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Of the other known sites of ATP utilization, membrane-bound ATPase is inhibited by fluoride, but the incorporation of fatty acids into membrane phospholipids is unaffected until ATP is depleted.

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## I. EFFECTS OF SODIUM FLUORIDE

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ABSTRACT Exposure of red cells to fluoride produces a variety of metabolic alterations, most of which are based upon the secondary effects of enolase inhibition, which reduces pyruvate synthesis and interferes with the regeneration of diphosphopyridine nucleotide (NAD). Adenosine triphosphate (ATP) is consumed in the hexokinase and phosphofructokinase reactions but is not regenerated since the deficiency of NAD limits glyceraldehyde phosphate dehydrogenase. ATP depletion in the presence of fluoride and calcium induces a massive loss of cations and water.

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## INTRODUCTION

In the search for improved methods of management of patients with congenital hemolytic anemia, the correction of induced disorders of erythrocyte metabolism may be instructive. The inhibition of glycolysis at the level of enolase by fluoride (1) has been used as a model system with particular reference to inherited erythrocyte pyruvate kinase deficiency (2). This paper presents an analysis of the metabolic perturbations produced by fluoride in the red cell and also describes their partial correction by the introduction of an oxidant, methylene blue.

## METHODS

Human blood samples were obtained from nonfasting normal subjects and patients with young red cell populations. These included patients recovering from iron deficiency, pernicious anemia, and acute hemorrhage, as well as a patient with an unstable hemoglobin hemolytic anemia and another with an acquired hemolytic anemia. They were collected in preservative-free heparin (0.1 mg/ml) and washed three times at 4°C in Krebs-Henseleit buffer containing albumin (1 g/100 ml). The final hematocrit was adjusted to approximately 30% with the same buffer. Measurements of packed cell volume, red cell count, hemoglobin concentration, and reticulocyte count were performed by standard methods (3) and in a Coulter model S counter. Glutamic oxaloacetic transaminase activity of hemolysates was performed by the method of Karmen, Wróblewski, and LaDue (4).

Cell suspensions (3-8 ml in 25-ml Erlenmeyer flasks) were gassed with a mixture of air and 5% $CO_2$ to bring the pH to 7.5 ±0.1. Additives were prepared to provide desired final concentrations by the addition of 10 $\mu$l to the incubation flask. Incubations were carried out in a 37°C water bath at 60 oscillations/min. $NaH_2PO_4$ was added to give a final concentration of 10 mmoles/liter of incubation mixture. The pH was measured when samples were obtained. If the pH had changed by more than 0.1 U, the samples were gassed with 5% $CO_2$ or left open briefly to bring the pH back to 7.5. Filtrates were prepared at 4°C in two volumes of 1 N perchloric acid before and during the incubations. These were centrifuged at 4°C twice to remove the sediment, neutralized with 5 M $K_2CO_3$, and frozen within 2 hr. These were later analyzed for ATP (5), glucose (6), lactate (7), and 2,3-diphosphoglycerate (2,3-DPG) (8).[1]

[1] *Abbreviations used in this paper:* DHAP, dihydroxy acetone phosphate; 2,3-DPG, 2,3-diphosphoglycerate; FDP, fructose diphosphate; G3P, glyceraldehyde-3-phosphate; GPD, glyceraldehyde-3-phosphate dehydrogenase; 2PG, 2-phosphoglycerate; 3PG, 3-phosphoglyceric acid.

