however, that a clear distinction between the selective response of the enzyme to various alkali cations and the selective binding of these ions to the enzyme should be made.

Interactions of ATP and orthophosphate with the enzyme were investigated. It was shown that, in the presence of the two anions, decrease in ATP concentration and increase in orthophosphate concentration could have synergistic inhibitory effects on the enzyme activity. Possible significance of such effects in relation to the mechanisms for the control of adenine nucleotide content of erythrocyte was pointed out.

INTRODUCTION

The assumption of the presence of one or more cellular constituents with high degree of selectivity for the binding of alkali cations is common to most of the theories of the origin of the cellular alkali-cation selectivity. Of the many compounds of biologic origin which are known either to bind selectively or to respond selectively to various alkali cations, only a few enzymes have selectivities whose magnitude approach that of the whole cell (1, 2). Therefore, the study of the molecular and submolecular basis for the selective activation or inhibition of any one of these enzymes will contribute to the better understanding of the mechanism of the cellular alkali-cation selectivity and of the many physiologic and pharmacologic phenomena related to this cell property.[1]

Previous work from this laboratory has shown the absolute requirement of human erythrocyte AMP deaminase (AMP aminohydrolase, EC 3.5.4.6) for certain monovalent cations (3). It was also demon-

[1] A preliminary report of this work was presented before the Division of Biological Chemistry at the 150th National Meeting of American Chemical Society, Atlantic City, New Jersey, September 1965.

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strated that ATP produces both qualitative and quantitative changes in the selective response of this enzyme to various alkali cations (4). Since no chemical alteration of ATP could be detected in its interaction with AMP deaminase from various tissues, it was suggested (4) that the binding of the highly charged ATP anion to the enzyme protein induces changes in protein fixed-charges and leads to variations in the amount and the nature of the counterions associated with the protein. The basis for such a mechanism of action of ATP was sought within the framework of the theories of selective binding of various counterions to polyelectrolyte fixed-charged systems (5, 6). A similar mechanism of action of ATP has also been postulated to explain the effects of ATP on the selective binding of Na^+ and K^+ to membrane-bound ATPase of various tissues (7, 8).

The argument for considering ATP as an anionic modifier of AMP deaminase would become stronger if other anions could also be shown to have effects on the selective response of the enzyme to alkali cations. This work was undertaken to test such a possibility. It should be noted that prior to the discovery of the alkali-cation requirement of this enzyme its activation or inhibition by some anions had been reported (9, 10). However, it was not certain whether these observed anion effects were real or due to variations in the nature and amounts of alkali cations in the assay solutions.

MATERIALS AND METHODS

AMP and the sodium salt of ATP were obtained from Sigma Chemical Co., St. Louis, Missouri. Tris-ATP was prepared by the passage of the sodium salt through a column of Dowex 50 in Tris form. All other chemicals were of Reagent Grade quality and were used without further purification. The various $Tris^+$-anion buffers were prepared by the following methods: (a) If the purified acid were available, e.g., HCl or H_2SO_4, the buffer was made by the direct titration of Tris base with the acid. (b) When it was difficult to work with the pure acid, e.g., HI, a column of Dowex 1 in the desired anion form was prepared by the passage of a purified salt of the anion through the column and subsequent washing with water. Tris-HCl was then passed through the column to exchange the Cl^- with the desired anion.

Methods for the preparation of the enzyme source and the determination of enzyme activity have been described (4). The enzyme activities mentioned in this paper are all initial velocities. Under any given condition enzyme activity was determined at three time intervals. The reaction times were chosen to obtain no more than 10–20% disappearance of the substrate. Within these limits the substrate disappearance was a linear function of time in most of the experiments. When slight deviations from linearity were obtained, the initial velocities were calculated from the tangents to the curves. All enzyme activities are expressed as $-\Delta A_{265}$/ml enzyme/h (ref. 4), unless otherwise stated.

