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# REGULATION OF ERYTHROCYTE GLYCOLYSIS

## MEMBRANE-MEDIATED ACTIVATION INDUCED IN LOW-ELECTROLYTE MEDIUM

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SUMMARY

Human erythrocyte suspensions prepared in low-electrolyte medium maintain a high rate of glycolytic flow, which may exceed by 3-fold the normal rate of glucose utilization by these cells. Rapid redistribution of permeable cation and anion occurs in low-electrolyte media to new Donnan equilibria with changes in both intramembrane and intracellular ionic environment indicated. A primary consequence of the ionic redistribution involves proton loss with intracellular alkalinization accounting for a part, but not all, of the resultant glycolytic stimulation. Stimulation of glycolytic rate in response to pH includes an apparent highly coordinated dual mechanism involving both pH-induced acceleration of ATP degradation and pH activation of the regulatory enzyme phosphofructokinase. A second pH-independent, membrane-mediated, stimulation of glycolytic rate is also indicated in low-electrolyte media. The basis of this additional stimulation involves again an induced acceleration of ATP-degradation rate, with changes in intramembrane ionic environment and resultant effects on the associated ATPase of apparent contributing significance.

## INTRODUCTION

The human erythrocyte engages in only a limited metabolism, directed to little other than its immediate survival. Energy production by these cells is restricted to glucose degradation with the Embden–Meyerhof pathway of enzymes providing solely for their ATP requirement. Cell-energy expenditure has been delineated to the extent of an estimated 15–20 % of the basal glycolytic rate, which is regulated by and directed to the active transport of $K^+$ into and $Na^+$ out of the cell [1, 2]. The regulation of and the purpose to which the balance of the basal ATP-energy production is directed remains to be accounted for; although an ATP-dependent energization of

Abbreviations: DMO, 5,5-dimethyl-2,4-oxazolidinedione; TES, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid.

the cell membrane has been proposed [3] in the maintenance of discoid cell shape [4] and the preservation of membrane deformability [5].

Due to its unique metabolism, the erythrocyte has been utilized frequently in investigations of the glycolytic process and has proven particularly useful in the study of regulatory mechanisms associated with this multienzyme pathway. (For recent reviews, see refs 6–8.) Information has been reported previously from this laboratory concerning the limiting role of adenine nucleotide [9] and the non-limiting nature of pyridine nucleotide [10], and on the mechanism of stimulation by inorganic phosphate [11] in the glycolysis of the human erythrocyte. More recent study of regulatory processes has examined mechanisms associated with a marked stimulation of glycolytic activity observed [12] in erythrocyte suspensions prepared in isotonic media containing apparent inert solutes of limited cell permeability. Since glycolytic stimulation of this nature had not been reported previously and an associated unknown work function implied, extended investigation of the observed effect was undertaken.

In this report are summarized experiments demonstrating membrane-mediated direct and indirect effects of low-electrolyte media on the erythrocyte with stimulation of the membrane-associated ATPase resulting in each case. A mechanism of erythrocyte glycolytic stimulation involving primarily induced increases in cell ATPase activity in low-electrolyte media is indicated on the basis of these studies.

## EXPERIMENTAL PROCEDURES

### *Cell incubation*

Red cells were prepared from heparinized, freshly drawn human blood from healthy donors. The cells were separated and washed three times with normal saline and the top layer of leucocytes discarded. The packed, washed cells were added directly to reaction tubes at suspension concentrations of 2–5% and incubated at 37 °C.

### *Analyses of cell constituents and metabolites*

Red-cell incubation mixtures were variously analyzed. Aliquots were removed directly for hemoglobin determination [13], for deproteinization, and for cellular pH and $K^+$ measure. Deproteinization was carried out by addition of 0.1 vol. 5.0 M $HClO_4$ with the resulting extract partially neutralized by addition of 0.1 vol. of 4.5 M KOH. The neutralized extract was assayed for one or more of the following: lactate, fructose diphosphate, triose phosphates and ATP. Assays were by enzyme-coupled pyridine nucleotide oxidation or reduction measured fluorimetrically [14, 15], except in the case of lactate which was measured by absorbance at 340 nm [16]. Measure of cell pH and $K^+$ was carried out on hemolysates prepared by addition of 5–10 vol. of 0.1% Triton X-100 to cells following their separation by centrifugation from suspending media. The pH of the resulting hemolysate was measured at 37 °C with little differences observed within the range of hemolysate dilutions employed. Measurements of intracellular pH of human erythrocytes have been found to yield essentially similar values whether determined by glass electrode on the hemolysate (hemolysis induced by either freezing and thawing or saponin) or as calculated values derived from extra- and intracellular distribution of $CO_2$, 5,5-dimethyl-2,4- oxazolidinedione (DMO) or $Cl^-$ (for review of extensive literature see ref. 17). Separate aliquots of the hemolysate were taken for hemoglobin measure and $K^+$ analysis (flame photometer).