Other glycolytic intermediates in the filtrates were measured in assays of 1 ml final volume by modifications of the fluorometric methods of Lowry, Passonneau, Hasselberger, and Schulz (9) with an Eppendorf Fluorimeter, model 1100 M. For hexose monophosphates, 0.1–0.4 ml of neutralized filtrate was used in a system containing 100 mM Tris buffer (pH 8.0), 80 $\mu$M NADP, and 10 mM MgCl$_2$. The increase in fluorescence was always less than that produced by a standard of 4 m$\mu$moles of NADPH. For fructose diphosphate (FDP), dihyroxy acetone phosphate (DHAP), and glyceraldehyde-3-phosphate (G3P), 0.05–0.2 ml of neutralized filtrate was used in a system containing 16 mM imidazol buffer (pH 7.0) and 4–16 $\mu$M NADH. For 3-phosphoglyceric acid (3 PG), 0.2 ml of neutralized filtrate was used in a system containing 16 mM imidazol buffer (pH 7.0), 5 mM 2-mercaptoethanol, 5 mM MgCl$_2$, 0.3 mM ATP, and 4 $\mu$M NADH. For pyruvate, perchloric acid filtrates were prepared from buffer, the red cells having been eliminated by centrifugation (10). 0.05–0.2 ml of a neutralized filtrate was used in a system containing a final concentration of 40 mM phosphate buffer (pH 7.0), 4 mM MgCl$_2$ and 4–16 $\mu$M NADH. With the methodology used, fluoride interfered with the accurate measurement of 2-phosphoglycerate (2PG), and this intermediate was not estimated. Neither fluoride nor methylene blue interfered with the other enzymatic determinations. The release of potassium from incubated red cells was estimated by the change in the potassium ion concentration in the incubation medium measured with an internally standardized flame photometer (model 143; Instrumentation Laboratory Inc., Watertown, Mass.). All of the above concentrations were expressed per liter of red cells based upon the initial hematocrit of the cell suspension.

The activity of membrane-bound ATPase (11) was measured in ghosts prepared by the Nakao, Kurashina, and Nakao (12) modification of the method of Dodge, Mitchell, and Hanahan (13).

The incorporation of radioactive fatty acid into red cell membrane phospholipid was also measured. Red cells were washed three times in isotonic saline, lysed in 10$^{-4}$ M EDTA, and resuspended to their original volume in 300 mOsm phosphate buffer (pH 7.4). These ghosts were incubated in the presence of 10 mM ATP, 10 mM MgCl$_2$, 1 mM lysolecithin, 0.1 mM coenzyme A, and 40 $\mu$M linoleic acid–$^{14}$C (1.5 mCi/mmole). Lipid extracts were analyzed for the incorporation of label into phosphatidylcholine as previously described (14). To assess the process in intact cells, approximately 4 $\mu$Ci (0.1 $\mu$mole) linoleic acid–$^{14}$C per ml of incubate was added to the incubation flask at the onset of the study. At intervals, specimens were removed, washed three times in saline, lysed in water, and extracts were analyzed for the incorporation of label into phosphatidylcholine (14).

## RESULTS

### Effects of fluoride on red cell ATP and potassium content

As has been previously observed (15) the addition of NaF (10 mmoles/liter) resulted in a rapid decrease in red cell ATP concentration both in mature and immature cells (Fig. 1). 5 mM NaCN was also added to inhibit the small contribution of mitochondrial ATP synthesis which occurs in immature cells (2). Cyanide

had no discernible effect on ATP and 2,3-DPG concentrations when used alone, and NaCN increased lactate production in immature cells less than 10%. The rate of ATP decline seemed faster in immature cells, but the difference was not statistically significant. Stable levels were attained in both in less than 1 hr. No further decline was noted at 3 or 4 hr. The final ATP concentration, 0.49 ±0.13 mmoles/liter cells, was approximately one-third of the initial value.

Fig. 1 also shows the rate of loss of potassium from fluoride-treated cells. After a lag period, during which ATP fell, a dramatic loss of potassium ensued, especially in immature cells. We also confirmed earlier findings (16–19) that this massive effect on potassium permeability was inhibited by 5 mM EDTA and was not obfree buffer (data not shown). In spite of this protection against potassium loss, ATP stability was unafserved when the incubation was performed in a calcium-iected, confirming previous observations (15). The addition of 2 mM ATP to the incubation mixture did not protect fluoride-treated cells against potassium loss.

Since the effect of fluoride is known to be dependent upon phosphate concentration (20), cells were incubated in 10 mM sodium fluorophosphate to determine whether that complex might be directly involved in the massive potassium loss. Neither potassium loss nor inhibition of lactate production were observed under these conditions.[2]

### The effect of fluoride on ATP metabolism

The dramatic loss of red cell ATP encouraged an inquiry into the pathways of ATP utilization in the fluoride-treated erythrocyte.