RESULTS

Anion Effects

Table 1 shows the results of experiments in which the AMP deaminase activities in the presence of various anions and at fixed concentrations of Na^+ and K^+ were measured. Examination of the data reveal the following specific points: (a) Regardless of the nature of the activating cation, a change in the major anion of the system greatly influences the enzyme activity. (b) Under the conditions of this experiment K^+ is a better activator than Na^+ regardless of the nature of the anion. However, the relative effectiveness of the cations varies depending on the nature of the anion: e.g.,

$$\frac{v(KCl)}{v(NaCl)} = 7.3 \quad \text{and} \quad \frac{v(K_2SO_4)}{v(Na_2SO_4)} = 2.0$$

(c) A corollary of the above is that the relative effects of anions are influenced by the nature of the cations. This is best illustrated by the comparison of the chloride and the nitrate data. Whereas v (Cl^-) $> v$ (NO_3^-) in the presence of K^+, the order is reversed in the presence of Na^+. From this simple experiment the general

conclusion may be drawn that anions modify the activity of AMP deaminase and that there is an interaction between the anion and the cation effects on the enzyme.

In the hope of gaining better insight into the nature of the anion and cation effects on the enzyme, further kinetic studies were attempted. Before the presentation of the data the following clarifications are in order. Because of the method of enzyme assay used in these studies the use of a relatively large amount of buffer (Tris) in the initial reaction mixture was necessary. The presence of Tris cation was of no consequence since it could not replace an alkali cation as a required activator (3). However, the initial presence of an anion in the system created special problems. Thus, it was not possible to study the effect of varying anion concentration on the enzyme activity at concentrations below the buffer anion concentration. Since the activator cations had to be added with a modifier anion, at first it also seemed that the enzyme activity as a function of cation concentration could not be studied independent of anion effects. Preliminary experiments showed, however, that at constant substrate and activator cation concentrations, variation of anion concentration between 100 and 500 mM did not affect the enzyme activity. In Fig. 1 two examples of such experiments are shown. These results proved that by choosing an appropriate initial buffer concentration it was possible to study the effects of both the substrate concentration and the activator-cation concentration under conditions of constant and maximal anion effects.

Three anions (sulfate, chloride, and nitrate) were chosen for further studies. Figure 2 shows the activator-velocity curves over a wide range of Na^+ and K^+ concentrations in the presence of different anions. Some features of these curves are worthy of consideration. (a) In most of the curves inhibition at high concentration of activator is quite evident. Although not shown in the graph, this type of inhibition is obtained in all cases when higher concentrations of

TABLE 1

Effects of various anions on AMP deaminase activity of human erythrocyte

Each assay system contained 10 μmoles of AMP, a fixed amount of enzyme solution, and 100 μmoles of $Tris^+$-anion (pH 7.2). The indicated amounts of Na^+ and K^+ were added as appropriate salts. Sodium and potassium phosphates were added as buffers (pH 7.2). Final volume was 2.5 ml. Solutions were incubated at 37° and then deproteinized by the addition of 1.5 ml of 8% $HClO_4$. Absorbancies of appropriate dilutions of the reaction solutions and controls were measured at 265 mμ. Enzyme activity in the presence of K_2SO_4 was the highest ($-\Delta A_{265}$/ml of enzyme/h = 0.59). The arbitrary value of 100 is assigned to this activity in the table. All other activities are expressed relative to the highest activity.

Anions of the assay medium	Relative enzyme activity in the presence of:	
	K^+ (200 mM)	Na^+ (200 mM)
Sulfate	100	48
Chloride	73	10
Bromide	70	11
Nitrate	42	38
Iodide	8	5
Phosphate	1	0

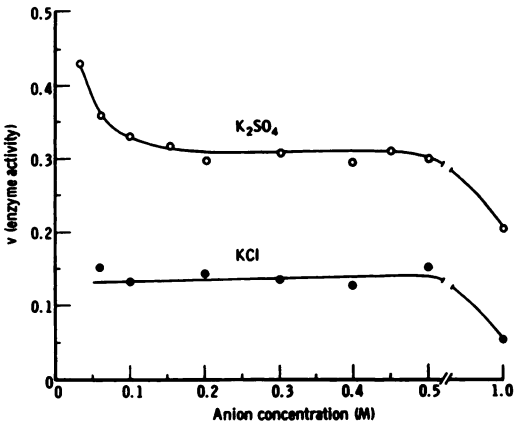

FIG. 1. *Effect of varying anion concentration on AMP deaminase activity at fixed concentrations of substrate and activator cation*

The initial $Tris^+$-anion concentration was 40 mM. [AMP] = 2 mM, and [K^+] = 20 mM. Increasing anion concentrations were obtained by the addition of $Tris^+$-anion buffers (pH 7.2). Other conditions were the same as described for Table 1.