*Membrane-ATPase preparation and measure*

Various methods were examined in the preparation of membrane ATPase, including the widely applied method of Post et al. [18]. The preparative procedure employed in the present study is described and was selected on the basis of a consistent yield of a highly active product. Washed cells were hemolyzed by addition of 10 vol. of saponin solution (0.1 mg/ml). Saponin action is thought to involve the solubilization of membrane-associated cholesterol [19]. Insoluble membrane fragments were separated by centrifugation (20 000 $\times g$, 30 min) and washed successively with 0.05 M then twice with 0.005 M histidine-imidazole at pH 8.0. The nearly colorless product was resuspended in the dilute buffer to five times the initial cell volume. The resulting suspension contained 5–7 mg protein per ml and retained considerable residual adenylate kinase activity, as did all other membrane preparations tested*. Storage resulted in 10–30% loss of ATPase activity over a 5-day period. Preparation of the enzyme was carried out at near 0 °C. The preparation demonstrated an absolute requirement for $Mg^{2+}$ at an optimal 1 : 1 ratio with ATP for activity. ATPase activity determined in the presence of both $K^+$ (75 mM) and $Na^+$ (25 mM) was reduced 10–20% on addition of ouabain as an index of that fraction of the activity representing the transport enzyme.

ATPase activity was determined by $P_i$ liberation, using either a colorimetric measure or radioactive label ($^{32}P$). $P_i$ was separated as the phosphomolybdate complex in iso-butanol [20] for radioactivity determinations and prior to colorimetric determination when interfering test substances (sugar alcohols, organic anions) were present in the reaction mixtures. ATP containing $^{32}P$ label in both $\beta$- and $\gamma$-positions was employed in view of contamination by adenylate kinase of the ATPase preparations employed. $^{32}P$ was measured in a liquid scintillation counter.

*Reagents*

Enzymes employed in the present study were products of Boehringer or Sigma Chemical Co. Radioactive ATP containing $^{32}P$ in the $\beta$- and $\gamma$-positions was purchased from Schwarz Bioresearch. Tris–ATP was prepared from the sodium salt of ATP by ion exchange, using Dowex 50, $H^+$ form, and subsequent neutralization of the free acid form of ATP with Tris. The buffer reagent *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES) and the disaccharide cellobiose (4-($\beta$-D-glucoside)-D-glucose) were products of Calbiochem. Tris (hydroxymethyl aminomethane) and sucrose were the "ultra pure" grade from Mann Research Laboratories. D-Mannitol, dulcitol, imidazole, and L-histidine were products of Sigma Chemical Co. Renografin-60, a pharmaceutical organic iodide preparation, was a product of Mallinckrodt Chemical Works.

## RESULTS

*Suspending media and glycolytic activity*

Erythrocyte suspensions demonstrate marked variation in glycolytic rate in response to selective alteration of media composition. In addition to the well-known

* Extraction of membrane preparations in salt solutions of high ionic strength (e.g. 0.3 M KCl) was found, later, to remove adenylate kinase contamination effectively.

rate changes in response to alteration of media pH [9, 21, 22], $P_i$ [9, 11, 23–25], and cation composition [26, 27], a remarkable acceleration of glycolytic rate was found to result in isotonic media containing apparent inert solutes of limited permeability [12]. Although acceleration of glycolytic rate could be demonstrated by both non-electrolyte and anionic impermeates, the identity of the respective activating mechanism(s) has not been established.

The present study has considered activating mechanisms associated with stimulation of erythrocyte glycolytic rate in low-electrolyte media containing non-electrolyte impermeates. Among a number of such non-electrolytes examined, including sucrose, lactose, maltose, cellobiose, sorbitol, mannitol and dulcitol, each was found to induce similar stimulatory effects on erythrocyte glycolytic rate. In most cases, experiments employing dulcitol media have been selected for presentation, since contamination by and conversion to metabolizable hexose were found to be minimal.

