

# The Role of Oxidized Nicotinamide Adenine Dinucleotide in Fluoride Inhibition of Active Sodium Transport in Human Erythrocytes

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**ABSTRACT** The rate coefficient for  $^{22}\text{Na}$  release from previously labeled human erythrocytes was determined in the presence of 0.1–10 mM sodium fluoride (F). The oxidized nicotinamide adenine dinucleotide ( $\text{NAD}^+$ ) level at the end of 2 hr of incubation in tris(hydroxymethyl)aminomethane (Tris)-Ringer medium was also measured. Both parameters decreased proportionately as F concentration was raised. Both F-induced changes were immediate and were reversed by 10 mM pyruvate. The decrease in  $\text{NAD}^+$  concentration following enolase inhibition by F is attributed to a diminished rate of formation in the reaction catalyzed by lactic dehydrogenase (LDH) with undiminished continued utilization in the reaction catalyzed by glyceraldehyde-3-phosphate dehydrogenase (GAPDH). It is postulated that the  $\text{NAD}^+$  lowering limited the GAPDH step, resulting in proportionate decreases in the rates of phosphoglycerate kinase (PGK) and Na,K-dependent adenosine triphosphatase (Na,K-ATPase), a reaction sequence thought to link glycolysis with active Na extrusion. Adding pyruvate with F increased  $\text{NAD}^+$  production at the LDH step, thus reactivating GAPDH, PGK, and Na,K-ATPase and leading to the observed restoration of  $^{22}\text{Na}$  release. The results suggest, therefore, that F inhibits active Na transport in intact human erythrocytes indirectly through a lowering of  $\text{NAD}^+$ , although, direct inhibition of the Na,K-ATPase by F may possibly occur simultaneously.

## INTRODUCTION

The active extrusion of Na ions from human erythrocytes is inhibited by low concentrations of fluoride ion (Maizels, 1951). This has been attributed to glycolytic inhibition caused by fluoride ion (F) (Embden and Haymann, 1924), which leads to a decline in cellular concentration of adenosine triphosphate (ATP), the presumed energy source for the Na pump (Gardos and

Straub, 1957; Eckel, 1958; Burn, 1962). During the past several years, evidence has emerged to suggest direct inhibition of the Na, K-dependent adenosine triphosphatase (Na, K-ATPase) by F (Kirschner, 1964; Opit et al., 1966; Yoshida et al., 1968; Quigley et al., 1969). Most of this recent evidence, however, has been obtained from studies with membrane fragments and leaves open the possibility that alternate mechanisms may account for the F effect in intact erythrocytes. In the present study, the relationship between the cellular oxidized nicotinamide adenine dinucleotide (NAD<sup>+</sup>) concentration and the rate of release of previously incorporated <sup>22</sup>Na from intact human erythrocytes has been evaluated in the presence of different F concentrations. A correlation between these two factors has been found which appears to permit new insight into the mechanism by which F affects active Na extrusion from the intact human red cell.

#### MATERIALS AND METHODS

The following substances were obtained from commercial sources: oxidized nicotinamide adenine dinucleotide (NAD<sup>+</sup>) and sodium pyruvate (Sigma Chemical Co., St. Louis, Mo.), sodium fluoride (Fisher Scientific Company, Springfield, N. J.), ouabain (Calbiochem, Los Angeles, Calif.), and alcohol dehydrogenase (Boehringer & Soehne, Mannheim, Germany). <sup>22</sup>NaCl was purchased from Abbott Laboratories, North Chicago, Ill.

Whole blood from normal, young adult males was generally collected in plastic bags containing acid-citrate-dextrose (National Institutes of Health, Formula B). On the day of collection or after different periods of cold storage up to a maximum of 29 days, a portion of the blood was centrifuged for 7 min at 800 *g* and 5°C. Following removal of the plasma and buffy coat, red cells were washed twice with 1 vol of a solution containing, in millimoles per liter: NaCl, 120; KCl, 5.6; MgCl<sub>2</sub>, 2; Na<sub>2</sub>HPO<sub>4</sub>, 1; tris(hydroxymethyl)aminomethane (Tris), 24; and glucose, 10. The pH of the medium was adjusted using a Beckman Model G meter (Beckman Instruments, Inc., Fullerton, Calif.) so as to read 7.4 at 37°C. The cells were washed once more with the same solution except that it now contained 2 mM CaCl<sub>2</sub> (Tris-Ringer medium).

Approximately 10-ml portions of packed cells were placed in several 50-ml beakers containing equal volumes of Tris-Ringer medium to which 0.25 μCi of <sup>22</sup>NaCl had been added per milliliter of medium. The blood suspensions were incubated for 1.5–2 hr at 37°C in a Dubnoff shaking incubator cycling at 100/min. The suspensions were combined and allowed to stand overnight at 5°C. The next day, the cells were separated by centrifugation and were washed twice with 2 vol of cold Tris-Ringer medium to remove the bulk of the trapped <sup>22</sup>Na.