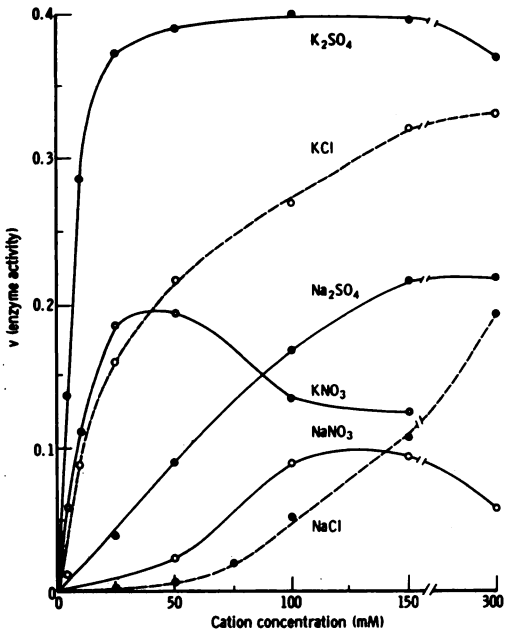

FIG. 2. *Effects of varying Na^+ and K^+ concentrations on AMP deaminase activity in the presence of different anions*

In each reaction system, the concentration of $Tris^+$-anion (pH 7.2) was 60 mM. [AMP] = 4 mM Na^+ and K^+ were added as indicated salts. Other conditions were the same as described for Table 1.

activator cations are used. (b) The rising portions of the Na^+-curves are not hyperbolic and do not conform to simple Michaelis-Menten kinetics. (c) Disregarding the inhibition at high activator concentrations, all the curves of Fig. 2 would fit the familiar Hill equation (11)

$$\log\left(\frac{v_m}{v} - 1\right) = \log K - n \log [A]$$

in which v is velocity, v_m is maximum velocity, [A] is activator concentration, and K and n are constants. In the presence of K^+, $n = 1$ in all cases, whereas $n > 1$ in the presence of Na^+ regardless of the anion used.

When experiments similar to those of Fig. 2 were performed in the presence of 2 mM ATP, the nature of the major anion of the system became irrelevant. Only two curves, one for Na^+ and one for K^+, were obtained. When fitted into the Hill equation the value of n in each case was one. Results of these experiments are shown in Fig. 3 in which log $[(v_m/v) - 1]$ is plotted against log (cation). The slope of each line is equal to $-n$. The data from experiments with NaCl and KCl in the absence of ATP are also included in Fig. 3 for comparison.

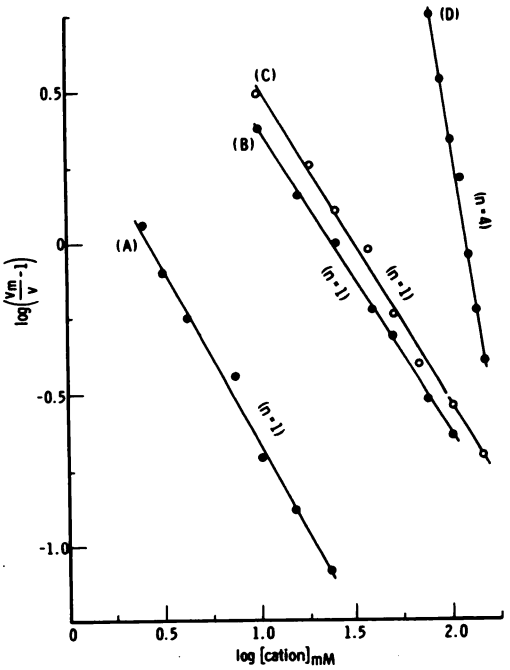

FIG. 3. *Hill plots of AMP deaminase activity as a function of activator-cation concentration*

(*A*), K^+ in the presence of ATP; (*B*), K^+; (*C*), Na^+ in the presence of ATP; (*D*), Na^+. In each reaction the major anion was Cl^-. Other reaction conditions were the same as described for Fig. 2 and Table 1.