In Fig. 1 are shown comparative glycolytic rates of erythrocyte suspensions prepared in Tris- or TES-buffered isotonic media containing NaCl or dulcitol as the primary solutes. Glycolytic rate, as measured by lactate production, was enhanced approx. 3-fold in the presence of dulcitol, in comparison with NaCl. Lactate production was maintained in the dulcitol medium at accelerated rate over the 4-h incubation period examined and varied slightly with the buffer mixture employed (lesser rates were observed in Tris–HCl, which contains the freely permeable $Cl^-$). Although conversion of glucose to lactate was essentially stoichiometric in the NaCl medium at the investigated pH, a characteristic deficiency of lactate production relative to glucose utilization was maintained in the dulcitol medium. Examination revealed the deficiency of lactate production to be accountable by an accumulation of glycolytic intermediates, including principally fructose diphosphate and triose phosphates. (See Fig. 1.) Accumulation of these particular intermediates has been described [11, 24, 25] in association with stimulation of erythrocyte glycolytic rate by elevation

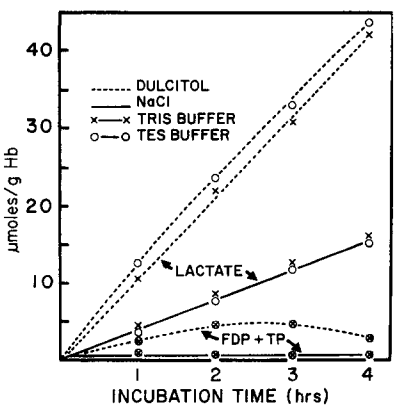


Fig. 1. Effect of medium solutes on erythrocyte glycolytic rate. Erythrocyte suspensions contained 5 % cells, 20 mM Tris or TES buffers (pH 7.4 at 37 °C), 0.7 mM glucose, and either 130 mM NaCl alone (NaCl system) or 15 mM NaCl plus 230 mM dulcitol (dulcitol system). Following specified incubation periods at 37 °C, lactate production and fructose diphosphate (FDP) and triose phosphates (TP) were determined.

of media pH and $P_i$ concentration, and has been attributed to selective activation of the enzyme phosphofructokinase by these agents.

*Suspending media and cell pH and $K^+$*

It has been known for a number of years [28, 29] that erythrocyte suspensions prepared in isotonic media of low-electrolyte content undergo marked shifts in ion distribution, including changes in cell pH and $K^+$ [29, 30]. Effects of variation of media composition with respect to non-electrolyte impermeate on cell pH and $K^+$ are illustrated by the experiment shown in Fig. 2. Dilute cell suspensions incubated in buffered isotonic NaCl media (Tris or TES buffers, pH 7.4) maintained, in the presence of glucose, an intracellular pH at 0.2 unit less than the media pH and lost little $K^+$ over extended incubation periods (Fig. 2). A similar pH difference has been shown by various methods (e.g. ref. see 17) to exist between the erythrocyte and blood plasma in vivo. Substitution of NaCl with an osmotic equivalent of dulcitol in the incubation media induced, on the other hand, marked shifts in intracellular pH and rapid $K^+$ loss (Fig. 2, closed circles). Addition of small amounts of the permeable $Cl^-$ to the dulcitol media (as NaCl or Tris–HCl buffer), served to moderate the rate of cell-$K^+$ loss, as shown previously by others [29, 30], and to stabilize intracellular pH (Fig. 2, open circles) at alkaline levels relative to media pH.

Extra- and intracellular pH differences exhibited by erythrocyte suspensions prepared in either NaCl or dulcitol media are illustrated further in Fig. 3. Cell suspensions prepared over a range of media pH maintained differences in pH between media and cell in the illustrated manner. Intracellular pH was maintained at less than media pH in the presence of NaCl and alkaline to the media pH in the presence of dulcitol over the pH range tested. Intracellular pH differences of approx. 0.4 unit

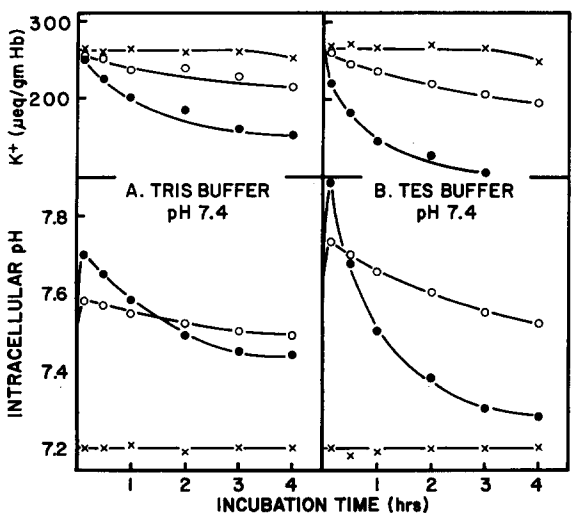


Fig. 2. Effect of medium solute composition on intracellular $K^+$ concentration and pH determined in the presence of Tris (A) and TES (B) buffers. Erythrocyte suspensions contained 5 % cells, 20 mM Tris or TES buffers (pH 7.4 at 37 °C), 0.7 mM glucose, and either 130 mM NaCl (×–×) or 260 mM dulcitol (●–●) or 230 mM dulcitol plus 15 mM NaCl (○–○). Following specified incubation periods at 37 °C, cell-$K^+$ concentrations and pH were determined.