1-ml portions of the labeled, packed cells were pipetted into 25-ml Erlenmeyer flasks containing 2 ml of Tris-Ringer medium. In the experimental flasks, the medium also contained either different concentrations of F or 0.1 mM ouabain. In some cases, 10 mM pyruvate was added either at time zero or at a later time. The suspensions

were incubated once again at 37°C. Flasks were removed at time zero and at 30-min intervals thereafter, and the contents were poured into precooled tubes which were allowed to stand in ice for 5 min before centrifugation in the cold. 0.3 ml portions of supernatant fluid and uncentrifuged suspensions were plated in triplicate on concentrically ringed planchets and allowed to air-dry overnight for counting in a gas flow counter (Nuclear-Chicago, Des Plaines, Ill., Model D47). The amount of radioactivity present in the labeled cells was calculated from the  $^{22}\text{Na}$  in the medium and in the whole suspension and from the suspension hematocrit, as described by Jacob and Jandl (1964). A correction for mass absorption was included.

After 2 hr of incubation, acid extracts containing oxidized NAD were prepared from packed cells according to the method of Burch et al. (1967). Hematocrit measurements of the packed cells were made with an International Microcentrifuge (International Equipment Co., Needham Heights, Mass.) in order to assess true cell volumes. Standard nucleotide was extracted along with the cells in every assay. The procedure of Gupta (Gupta et al., 1966; Marshall, 1966) was used to analyze the  $\text{NAD}^+$ . This method is based on a coupled oxidoreduction between ethanol and lactaldehyde, producing acetaldehyde and propanediol, respectively, in the presence of horse liver alcohol dehydrogenase.  $\text{NAD}^+$  is cycled in the process so that the over-all reaction may be driven by rate-limiting amounts of this nucleotide. The acetaldehyde formed is transferred out of the reaction vessel by a stream of  $\text{N}_2$  into a second flask containing semicarbazide and the resulting semicarbazone is read at 224  $m\mu$ . Nucleotide recoveries were at least 95%. Since similar recoveries were obtained in the presence of F or pyruvate, these agents did not appear to interfere with the  $\text{NAD}^+$  assay.

Additional flasks containing suspensions of unlabeled cells were also run in identical fashion and various other measurements were made with these duplicate suspensions. The final pH varied from 7.1 to 7.4 in different experiments. The metabolically inhibited cell suspensions were generally more alkaline by as much as 0.1 pH unit. Final hematocrits were generally 25–30% and did not vary greatly in different experiments or as a result of metabolic inhibition.

Cell water content was determined from the weights of suspension samples (1 ml) in weighing bottles before and after heating overnight at 90°C. Cell Na determinations were made with a flame photometer (Instrumentation Laboratory, Inc., Lexington, Mass., Model 143) on trichloroacetic acid extracts prepared according to a procedure described by Kirschner (1964). Cell Na content was similar to that reported previously for cold-stored erythrocytes, ranging from 13.7 to 55.3 mEq Na/liter of cell water depending on storage age. During the 2 hr incubation, an average decrease of 19% or  $5.5 \pm 0.9$  mEq Na/liter of cell water occurred ( $P < 0.001$ ). The average increase of 3% or 0.9 mEq Na/liter of cell water in F-poisoned cells was not significant. Lactic acid determinations were performed on perchloric acid extracts of the suspensions with a method utilizing lactic dehydrogenase (Hohorst, 1963). Lactate production ranged from 1.61 to 4.63  $\mu\text{moles/ml}$  of erythrocyte per hr and was inhibited 53–100% by 5 mM F, the inhibition being greater in cells that had been stored for longer periods (see Omachi et al., 1970).

## RESULTS

A typical experiment in which  $^{22}\text{Na}$  release from prelabeled human erythrocytes was followed is shown in Fig. 1 A. As can be seen, the release of radioactivity into the medium continued at a diminishing rate with time in each curve. Moreover, the rate of  $^{22}\text{Na}$  release became less as F concentration in the medium was raised from 0.1 to 10 mM. In Fig. 1 B, the radioactivity present in the cells at any time,  $t$ , is expressed as the fraction of initial cell  $^{22}\text{Na}$  and is plotted on a logarithmic scale against incubation time. Straight lines were obtained, reflecting the well-known first-order rate process for the release. Rate coefficients, to be discussed later, were obtained by measuring the slopes of these straight lines. The decreasing release rate with increasing F concentration is reflected here as a slower decline in the fraction of  $^{22}\text{Na}$  remaining in the cells. Maximum inhibition was achieved with 10 mM F, since 15 and 20 mM F (not shown) did not depress  $^{22}\text{Na}$  release further. A gradual decrease in control rate coefficients was noted as the storage time of the blood prior to experimental use was progressively increased. After 4 wk of storage, the rate coefficients were about 60% of the value in fresh blood. The degree of inhibition of  $^{22}\text{Na}$  release by F, however, remained proportionately the same throughout this interval.