The substrate-velocity curves in the presence of various anions and at fixed concentrations of Na^+ and K^+ were also determined. Depending on the concentration of the activator cation, curves of various shapes and degrees of complexity were obtained with each anion. However, in their general features such curves were very similar to the activator-velocity curves. In Fig. 4 one series of substrate-velocity curves are plotted in a manner similar to that of Fig. 3. In all cases $n > 1$. In the presence

of ATP, as in the case of the activator curves, only one curve with $n = 1$ is obtained regardless of the nature of the major anion. This is also shown in Fig. 4.

It has previously been shown (3) that ammonium ion, a product of AMP deamination, is also an activator of AMP deaminase. To avoid further complications due to product activation, the highest substrate concentration used in the above experiments was 8 mM. Under these conditions inhibitory effects at higher substrate concentrations were not evident in most instances. Only in the presence of KNO_3 was this type of inhibition observed. In Fig. 5 the substrate-velocity curves in the presence of KNO_3 and $NaNO_3$ are presented. The results clearly show the dependence of relative activating effects of Na^+ and K^+ on the substrate concentration.

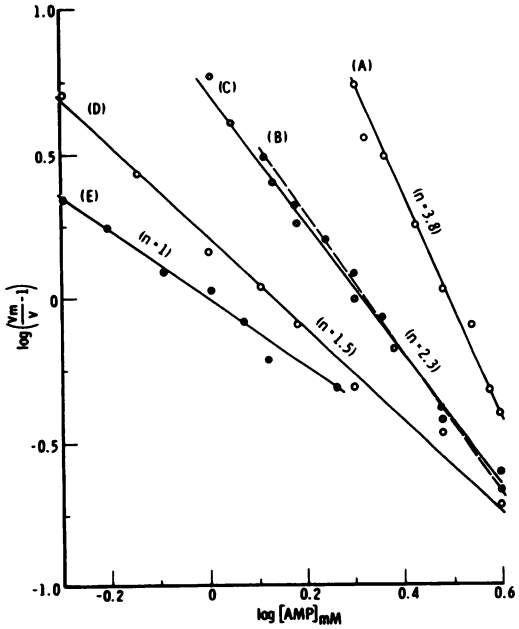

FIG. 4. *Hill plots of AMP deaminase activity as a function of AMP concentration in the presence of various anions*

(*A*), NaCl; (*B*), KCl. (*C*), Na_2SO_4; (*D*), K_2SO_4; (*E*), 2 mM ATP (major anion: either $SO_4^{=}$ or Cl^-; activator cation: either Na^+ or K^+). Other conditions were the same as described for Table 1.

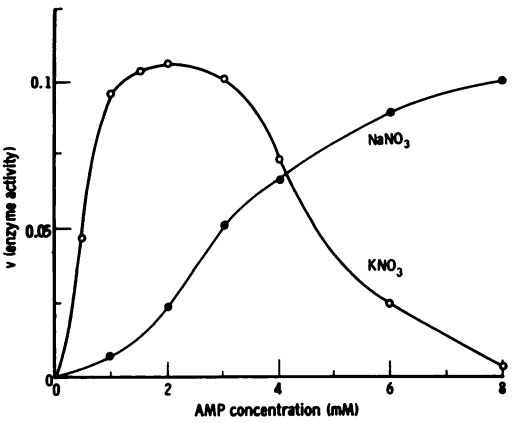

FIG. 5. *Effect of varying AMP concentration on AMP deaminase activity in the presence of KNO_3 and $NaNO_3$*

Concentration of Tris-HNO_3 (pH 7.2) was 100 mM. $[NaNO_3] = 200$ mM. $[KNO_3] = 100$ mM. Other conditions were the same as described for Table 1.

pH Effects

The pH-velocity curves were determined in the presence of various anions and at fixed concentrations of activator and substrate. In all cases the pH optimum (7.0) was the same as that previously determined in a chloride-containing medium (3).

Interactions of ATP and Orthophosphate with the Enzyme

As evident from the data of Table 1, the enzyme activity in the presence of orthophosphate is lower than the activity in the presence of any other tested anion. Considering the sensitivity of our method of assay, it was not possible to do studies with all-phosphate media comparable to those reported in previous sections for other anions. However, in view of possible theoretical significance of the effects of simultaneous presence of ATP and orthophosphate on the enzyme (see Discussion), the experiments indicated by Fig. 6 were performed in media containing chloride as the major anion. As expected from previous results, in a chloride-containing medium ATP may be considered as activator and orthophosphate as inhibitor of the enzyme. The interesting aspects of the data of Fig.