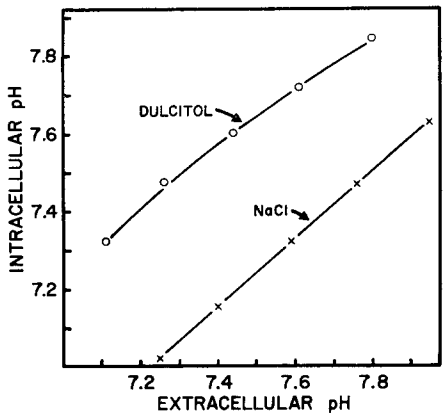


Fig. 3. Relationship between extracellular and intracellular pH of erythrocyte suspensions prepared in Tris-buffered media containing NaCl or dulcitol as principal solute. Cell suspensions were prepared at 5 % in media containing 20 mM Tris buffers ranging from pH 7–8 (at 37 °C) and either 130 mM NaCl (NaCl system) or 230 mM dulcitol plus 15 mM NaCl (dulcitol system). Following 1 h incubation at 37 °C, extracellular and intracellular pH were determined (at 37 °C).

were recorded between cell suspensions prepared in identically buffered salt or dulcitol media. The pH plots shown in Fig. 3 reveal generally linear relationships over the examined range between extra- and intracellular pH in both salt and dulcitol media, yielding in each case approximately straight lines of similar slope.

*Media composition, intracellular pH and glycolytic rate*

The effect of pH on erythrocyte glycolytic rate has been considered previously

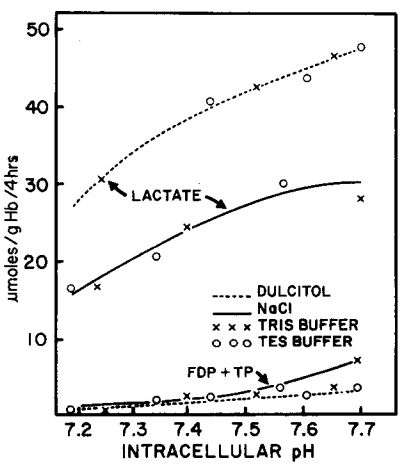


Fig. 4. Relationship between erythrocyte lactate-production rates and intracellular pH in dulcitol and NaCl media. Cell suspensions were prepared at 5 % in media containing 0.7 mM glucose, 20 mM Tris or Tes buffers ranging from pH 7–8 (at 37 °C) and either 130 mM NaCl (NaCl system) or 230 mM dulcitol plus 15 mM NaCl (dulcitol system). Lactate production and fructose diphosphate (FDP) and triose phosphate (TP) accumulation were determined after 4 h incubation at 37 °C. Intracellular pH was monitored hourly with approx. 0.1 pH-unit drop recorded in dulcitol medium over the 4-h incubation period. (Also see Fig. 2.)

in relation to the pH of the suspending medium rather than cell pH [21, 22]. Since considerable differences may result between intra- and extracellular pH on variation of composition of suspending media, pH effects in relation to glycolytic rate should more properly be considered in relation to the existing cellular rather than medium pH. Erythrocyte suspensions respond to elevation of their intracellular pH by acceleration of glycolytic rate in the manner illustrated in Fig. 4. Erythrocyte suspensions incubated in buffered NaCl media increase lactate production approx. 2-fold on elevation of intracellular pH from 7.2 to 7.6 and demonstrate pH-dependent accumulation of fructose diphosphate and triose phosphates. A comparative plot of lactate-production rates of erythrocyte suspensions prepared in dulcitol media and at various buffer pH values selected to provide a similar range of intracellular pH (Fig. 3) is also included in Fig. 4. Comparison of lactate-production rates in the two media, over a comparable range of intracellular pH, demonstrates clearly a further pH-independent, selective stimulation of erythrocyte glycolytic rate in the dulcitol medium, extending over the entire range of intracellular pH tested.