Fig. 2 shows the effect of F on the  $\text{NAD}^+$  concentration in erythrocytes incubated in Tris-Ringer medium for 2 hr at  $37^\circ\text{C}$ . In this graph, the ordinate represents per cent change in cellular  $\text{NAD}^+$  concentration and the abscissa represents F concentration ranging from 0.1 to 10 mM plotted on a logarithmic scale. It was necessary to normalize the  $\text{NAD}^+$  data in this manner since the control levels in incubated erythrocytes varied according to the storage age of the cells, i.e. these values decreased during the early weeks of storage and then rose, as reported in detail elsewhere (Omachi et al., 1972). It has previously been shown in our laboratory that human erythrocytes exhibit a decrease in  $\text{NAD}^+$  content when exposed to 5 mM F (Omachi et al., 1969). It may be noted in Fig. 2 that the per cent decrease in  $\text{NAD}^+$  was found to be greater as F concentration was raised. As with  $^{22}\text{Na}$  release, maximum depression of the  $\text{NAD}^+$  concentration occurred with 10 mM F, since 15 and 20 mM F (not shown) did not decrease  $\text{NAD}^+$  further. Also, as in the case of Na efflux, in spite of the variation in control  $\text{NAD}^+$  values noted above, the lowering of  $\text{NAD}^+$  concentration by F remained proportionately the same regardless of storage time of the blood.

The seemingly parallel decline in both  $^{22}\text{Na}$  release rate and oxidized  $\text{NAD}$  level with increasing F concentration suggested a cause and effect relationship between these two parameters. Experiments were therefore conducted in which  $^{22}\text{Na}$  release and cellular  $\text{NAD}^+$  levels were both measured in the same experiment. Rate coefficients for  $^{22}\text{Na}$  release were obtained, which varied

from 0.30/hr for cells incubated in the control medium to 0.04/hr for cells incubated with 10 mM F.  $\text{NAD}^+$  content in this series ranged from 91 to 4 nmoles/ml of erythrocyte, for control and maximally inhibited cells, respectively. In Fig. 3, these rate coefficients, expressed as per cent of control, are plotted against cellular  $\text{NAD}^+$  concentration, also expressed as per cent of control. Each point represents a different F concentration, with the latter

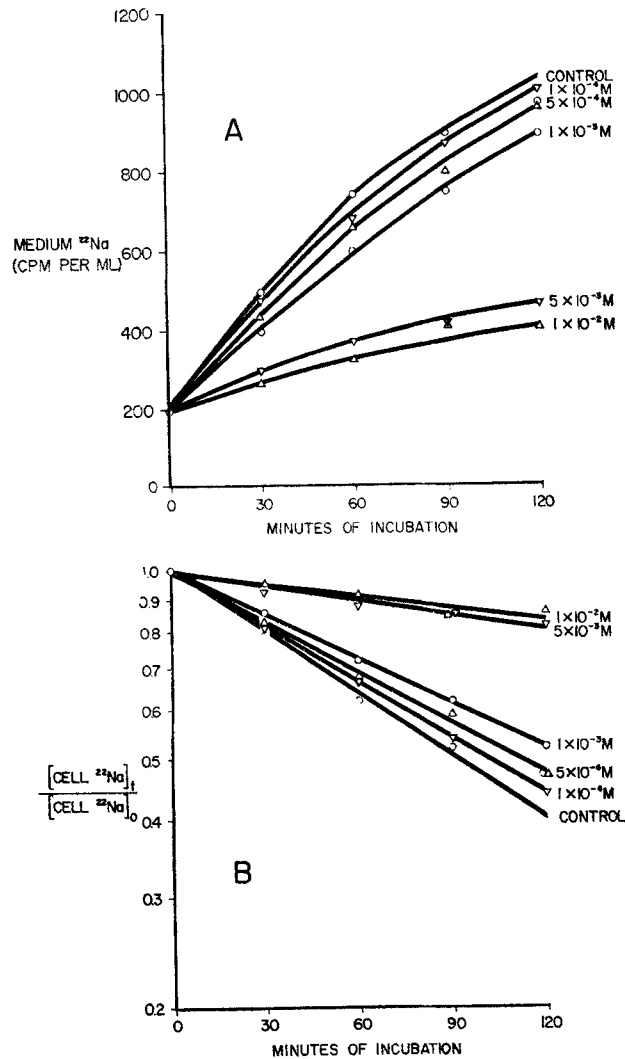


FIGURE 1 A. Effect of fluoride on  $^{22}\text{Na}$  release. Previously labeled erythrocytes were suspended at  $37^\circ\text{C}$  in a nonradioactive, Tris-Ringer medium containing the indicated fluoride concentrations.