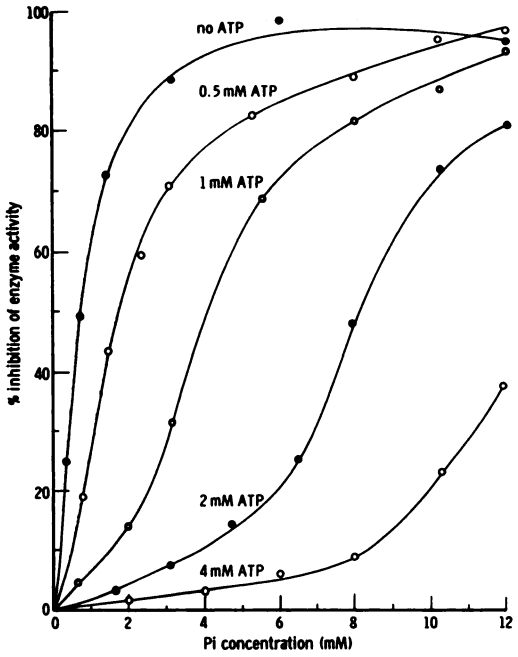

FIG. 6. *Inhibitory effects of orthophosphate on AMP deaminase activity in the presence of various concentrations of ATP*

In each reaction the concentration of Tris-HCl (pH 7.2) was 100 mM. [AMP] = 2 mM. [KCl] = 100 mM. Phosphate was added as buffered potassium salt (pH 7.2). Other conditions were the same as described for Table 1.

6 are the sigmoid shapes of the phosphate inhibition curves in the presence of ATP. These indicate that the per cent inhibition of enzyme by a fixed concentration of orthophosphate (especially at the lower range of phosphate concentrations) increases as the ATP concentration is decreased. Thus, in a situation where the enzyme is functioning in the presence of both ATP and orthophosphate, decrease in ATP concentration and increase in orthophosphate concentration could have synergistic inhibitory effects on the enzyme activity.

DISCUSSION

The main object of this work was to determine whether anions other than ATP could modify the activity of the alkali-cation-dependent AMP deaminase. The results clearly show such effects of some simple anions. The kinetic data obtained, however, are of such complicated nature that no definite conclusions about the mechanisms of the cation and anion effects may be based on them. In conformity with current trends, the easiest, if not the simplest, way of interpreting the agreement of our data with the Hill equation would be to say that both cations and anions (including ATP) are "allosteric effectors" of the enzyme (11). Since the substrate is also an anion, the reaction scheme based on such assumptions would certainly be complicated enough to conform to almost any type of kinetic data. A reaction scheme, more in accordance with the theoretical considerations referred to in the Introduction, could also be devised on the assumption of the presence of cooperative interactions only between the anion binding sites and the activator-cation binding sites. Under steady-state conditions, this scheme would also lead to velocity equations sufficiently complicated to be consistent with the data. However, considering that a relatively simple reaction scheme involving only one modifier could result in velocity equations and types of curves as those described by Botts (12), it becomes obvious that in the absence of independent evidence it would be useless to attempt the interpretation of complex kinetic data on the basis of a chosen reaction model.

Regardless of the value of our kinetic data for mechanistic interpretations, certain general conclusions of interest may be drawn from them. The most notable is that a clear distinction between the selective response of AMP deaminase activity to various alkali cations and the selective binding of these ions to the enzyme should be made. Consider, for example, the data of Fig. 5. At 1 mM substrate concentration the enzyme activity in the presence of K^+ is at least twenty times greater than that in the presence of Na^+. At 8 mM substrate concentration the activating effect of Na^+ becomes about twenty times greater than that of K^+. However, it is clear that an estimation of relative binding of Na^+ and K^+ to the enzyme under the above

conditions cannot be made. The problem of the relation of the activation of an enzyme by alkali cations to the binding of these ions to some enzyme sites has recently become of some interest in relation to the mechanism of active transport of alkali cations. Evidence has accumulated which suggests a relation between a membrane-bound ATPase, activated by Na^+ and K^+, and the process of active transport of these ions by the intact cells (13). On this basis some attempts have been made to formulate models for the mechanism of active transport involving this enzymic activity or its various components (7, 8, 14, 15). In all these cases, based on very limited kinetic data, the relative selectivity of an enzymic reaction for Na^+ and K^+ has been equated to the selectivity for the binding of these cations. Recent studies (16) suggest, however, that the kinetics of activation of ATPase by Na^+ and K^+ may be just as complex as those we have presented for AMP deaminase. It should be clear then that in trying to correlate the properties of ATPase to any model for active transport the kinetic data must be interpreted with some caution.