*Effect of pH and dulcitol on cell ATP*

Regulation of erythrocyte glycolytic rate induced by changes in medium pH has been attributed to specific pH activation of phosphofructokinase [8, 22]. It is evident, however, that stimulation restricted to one (or more) of the glycolytic enzymes only cannot sustain an accelerated glycolytic flow in the absence of a cor-

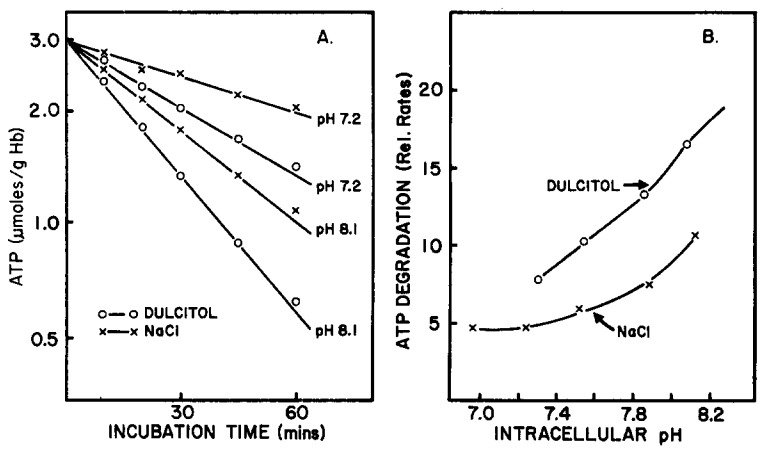


Fig. 5. (A) Relative degradation rates of cell ATP at low (pH 7.2) and high (pH 8.1) intracellular pH in (a) NaCl or (b) dulcitol media, containing $F^-$ and without glucose present. Washed erythrocytes from freshly drawn blood were preincubated for 15 min at 37 °C in normal saline containing 20 mM Tris buffer at pH 7.2. The preincubated cells were separated and reincubated at 37 °C at 5 % suspension in 20 mM Tris buffer at appropriate pH to yield intracellular pH of 7.1 or 8.2 (Fig. 3), 5 mM $P_i$, 5 mM NaF, and either NaCl at 122 mM or dulcitol at 210 mM plus 15 mM NaCl. Aliquots of the incubation mixture were removed at the indicated times to monitor cell pH and for ATP determinations. (B) Relative degradation rates of cell ATP in (a) NaCl and (b) dulcitol media as a function of intracellular pH. The relative degradation rates were obtained from the slopes of the straight lines resulting from semi-log plots similar to those shown in Fig. 5A. The experiments were carried out over a range of pH (Tris buffer) under conditions otherwise identical to those described in (A).

responding increase in rate of ATP turnover. Since an accompanying increase of ATP synthesis would be expected, an accelerated glycolytic flow to be maintained would require that ATP degradation proceeds at a corresponding accelerated rate. Stimulation of erythrocyte glycolytic rate induced by pH and dulcitol was, therefore, considered further in relation to the specific effect of these agents on the rate of cell-ATP degradation.

Rapid dephosphorylation of cell ATP accompanies incubation of erythrocytes in the absence of added glycolyzable substrate and in the presence of $F^-$. $F^-$ addition blocks effectively ATP resynthesis via metabolism of endogenous substrates (primarily 2,3 diphosphoglyceric acid), as evidenced by the finding of little or no accumulation of pyruvate or lactate, allowing a reliable measure of cell-ATP degradation rates. Cellular ATP degradation under these conditions was found to follow first-order kinetics generally, and demonstrated considerable differences in rates by alteration of media composition with respect to pH and solutes. In Fig. 5A are shown comparative ATP-degradation rates demonstrated by erythrocyte suspensions incubated in either NaCl or dulcitol media, buffered to yield equivalent low (pH 7.2) and high (pH 8.1) intracellular pH. The results demonstrate clearly both a pH-associated as well as a separate dulcitol-specific acceleration of intracellular ATP-degradation rates. The effect of intracellular pH and the combined effects of pH and dulcitol on ATP-degradation rate are illustrated further in Fig. 5B. In NaCl media, degradation of cell ATP increased approx. 2-fold on elevation of intracellular pH from 7–8 as shown. In dulcitol media, ATP-degradation rates were considerably higher than in NaCl media at all comparable intracellular pH. The combined effects of elevation of intracellular pH and substitution of dulcitol for NaCl in the suspending media served to induce a greater than 3-fold acceleration of cellular ATP-degradation rates in the erythrocyte.

*Media composition and cell-membrane ATPase activity*

ATP loss during in vitro incubation of erythrocytes in the absence of substrate was found to be accompanied by a nearly stoichiometric release of $P_i$, indicating selective involvement of an ATPase(s). Demonstrable ATPase activity was found to be associated wholly with the cell membrane, localizing the sites of ATP breakdown and presumably of energy transfer. Further study was directed to examination of the membrane ATPase with particular reference to activating mechanisms.