FIGURE 1 B. The fraction of initial  $^{22}\text{Na}$  remaining in the cells after different periods of incubation is plotted on a logarithmic scale against time.

increasing as the line moves from high to low values on the graph. As can be seen, a straight line is obtained in this plot, showing a direct proportion to exist between rate coefficient and  $\text{NAD}^+$  level under these conditions.

Inhibition of  $^{22}\text{Na}$  release by F takes place rapidly, judging from the curves in Fig. 1. The time-course of the change in  $\text{NAD}^+$  content in red cells exposed to 5 mM F was also determined. This change was found to be virtually com-

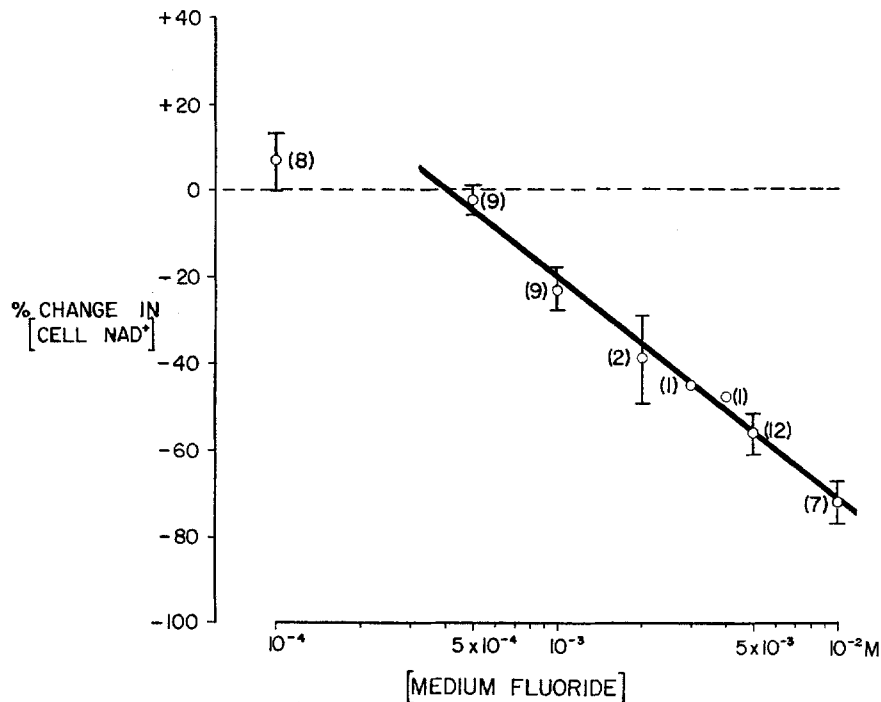


FIGURE 2. Effect of fluoride on cellular  $\text{NAD}^+$  concentration. The per cent change in  $\text{NAD}^+$  is plotted against the fluoride concentration on a logarithmic scale. The dashed line represents the control situation. Vertical lines above and below each point represent plus and minus one standard error of the mean, respectively. The numbers in parentheses indicate the number of experiments performed for that point. The mean and standard error of the cell  $\text{NAD}^+$  concentration in 19 control suspensions is  $78 \pm 7$  nmoles/ml erythrocyte. Incubation conditions were the same as in Fig. 1.

plete within the first few minutes of incubation (Fig. 4). This rapid decline to new steady-state levels could have been predicted from a consideration of normal glycolytic rates and the average change in  $\text{NAD}^+$  content in the presence of F. Lactate production is normally about  $3 \mu\text{moles/ml}$  of erythrocyte per hr, so that a turnover of 3000 nmoles of  $\text{NAD}^+$  may be associated with this activity. This is a large figure compared to the average change in  $\text{NAD}^+$  observed in the presence of 5 mM F, i.e., approximately 30 nmoles/ml of erythrocyte. Under these conditions, the observed  $\text{NAD}^+$  difference between

control and F-treated cells would be established in 0.01 hr, or in less than 1 min.

Fig. 5 illustrates the effect of added pyruvate on  $^{22}\text{Na}$  release and on  $\text{NAD}^+$  concentration in cells exposed to F. The amount of radiosodium released