In addition to the above considerations, the modification of the activity of red cell AMP deaminase by ATP and other anions is of interest in relation to the possible role of this enzyme in the control of cell metabolism. It has been established that the mature human red cell is not able to synthesize the adenine portion of adenosine and its nucleotides (17). Since both AMP deaminase and adenosine deaminase are present in the red cell, the maintenance of ATP and other adenosine nucleotide levels in the cell must depend on the supply of adenine or adenosine from plasma, and on the control of the activities of the deaminases. Nothing is known about the regulation of adenosine deaminase in the red cell. However, the effects of ATP and orthophosphate on AMP deaminase suggest the possibility of a mechanism for the regulation of the adenine nucleotide content of the cell. Consider the following simplified scheme for the formation and breakdown of AMP in intact erythrocytes *in vitro:*

$$ATP \xrightarrow[\text{adenylate kinase}]{\text{ATPase and}} 2P_i + AMP \rightarrow IMP + NH_3$$

If the breakdown of ATP is accelerated (e.g., when the rate of active transport of cations is increased), the resulting decrease in ATP concentration and the increase in orthophosphate concentration would both tend to diminish the rate of AMP deamination (Fig. 6). Thus, when a demand for increased rate of ATP synthesis is created, the adenosine nucleotides of the cell are conserved. Conversely, when the rate of breakdown of ATP is diminished (e.g., by inhibition of active transport), the opposite changes in ATP and orthophosphate concentrations accelerate the rate of AMP deamination. In this case a decrease in the demand for ATP synthesis leads to decrease in total adenosine nucleotide content. Since adequate information about the relative activities of the enzymes involved in the breakdown of AMP is not available, it is difficult to ascertain the significance of the above control mechanism to red cell metabolism.

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REFERENCES

1. H. B. Steinbach, *in* "Comparative Biochemistry" (M. Florkin and H. S. Mason, eds.), Vol. IV, p. 677. Academic Press, New York, 1962.
2. H. H. Ussing, Handbuch der experimentellen Pharmakologie, Vol. 13, p. 1. Springer, Berlin, 1960.
3. A. Askari, *Science* **141**, 44 (1963).
4. A. Askari and J. E. Franklin, Jr., *Biochim. Biophys. Acta* **110**, 162 (1965).
5. G. N. Ling, "A Physical Theory of the Living State." Blaisdell, New York, 1962.
6. G. Eisenman, *Biophys. J.* **2**, Suppl., 259 (1962).
7. J. C. Skou, *Progr. Biophys.* **14**, 131 (1964).

8. L. J. Opit and J. S. Charnock, *Nature* **208**, 471 (1965).
9. G. Nikiforuk and S. P. Colowick, *J. Biol. Chem.* **219**, 119 (1956).
10. E. J. Conway and R. Cook, *Biochem. J.* **33**, 457 (1939).
11. J. Monod, J. Changeux and F. Jacob, *J. Mol. Biol.* **6**, 306 (1963).
12. J. Botts, *Trans. Faraday Soc.* **54**, 593 (1958).
13. J. C. Skou, *Physiol. Rev.* **45**, 596 (1965).
14. R. L. Post, A. K. Sen and A. S. Rosenthal, *J. Biol. Chem.* **240**, 1437 (1965).
15. R. W. Albers, S. Fahn and G. J. Koval, *Proc. Natl. Acad. Sci. U. S.* **50**, 474 (1963).
16. R. F. Squires, *Biochem. Biophys. Res. Commun.* **19**, 27 (1965).
17. C. Bishop, *in* "The Red Blood Cell" (C. Bishop and D. M. Surgenor, eds.), p. 147. Academic Press, New York, 1964.