*The cation pump.* In the presence of 2.7 mM NaF, potassium loss from mature cells was linear and similar in magnitude to that produced by 0.1 mM ouabain (Fig. 2), indicating that this concentration of fluoride inhibited the cation pump but did not create the additional membrane damage and massive potassium loss associated with 10 mM fluoride. 10 mM fluoride totally abolished all erythrocyte ATPase activity, confirming the observations of Kirschner (21). Thus, no part of the ATP loss in cells exposed to 10 mM NaF could be attributed to the cation pump.

*Acylation.* A small fraction of newly synthesized red cell ATP is utilized for membrane phospholipid renewal (14). This process was not totally inhibited in fluoride-treated red cells until ATP had fallen to basal levels (Fig. 3). 1 mM CuCl$_2$, a potent inhibitor of the acylase system in red cell ghosts (22), immediately and virtually completely inhibited the incorporation of linoleic acid–$^{14}$C into the phospholipids of intact
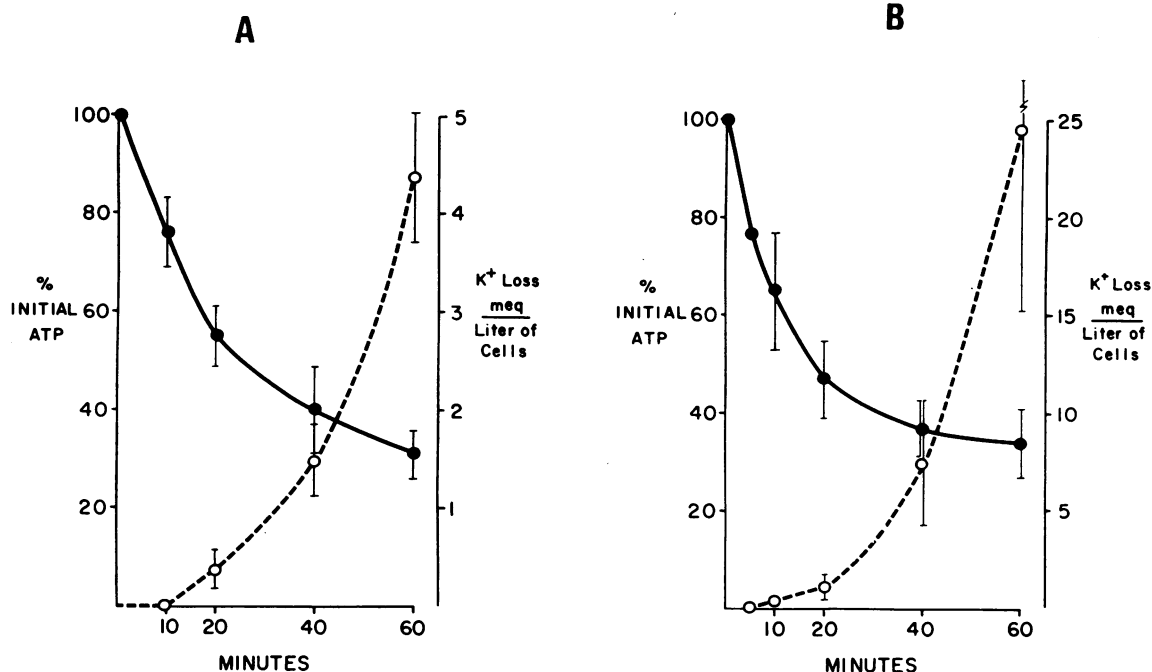
FIGURE 1  Effect of 10 mM NaF and 5 mM NaCN on mean ATP concentration and potassium loss in mature (A) and immature (B) populations of red cells. The reticulocyte counts of B ranged from 5 to 50%. Glutamic oxaloacetic transaminase activities for A were 0.4–0.6 U and for B were 1.1–3.1 U. Note the fivefold scale compression for potassium loss in B. ●——●, ATP ±SD; ○——○, potassium loss ±SD.

cells while the concentration of ATP was stable. The incorporation of labeled linoleic acid into red cell ghosts was inhibited by copper, while fluoride had no such effect (Table I). When copper and fluoride were added simultaneously to intact cells, the initial fall in ATP concentration was 10–20% less rapid than with fluoride alone (Fig. 4). These curves represent the means of five experiments and show that the mean ATP levels during the first 20 min of incubtion were significantly different ($P < 0.01$). The presence of copper did not alter the potassium loss induced by fluoride.