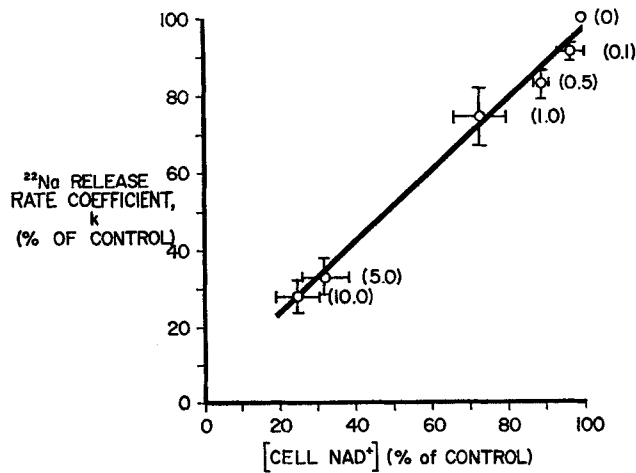


FIGURE 3. The relationship between the  $^{22}\text{Na}$  release rate coefficient,  $k$ , and cellular  $\text{NAD}^+$  concentration in fluoride-inhibited erythrocytes. Values for both parameters are expressed as per cent of control. The numbers in parentheses are fluoride concentrations, in millimoles per liter. The vertical and horizontal lines bracketing each point represent plus and minus one standard error of the mean for the ordinate and abscissa, respectively. Measurements of rate coefficients and  $\text{NAD}^+$  levels are both subject to statistical variation, hence, the vertical and horizontal lines. Each point is the mean of five experiments. Incubation conditions were the same as in Fig. 1.

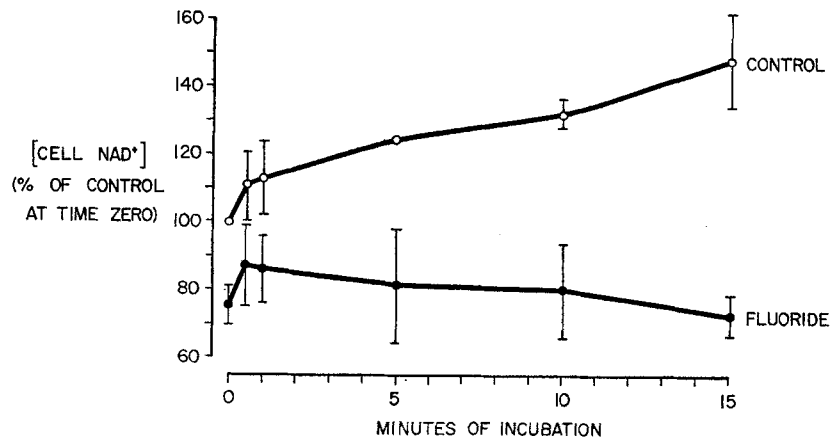


FIGURE 4. Time rate of change of  $\text{NAD}^+$  concentration.  $\text{NAD}^+$  concentration is expressed as per cent of control value at zero incubation time. Vertical bars above and below each point represent plus and minus one standard error of the mean, respectively. The number of experiments is three. Incubation conditions were the same as in Fig. 1, except that the only fluoride concentration used was 5 mM.

from the cells into the medium has been plotted against incubation time; to the right of the curves are shown the corresponding  $\text{NAD}^+$  levels after 2 hr of incubation. Again, 5 mM F was seen to lower both  $^{22}\text{Na}$  release rate and  $\text{NAD}^+$  level, when compared to the control values. By including 10 mM pyruvate in the F-containing medium, the  $^{22}\text{Na}$  release rate was changed toward the control rate and the  $\text{NAD}^+$  level was elevated to a value well