The membrane-ATPase preparations employed in the present study were found to respond to elevation of solution pH by an increase of activity, with some variation apparent due to specific buffer effects, as illustrated by the results obtained in Tris and imidazole buffers (Fig. 6). ATPase activity was minimal at acid pH and in media which did not contain added monovalent, inorganic cation. A generalized stimulation of ATPase activity resulted in the presence of $K^+$ (also $Na^+$, not shown) except at acid pH. An additional 10–20% increase in ATPase activity was demonstrable in the combined presence of $K^+$ and $Na^+$ when tested in the absence of ouabain (not shown). Incubation of membrane-ATPase preparations in the absence of $K^+$ but in the presence of sucrose (or other non-electrolytes) resulted in little or no change in enzyme activity. On the other hand, addition of both non-electrolyte and $K^+$ induced a remarkable increase in membrane-ATPase activity over the entire pH range examined (Fig. 6). The degree of stimulation varied somewhat with the

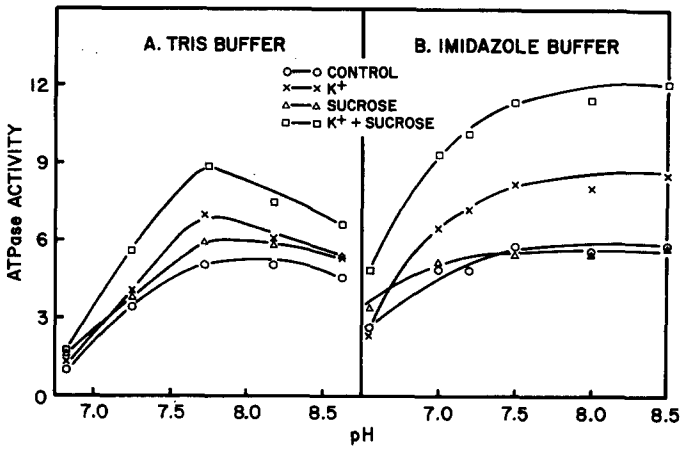


Fig. 6. Effect of medium pH and solute composition on the ouabain-insensitive ATPase activity of erythrocyte membrane. Reaction mixtures contained 0.1 ml of a fresh membrane preparation (see Experimental Procedure), 5 mM $MgCl_2$, 5 mM ATP, 50 mM Tris (a) or imidazole (b) buffer, 75 mM KCl where indicated, and 300 mM sucrose where indicated, in a final 1.0-ml volume. ATPase activity is expressed in $\mu$moles $P_i$ liberated per h at 37 °C by an amount of cell membrane originating from 1 ml erythrocytes.

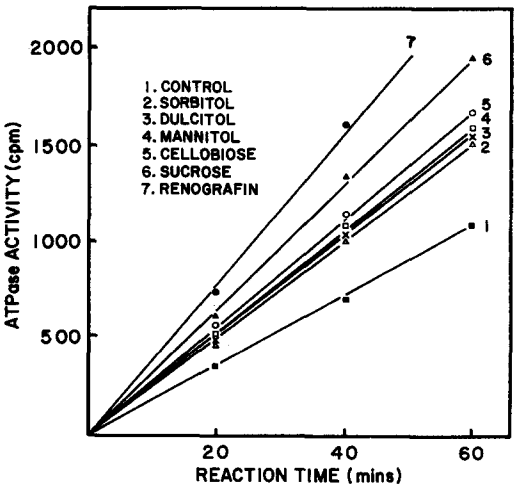


Fig. 7. Stimulation of erythrocyte membrane ATPase activity by organic cell impermeates. Reaction mixtures contained 0.1 ml of a fresh membrane-ATPase preparation (see Experimental Procedure), 5 mM ATP containing 0.04 $\mu$Ci $^{32}P$ label in the $\beta$- and $\gamma$-positions, 5 mM $MgCl_2$, 50 mM imidazole · HCl at pH 8.0, 75 mM KCl, and separately as indicated: 300 mM sucrose, sorbitol and mannitol; 150 mM dulcitol and cellobiose, 35 mM Renografin-60; all in a final 1.0-ml volume. $P_i$ liberation was determined by radioactivity measurement (see Experimental Procedure).

particular buffer employed (e.g. less in Tris), and was found to be clearly insensitive to ouabain. Among a variety of non-electrolyte and electrolyte impermeates of the erythrocyte, which were shown earlier to induce acceleration of glycolytic rate [12], many were found additionally to effect stimulation of membrane-ATPase activity as illustrated in Fig. 7. Maximal stimulation by non-electrolyte generally required concentrations in excess of 100 mM, a high grade of reagent purity, and the additional presence of $K^+$. Apparent differences in degree of stimulation induced among the non-electrolytes tested were presumed to be the result of variable reagent purity. Stimulation of membrane ATPase induced by the presence of an electrolyte impermeate in the suspending medium is also illustrated (Fig. 7) in the case of Renografin, an organic iodide of demonstrated limited erythrocyte permeability. Stimulation by Renografin appeared to exceed that induced by non-electrolyte impermeates.