These studies provide evidence that in the fluoride-treated cell, some of the ATP loss could be due to its utilization in the membrane acylation reaction. Copper is also known to inhibit membrane ATPase at this concentration (23), but since fluoride was present, the ATPase system was already inoperative. Other undefined ATP-utilizing membrane reactions in addition to the acylase system also may have been inhibited by the presence of copper.

*Glycolysis.*  Normal glycolysis both produces and consumes ATP. The pattern of glycolysis was investigated in order to determine whether the fluoride-induced block at enolase could have lead to unbalanced ATP metabolism. Table II summarizes metabolic data derived from an incubation of normal red cells in the

presence of fluoride and methylene blue. These data are representative of several such studies. ATP instability occurred upon exposure to fluoride as previously described. Lactate production was immediately and drastically curtailed while the rate of glucose utilization was reduced more gradually. A rise in 2,3-DPG did not occur, which suggested that glycolysis was inhibited above the level of 1,3-DPG specifically at glyceraldehyde-3-phosphate dehydrogenase (GPD). The levels of triose phosphates and FDP observed at the onset of the incubation exceed normal values for fresh cells. This is probably the metabolic result of the preparation
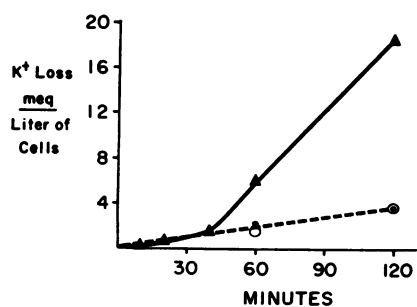


FIGURE 2  The effect of ouabain and sodium fluoride on potassium loss from normal red cells. ●——●, 2.7 mM NaF; ○——○, 0.1 mM ouabain; ▲——▲, 10 mM NaF.
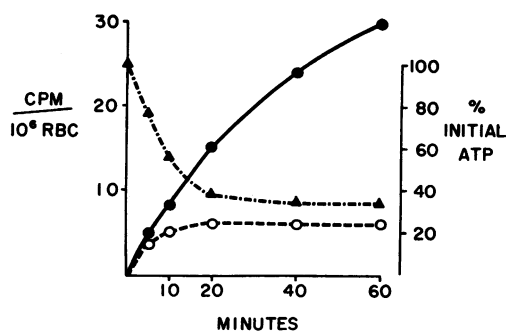
FIGURE 3 ATP stability and linoleic acid-$^{14}$C incorporation into membrane phospholipids in intact red cells. ●——●, incorporation, no addition; ○——○, incorporation (10 mM NaF) ; ▲——▲, ATP (10 mM NaF).

of the cells. Marked accumulation of FDP and trioses was noted during the incubation. Indeed, the glucose utilized was converted stoichiometrically to these intermediates and required net ATP utilization of the magnitude observed.

The apparent block at GPD could have been secondary to fluoride-induced inhibition of enolase, since a marked decrease in pyruvate production would have created a failure to regenerate NAD, a necessary cofactor for GPD (24–26). Accordingly, an attempt was made to regenerate NAD (and NADP) via a diaphorase system (27) utilizing methylene blue.

ATP instability due to fluoride was totally reversed by 1 mM methylene blue. Glucose utilization returned to normal, and lactate production improved markedly. Accumulation of FDP and trioses was reduced, attesting to. the fact that the block at GPD was overcome. Improved lactate and pyruvate production, most marked in the second time interval, demonstrated that the inhibition of enolase was partially overcome by mass action. This was accompanied by initial accumulation of 3PG. The terminal rise in triose and FDP probably reflects the inability of methylene blue to maintain the

oxidant effect. The concentration of 3PG which had been maintained earlier by the combination of increased glucose utilization, triose dehydrogenation, and partial enolase inhibition, fell at this time, presumably because of the inability to oxidize the trioses and the continued action of enolase at the elevated concentration of 3PG. Methylene blue also corrected the massive cation leak. The net potassium leak in the presence of fluoride and methylene blue was indistinguishable from that of 0.1 mM ouabain (Fig. 2).