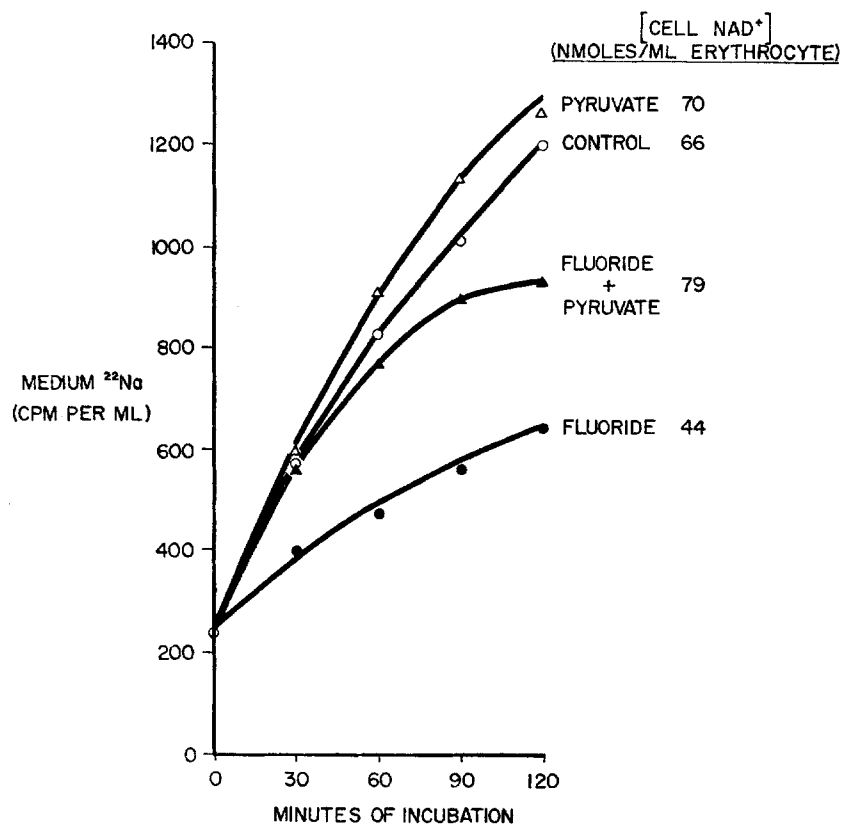


FIGURE 5. Effect of pyruvate on  $^{22}\text{Na}$  release and cellular  $\text{NAD}^+$  concentration in fluoride-treated and control erythrocytes. Fluoride and pyruvate concentrations were 5 and 10 mM, respectively. Essentially the same results were seen in six experiments. Incubation conditions were the same as in Fig. 1.

above its concentration in F-treated cells. In other experiments, pyruvate was added to the suspension 1 hr after incubation with F was begun (Fig. 6). The rate of  $^{22}\text{Na}$  release, as well as the  $\text{NAD}^+$  concentration, was clearly restored to near normal levels even after prior exposure of the cells to F for this period.

Since F is known to inhibit  $\text{Na}, \text{K-ATPase}$  directly, the ability of pyruvate to reverse the decline in  $^{22}\text{Na}$  release associated with ouabain, another well-known inhibitor of this ATPase, was also tested. A pyruvate reversal of the



ouabain-induced inhibition of  $^{22}\text{Na}$  release was not observed (Fig. 7). Inhibition of  $^{22}\text{Na}$  release by 5 mM F is also shown in this graph and it may be seen that the release curve with F is essentially the same as the one with ouabain. The ouabain concentration used, 0.1 mM, is one which is known to inhibit maximally Na efflux from human erythrocytes (Gill and Solomon, 1959).

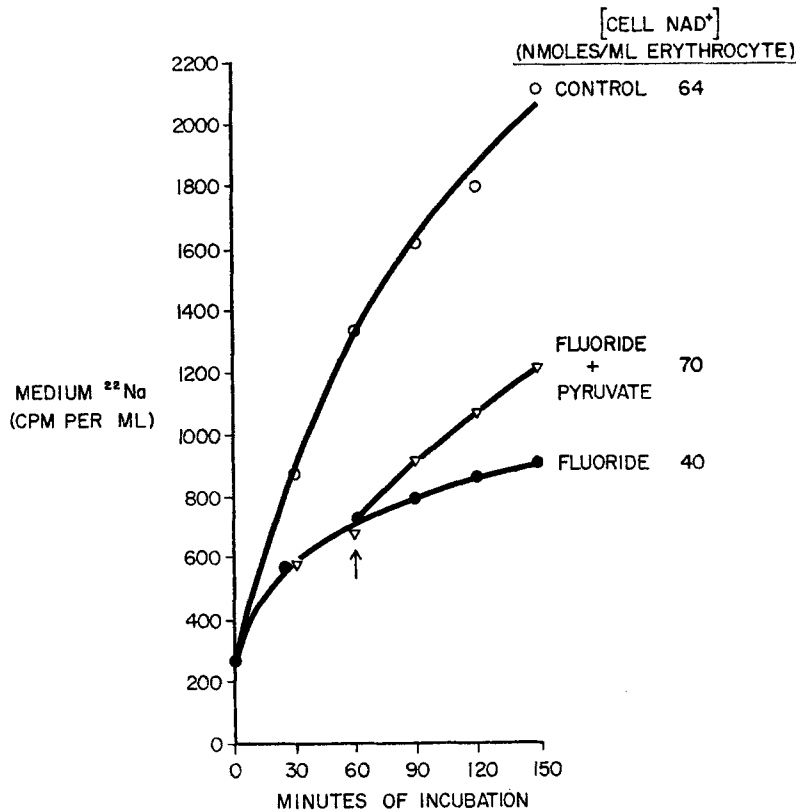


FIGURE 6. Effect of pyruvate on  $^{22}\text{Na}$  release and cellular  $\text{NAD}^+$  concentration in cells poisoned with fluoride 1 hr before addition of pyruvate (arrow). Fluoride and pyruvate concentrations were 5 and 10 mM, respectively. Similar results were observed in a total of six experiments. Incubation conditions were the same as in Fig. 1.