*Membrane ATPase and substrate–activity relationships*

The observed added stimulation of membrane-ATPase activity, induced in the presence of sucrose and similar non-electrolyte cell impermeates when tested with $K^+$ present, was variously examined with respect to the mechanism involved. Information relating to the activating mechanism was obtained on investigation of membrane-ATPase activity and substrate relationships as summarized in Fig. 8. Lineweaver–Burk plots of the initial velocity of $P_i$ liberation against the substrate concentration are shown when measured in the presence and absence of sucrose and $K^+$, tested singly and together. In the absence of $K^+$ (Curves 1 and 2), the initial velocity of the reaction approached a maximum at about 2 mM substrate and changed little on increasing to 5 mM substrate. A substrate concentration of 5 mM was routinely employed for ATPase activity measurements in the previously described studies. (See Figs 6 and 7.) At substrate concentrations exceeding 2 mM, inhibition of the reaction was thus indicated. Addition of sucrose had only a slight effect on the reaction velocity over the range of substrate concentrations tested. (Compare Curves

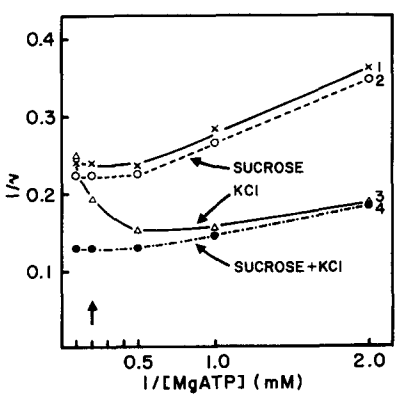


Fig. 8. Effects of sucrose and $K^+$ on substrate-velocity relationships of ouabain-insensitive membrane ATPase. Reaction mixtures contained in a final 1.0-ml volume, 0.1 ml of a fresh membrane preparation, 50 mM imidazole · HCl at pH 8.0, $MgCl_2$, and Tris–ATP at the indicated concentrations, 0.1 mM ouabain, and either: Curve 1, no other addition; Curve 2, 150 mM sucrose; Curve 3, 75 mM KCl; or Curve 4, 150 mM sucrose and 75 mM KCl. Velocity is expressed in $\mu$moles $P_i$ liberated per h (at 37 °C) by an amount of cell membrane originating from 1 ml erythrocyte.

1 and 2.) Addition of $K^+$ to the reaction mixtures induced, on the other hand, a marked stimulation over the lower substrate range and increasingly strong inhibition of the reaction at substrate levels exceeding 2 mM (Curve 3). At concentrations of substrate routinely employed for ATPase assay (5 mM), strong inhibition (see arrow) of the reaction was apparent with $K^+$ present. Addition of sucrose along with $K^+$ had little effect on the reaction velocity at the lower substrate concentrations; however, the intense inhibition resulting at higher substrate levels with $K^+$ present was found to be largely reversed in the presence of sucrose. (Compare Curves 3 and 4.) It was concluded from these studies that the apparent stimulation of membrane ATPase observed in sucrose medium at 5 mM substrate and with $K^+$ present (Figs 6 and 7) represents a mechanism, in fact, of partial reversal of a $K^+$-augmented substrate inhibition of the enzyme.

*Data analysis*

The data presented in Fig. 8 were analyzed by use of an IBM-360/50 time-sharing system using programs written in the PL/ACME language as described by Porter et al. [31]. Least-square fits of the data were obtained to the following velocity equation describing the usual Michaelis–Menten kinetics, including a term for substrate inhibition [31]:

$$v = \frac{VS}{K_m + S + \frac{S^2}{K_i}} \tag{1}$$

An average deviation of less than 5% was found between the experimental (Fig. 8) and calculated data sets. Estimated values of apparent Michaelis constants ($K_m$), maximal initial velocities ($V$), and substrate-inhibition constants ($K_i$) obtained for the membrane ATPase in the presence and absence of $K^+$ are shown in Table I. The extent of modification of the kinetic constants induced by $K^+$ is indicated from the values shown.