The reversal of the effects of fluoride was possible even when methylene blue was added 1 hr after exposure to fluoride, when ATP levels had already fallen.

## DISCUSSION

Fluoride exerts multiple effects on erythrocyte physiology and metabolism. A dual role in cation homeostasis has been previously demonstrated (15–19) and was confirmed in these studies. Fluoride inhibited membrane ATPase 40% at 1 mmole/liter and completely at 5 and 10 mmoles/liter. Therefore the cation pump could not be a source of ATP utilization in 10 mM fluoride-induced ATP instability. We have shown, as have others (18, 19), that fluoride reacts directly with red cell membranes if free calcium is present. The combination induces a massive cation leak. The present studies demonstrated a need for higher concentrations of fluoride ion (10 mmoles/liter) for this effect than for the inhibition of enolase or ATPase. The protection against the membrane damage afforded by EDTA (17–19, 28) or intracellular, but not extracellular, ATP (19) suggests a role for red cell ATP in the maintenance of the

TABLE I

Effect of Copper and Fluoride on Red Blood Cell Stromal Acylase Activity

| Additive | N | millimicromoles fatty acid incorporated per milliliter stroma per hour |
|---|---|---|
| None | 6 | 10.0 ±0.7 |
| 10 mM CuCl₂ | 6 | 0.6 ±0.6 |
| 1 mM CuCl₂ | 6 | 0.9 ±0.5 |
| 0.1 mM CuCl₂ | 2 | 1.5 ±0.5* |
| 10 mM NaF | 3 | 9.6 ±1.0* |

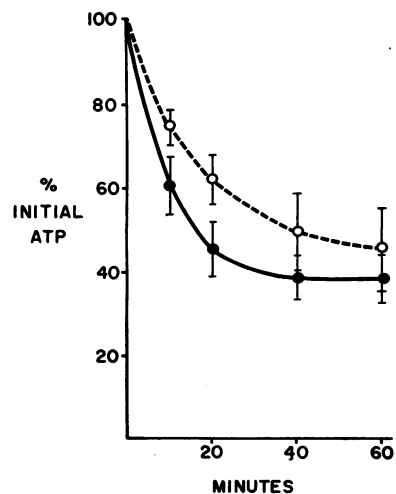* The value represents the mean and range.



FIGURE 4 Effect of copper on fluoride-induced ATP instability. ●——●, 10 mM NaF; ○——○, 10 mM NaF, 10 mM CuCl₂.

TABLE II
The Effects of Fluoride and Methylene Blue (MeB) on Glycolysis in Normal Erythrocytes

| | ATP | Glucose utilized | Lactate produced | Pyruvate produced | FDP | Triose phosphate | 2,3-DPG | 3PG |
|---|---|---|---|---|---|---|---|---|
| | mmoles/ liter cells | mmoles/ liter cells | mmoles/ liter cells | μmoles/ liter cells | μmoles/ liter cells | μmoles/ liter cells | mmoles/ liter cells | μmoles/ liter cells |
| No additive | | | | | | | | |
| 0 | 1.32 | — | — | — | 96 | 135 | 5.31 | 48.4 |
| 20 min | 1.39 | 0.56 | 1.20 | 2 | 53 | 227 | 5.31 | 46.2 |
| 60 min | 1.45 | 1.71 | 3.13 | 62 | 129 | 326 | 5.51 | 49.5 |
| 10 mM NaF | | | | | | | | |
| 0 | 1.34 | — | — | — | 91 | 190 | 5.46 | 57.5 |
| 20 min | 0.59 | 0.36 | 0.02 | 0 | 299 | 453 | 5.61 | 175.0 |
| 60 min | 0.14 | 0.72 | 0.22 | 0 | 495 | 695 | 5.36 | 282.0 |
| 10 mM NaF, 1 mM MeB | | | | | | | | |
| 0 | 1.66 | — | — | — | 90 | 188 | 5.41 | 67.0 |
| 20 min | 1.52 | 0.48 | 0.33 | 306 | 74 | 217 | 5.66 | 529.0 |
| 60 min | 1.57 | 2.15 | 1.93 | 960 | 216 | 512 | 5.71 | 192.0 |

physical integrity of the membrane through its interaction with calcium.