#### DISCUSSION

The results obtained in the present investigation permit us to offer a new explanation for the mechanism underlying F inhibition of active Na extrusion from intact human red cells. The principal observation which serves as the basis for the new hypothesis is the correlation between the  $\text{NAD}^+$  level and  $^{22}\text{Na}$  release from prelabeled red cells in the presence of different F concentrations (Fig. 3). This correlation suggests that the decline in the rate of  $^{22}\text{Na}$  release under these conditions may have been mediated through the observed

decrease in  $\text{NAD}^+$  concentration. This change, in turn, is thought to have been a direct result of the glycolytic inhibition caused by F through inhibition of the enzyme, enolase (Warburg and Christian, 1942). It may be recalled from the known reactions of the glycolytic pathway (Fig. 8 A) that enolase inhibition decreases pyruvate production and hence  $\text{NAD}^+$  formation at the lactic acid dehydrogenase (LDH) step.  $\text{NAD}^+$  utilization, however, continues relatively unaffected at the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) step, thus leading to the observed decline in  $\text{NAD}^+$  content. With

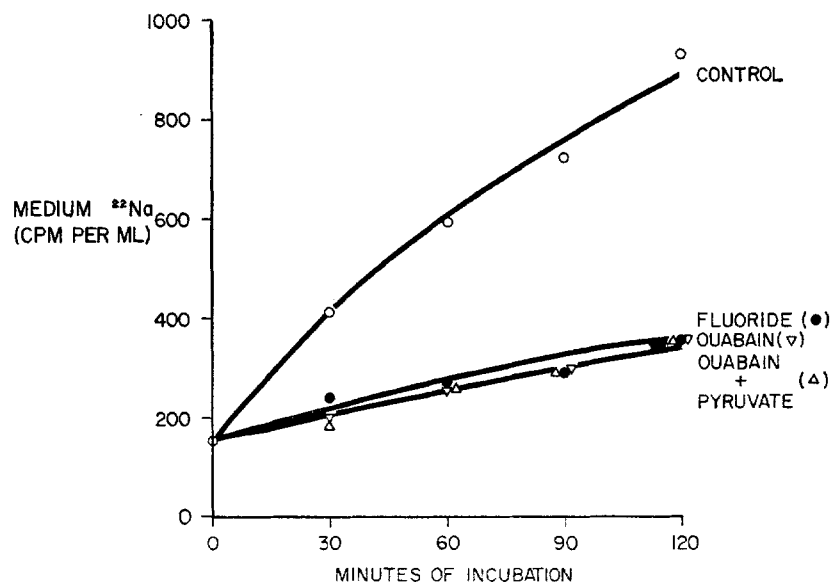


FIGURE 7. Absence of pyruvate reversal in ouabain-inhibited erythrocytes. Ouabain, fluoride, and pyruvate concentrations were 0.1, 5, and 10 mM, respectively. The same general pattern was seen in three experiments. Incubation conditions were the same as in Fig. 1.

increasing F concentrations, therefore, graded enolase inhibition resulted in proportionate decreases in  $\text{NAD}^+$  concentration (Fig. 2). Another observation consistent with this analysis was the progressively greater inhibition of lactate production as the F concentration was raised.

This change appears to be particularly significant when viewed in the light of recent work by Schrier (1966) and by Parker and Hoffman (1967), who implied a close linkage of membrane-bound phosphoglycerate kinase (PGK) with  $\text{Na, K-ATPase}$ , as illustrated in Fig. 8 B. The substrate for PGK is 1,3-diphosphoglycerate (DPG), the product of the preceding reaction in which  $\text{NAD}^+$  is enzymatically reduced by GAPDH. In view of the direct proportionality between  $\text{NAD}^+$  concentration and the rate coefficient for  $^{22}\text{Na}$  release in F-inhibited human red cells (Fig. 3), it seems reasonable to

propose that  $\text{NAD}^+$  may be a limiting factor in the series of reactions catalyzed by the enzymes, all of which are membrane bound: GAPDH, PGK, and Na,K-ATPase. These reactions lead to active ion transport which, therefore, must also be  $\text{NAD}^+$  limited under these conditions.

In Fig. 3, the rate coefficient, rather than the actual flux, has been used as a quantitative measure of the rate of Na extrusion. The flux is the product of the rate coefficient and the cell Na concentration. The rate coefficient

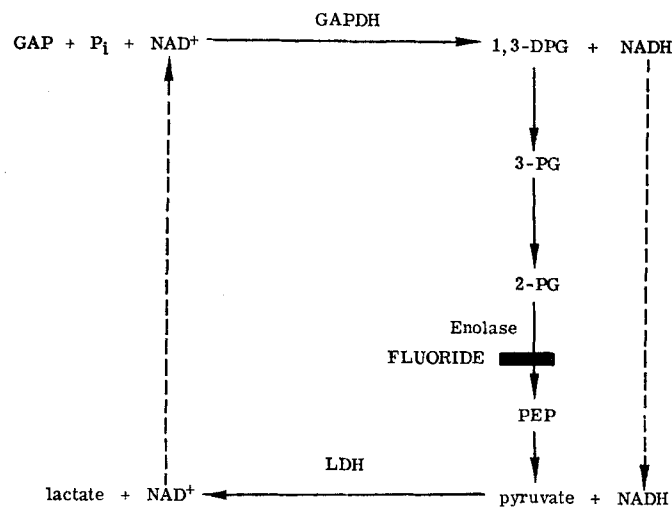


FIGURE 8 A. Relationship of  $\text{NAD}^+$  to erythrocyte glycolysis.