TABLE I

APPARENT KINETIC CONSTANTS FOR MEMBRANE ATPase (OUABAIN INSENSITIVE)

Conditions were 37 °C at pH 8.0. The constants are defined according to Eqn 1 and the numerical values are derived from data shown in Fig. 8.

| | $[K^+] = 0$ | $[K^+] = 75$ mM |
|---|---|---|
| $K_m$ | 0.10 mM | 0.036 mM |
| $K_i$ | 16.4 mM | 1.3 mM |
| $V$ | 7.3 | 11.0 |

DISCUSSION

Erythrocyte suspensions may respond to variation of media composition by altering their glycolytic rate. The extent to which such modification of glycolytic rate may reflect possible media-induced changes in the energy demand of the cell and the nature of the work function(s) which may be involved, remain, for the most part,

obscure. Among a number of media solutes known to effect alteration of erythrocyte glycolytic rate, only in the case of stimulation induced by the presence of transportable cations has an associated work function been clearly delineated. (See refs 1 and 2.) Variation of suspending media with respect to $H^+$ results in marked alteration of erythrocyte glycolytic rate, which has been found to parallel generally the pH-activation kinetics of the enzyme phosphofructokinase. On this basis, a mechanism of pH-induced modification of erythrocyte glycolytic rate has been proposed, in which phosphofructokinase is assumed to play a primary regulatory role [7, 8]. The proposed mechanism remains incomplete, however, failing to include an accounting of those changes in ATP turnover which would be expected to accompany a sustained modification of glycolytic rate. Thus any mechanism of alteration of steady-state glycolytic flow would be expected to involve reaction(s) inducing corresponding changes in ATP-degradation rate. It was on this basis that correlative effects of pH on glycolytic rate and ATP degradation were sought in the present study. Remarkably similar effects of pH on both glycolytic rate (Fig. 4) and ATP degradation could be demonstrated in intact-cell studies (Fig. 5) with parallel stimulation of each resulting from elevation of cell pH. Furthermore, generally similar pH effects on ATP-degradation rates were found in both the intact cell (Fig. 5) and by cell-membrane ATPase preparations (Fig. 6). A highly coordinated, dual mechanism of pH action was indicated on the basis of these studies, involving both membrane-mediated effects on ATP-degradation rate (due to pH-induced alteration of ATP-energy demand) and compensatory effects on ATP-production rate (primarily via pH-sensitive phosphofructokinase-mediated alteration of glycolytic rate), presumably as a product of the change in cell-adenylate energy charge [32].

Stimulation of erythrocyte glycolytic rate induced in low-electrolyte medium containing non-electrolyte impermeates was examined in the present study with respect to the mechanism involved. Both indirect and direct effects of the low-electrolyte medium were distinguishable, with each contributing additively to enhance glycolytic rate. An indirect effect of the low-electrolyte medium involved a rapid redistribution of permeable anions and protons between cell and medium with cell alkalinization a primary consequence (Figs 2 and 3), resulting in a corresponding pH-induced stimulation of glycolytic rate (Fig. 4). An additional, pH-independent stimulation of erythrocyte glycolytic rate in the low-electrolyte medium was also indicated (Fig. 4). This pH-independent glycolytic stimulation had as a basis an accelerated rate of cell-ATP degradation found to be induced in low-electrolyte media (Fig. 5). Thus, both pH-dependent and pH-independent stimulation of erythrocyte glycolytic rate, resulting in low-electrolyte media, appeared to be mediated at the cell-membrane level and to involve in each case selective stimulation of a membrane ATPase. Accelerated ATP-degradation rates resulting in cell suspensions prepared in low-electrolyte media, indicated the possibility of a required increase in energy expenditure for maintaining cellular homeostasis in such an environment.

Investigation of the mechanism of action of non-electrolyte impermeates on erythrocyte metabolism was extended to examination of their effects on the membrane ATPase. An apparent significant stimulation of the ouabain-insensitive ATPase activity of erythrocyte membrane suspensions was observed in the presence of non-electrolyte cell impermeates (Figs 6 and 7), consistent with enhanced ATP-degradation rates resulting in erythrocyte suspensions incubated in similar low-

electrolyte medium (Figs 5A and 5B). Further kinetic study of the membrane ATPase revealed that the apparent stimulation was rather the reversal of an intense inhibition of the reaction induced in low-electrolyte medium by excessive substrate in the added presence of $K^+$. The effect of the low-electrolyte medium has been assumed to involve a redistribution in the intramembrane ionic environment (by Donnan equilibrium), resulting in conformational changes of the membrane-associated ATPase and alteration of its kinetic properties.

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