The failure of acylation in fluoride-treated intact cells coincided with the attainment of basal ATP concentration. The ghost assay demonstrated that fluoride does not directly inhibit phospholipid renewal. The concentration of ATP may have become limiting for the reaction in intact cells as has been previously suggested by Fischer and his associates (29). The studies with copper and fluoride indicate that acylation may be a significant pathway for ATP catabolism in the erythrocyte. Shohet, Nathan, and Karnovsky estimated from stoichiometric analysis that only 5–10% of red cell ATP is directed toward phospholipid renewal (14), whereas copper, an inhibitor of the acylase system, decreased the rate of ATP utilization by nearly 20%. Whether acylation is more costly than appears from the studies of Shohet and coworkers or whether other copper-inhibitable ATP-utilizing reactions are present on the membrane is not established. In any case, acylation and the pump together account for approximately one-third of total red cell ATP catabolism (14, 30), and the pathway(s) whereby the remainder is utilized remains a subject of speculation and inquiry.

Fluoride alters the kinetics of enolase, increasing the requirement for 2PG (1). This effect requires the presence of inorganic phosphate (20). It is markedly potentiated by the secondary failure to regenerate NAD by pyruvate reduction, which results in inhibition of the GPD reaction with accumulation of FDP, DHAP, and G3P, and no change in the concentration of 2,3-DPG. When oxidized pyridine nucleotide is regenerated as with methylene blue, GPD functions, and enolase inhibition is partially overcome by mass action. The inhibition of lactate production can also be reversed by the addition of inosine, which, at low concentrations of inorganic phosphate, further reduces the intracellular phosphate to a level at which fluoride no longer inhibits enolase (20). At higher concentrations of phosphate, inosine is much less effective in restoring lactate production.

Several authors (24–26, 31) have demonstrated the central role of the NAD:NADH ratio in the control of glycolysis. Omachi, Scott, and Hegarty (25) have specifically noted a reduction in the concentration of NAD in red cells incubated with 5 mM fluoride. Under these circumstances one would expect glucose utilization to proceed up to the point at which the NAD depletion results in glycolytic obstruction at GPD, which is before the ATP regeneration steps. Such unbalanced glycolysis would result in stoichiometric depletion of ATP by hexokinase and phosphofructokinase. Ultimately, ATP becomes insufficient for hexokinase, and the cell's phosphate stores become trapped as FDP and triose phosphates.

Whittam has noted a "curious" effect of pyruvate that prevents massive loss of potassium from red cells exposed to high concentrations of fluoride (32). Our results may explain this "curious" effect since pyruvate, like methylene blue, produces regeneration of NAD, thereby providing the cofactor for GPD. ATP levels are maintained, and protection is afforded by the membrane insult which results in massive potassium loss. The direct inhibition of the cation pump is not affected by methylene blue. Recently, Oski, Travis, Miller, Papadopoulos, and Cannon have shown that high concen-

trations of pyruvate can restore 2,3-DPG levels and decrease triose concentrations in bank blood stored in ACD after 4 hr of incubation (33). Presumably, restoration of NAD levels is responsible for this effect.

Rose and Warms have suggested that erythrocyte pyruvate kinase deficiency may result in red cell ATP depletion through a secondary failure of GPD as a result of a reduction in the NAD:NADH ratio (24). The similarity of the fluoride-treated cell is apparent. The reversal of ATP instability in fluoride-treated cells by methylene blue suggests that treatment of pyruvate kinase–deficient red cells with an oxidant might be beneficial. Regeneration of NAD would increase levels of 2PG, 3PG, and PEP, but the success of such treatment might depend upon the presence of a high $K_m$ variant of pyruvate kinase (34) which would be more active at a higher than usual substrate concentration. The influence of methylene blue on the metabolism of PK-deficient red cells is now being investigated.

## ACKNOWLEDGMENTS

*Note added in proof:* We have recently appreciated that Keitt published pertinent studies of fluoride effects on red cells in abstract form in 1967 (35). He noted triose accumulation and a fall in the concentration of ATP. He also observed that methemoglobin protected the cells from the effects of fluoride. Finally he suggested that the imbalance of glycolysis was mediated by the limited production of NAD.

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