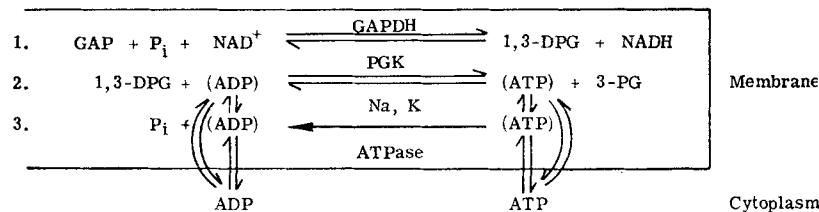


FIGURE 8 B. Proposed relationship of  $\text{NAD}^+$  to active Na transport.

seems to be the more appropriate term in the present context, since it is the parameter which appears to have been directly affected by the change in energy supply to the pump brought about by F through the above three-enzyme sequence. It may be noted, in fact, that cell Na concentration is much the same during the early phase of the release in inhibited as in control cells, because cells from the same pool were used in each experiment. Under these conditions, therefore, the observed changes in the rate of  $^{22}\text{Na}$  release must be due primarily to changes in the rate coefficient.

The effect of pyruvate in reversing the decline in  $\text{NAD}^+$  caused by F was undoubtedly related to its action at the LDH step (see Fig. 8 A). As would

be expected, pyruvate oxidized reduced NAD (NADH) to form lactate and  $\text{NAD}^+$ , and the newly formed  $\text{NAD}^+$  reactivated the GAPDH step. This facilitated production of 1,3-DPG for PGK, which then resynthesized ATP for active Na transport. Thus, the  $\text{NAD}^+$  level and the  $^{22}\text{Na}$  release rate in F-poisoned cells were augmented in the presence of pyruvate (Figs. 5 and 6). This result seems inconsistent with direct F inhibition of the Na, K-ATPase in intact cells, since it is difficult to envision how pyruvate might directly affect F binding to this enzyme. Furthermore, addition of 10 mM lactate had no effect on either the  $\text{NAD}^+$  concentration or the  $^{22}\text{Na}$  release rate observed in the presence of F. Pyruvate alone caused a slight activation of  $^{22}\text{Na}$  release which was accompanied by a small rise in  $\text{NAD}^+$  concentration. This suggests that the series of events described above for F-inhibited erythrocytes may also apply qualitatively to normal, unpoisoned cells. The possibility that the pyruvate effect is due to an increased passive permeability to Na can be ruled out considering the failure of this substrate to reverse the  $^{22}\text{Na}$  release inhibition caused by ouabain (Fig. 7).

The  $^{22}\text{Na}$  release curve for cells incubated with F and pyruvate appeared to plateau during the last 30 min of incubation (Fig. 5). This result suggests that pyruvate addition was not associated with complete reactivation of the enolase enzyme and that there may have been an accumulation of glyceric acid monophosphates. This could have retarded the PGK step and ATP synthesis by mass action or product inhibition. An observation which appears consistent with this view is the increase in 2,3-DPG concentration under similar conditions (Guest and Rapoport, 1941), presumably resulting, in part, from elevated 1,3-DPG levels. Furthermore, an increase in the latter could have led to product inhibition of GAPDH (Williamson, 1970) such that  $\text{NAD}^+$  utilization by this enzyme may also have been slowed. This is consistent with the higher  $\text{NAD}^+$  concentration observed with F and pyruvate compared with pyruvate alone (Fig. 5). In this instance, therefore, a higher  $\text{NAD}^+$  level was not associated with a greater rate of  $^{22}\text{Na}$  release.

It is assumed in this study that the effect of F to diminish the  $^{22}\text{Na}$  release was on the active component of Na efflux. F ions are known to have no influence on passive Na movements across the red cell membrane, although the passive transfer of K may be augmented (Lepke and Passow, 1968). Moreover, the effect on K is known to occur only at F concentrations higher than those generally employed in the present investigation (Eckel, 1958; Feig et al., 1971).

Our results do not absolutely rule out the possibility that direct inhibition of Na, K-ATPase by F may also have occurred in our experiments. Since this has been observed in broken cell preparations, it may be expected that this mode of F inhibition could occur in intact cells as well. It may be, therefore, that both direct and indirect inhibition of the Na pump occurred simul-

taneously. However, the close relationship between  $\text{NAD}^+$  and  $^{22}\text{Na}$  release in the presence of F, as well as the effect of pyruvate on F-inhibited cells, suggests that the indirect action through enolase poisoning may be more important.

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