Accumulation of Iron in the Rabbit Erythroid Cell As Affected by Ouabain, Sodium and Potassium Ions, and Temperature

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ABSTRACT Reticulocytosis was induced in rabbits with phenylhydrazine. The accumulation of a small part of ^{59}Fe in blood cells of these animals was inhibited by ouabain and related to changes in extracellular sodium and potassium concentrations. Sodium increases movement from the cell surface into the cell, whereas potassium and ouabain decrease this movement. ^{59}Fe movement was found to be temperature-dependent. Thus, the Na-K ATPase system appears to be important in the movement of iron from the cell membrane (stroma) to the cell interior, but influences only a small part of the total iron transport.

INTRODUCTION

Movement of iron into the developing red cell is a complex process characterized by (*a*) formation of an iron-transferrin complex in the plasma, (*b*) attachment of this complex to a specialized cell surface receptor, (*c*) release of iron to form a new iron-protein complex, possibly ferritin, and finally (*d*) the utilization of iron in hemoglobin or other iron compounds in the cell.

Katz and Jandl have reviewed the role of transferrin in the transport of iron to the developing red cell and indicate that iron is delivered by an iron-transferrin complex that becomes attached to the cell surface (1). The metal is actively removed and incorporated in the cell and apparently the transferrin is then displaced by other iron-bearing molecules. In earlier work Jandl and others showed that iron uptake by the reticulocyte is blocked by several metabolic inhibitors and low oxygen tension (2). Later Jandl and Katz concluded that, like the uptake of iron, the attachment of transferrin to the surface of the reticulocyte is blocked by such agents as cyanide and 2,4-DNP (3). Morgan has reported studies on interaction between rabbit, human, and rat transferrin and reticulocytes and observed that uptake of transferrin by cells takes place in two steps: first, the formation of a weak union probably by

physical absorption, and second, the development of a stronger union dependent on cell metabolism (4).

Preliminary experiments indicate that ouabain restricts accumulation of ^{59}Fe in the rabbit reticulocyte, raising speculation as to whether the sodium-potassium–activated ATPase enzyme system may be involved in some manner in iron transport (5). Recent studies indicate the possible participation of a Na^+-K^+–activated ATPase system in the transport scheme of a number of substrates, as well as transport of Na^+ and K^+. Evidence is based mainly on inhibition of transport by cardiac glycosides. The substrates and structures include Ca^{++}, Mg^{++}, inorganic phosphate, and glucose in the renal tubule of the dog (6), creatine in rat brain (7), and L-tyrosine, DL-phenylalanine, and uracil in frog small intestine (8). Also, significantly, Na^+ is required for transport of a variety of substances, such as Cl^- in frog cornea (9), sulfate in rabbit intestine (10), glycine in pigeon red blood cells (11), L-tyrosine and glucose in rat intestine (12) and, arbutin in chicken intestine (13). When ouabain was used, it was effective in inhibiting transport (7, 10, 12).

In the present experiments reticulocytosis was induced in rabbits with phenylhydrazine. With in vitro techniques, movement of ^{59}Fe into blood cells of these animals was found to be reduced by ouabain, related to changes in extracellular Na^+ and K^+ concentrations, and temperature-dependent.

METHODS

Young, adult New Zealand rabbits of both sexes were the experimental animals. Reticulocytosis was induced by injecting 0.25 ml/kg of 2.5% aqueous phenylhydrazine daily for 4 consecutive days. Blood was drawn by cardiac puncture on the 7th day. Reticulocytes (55-65% of blood cells) were identified with new methylene blue. Hematocrit readings were reduced about 65% in treated animals. Cells at the outset were washed three times with isotonic (0.28 M) tris [2-amino-2-(hydroxymethyl)-1,3-propanediol], brought to pH 7.2–7.4 with HCl. Isotonicity of the Tris-Cl (0.28 M) was verified by microscopic examination of the cells. All centrifugation was 1750 × *g*. This *g* force is sufficient to sediment the stroma completely from the rest of the hemolysate. ^{59}Fe was counted in the water hemolysate (total) and supernate fraction separately. Stroma, or particulate ^{59}Fe, was represented as the difference in counts. Duplicate determinations were always made. Each experiment was compared with a control of the same cells. For simplicity, all blood cell suspensions containing reticulocytes are referred to as "reticulocytes."

Cell Iron Fractionation, Method 1 After washing, 5.0 ml of packed reticulocytes were suspended in 95.0 ml of standard medium containing 125 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 19 mM Tris-Cl buffer, 0.5 ml rabbit serum, and 10^{-7} M iron ($^{59}FeCl_3$). The ^{59}Fe and serum were mixed first to bind the iron to the serum proteins. The amount of ^{59}Fe added to the serum was less than the iron-binding capacity of its transferrin and therefore the experiments follow the uptake by cells of iron from the iron-transferrin complex. In experiments in which Na^+ and K^+ were omitted, iso-

tonicity was maintained by the addition of the appropriate amounts of Tris-Cl. When ouabain was added it was 10^{-4} M. The suspension of cells, after shaking, was divided into three aliquots in flasks. One served as control. They were agitated in a water bath at 37°C for the respective time intervals. Two 1.0 ml samples of each preparation were then placed into each of two test tubes which were centrifuged for 1 min. The supernate was aspirated, and the cells were resuspended in 2.0 ml of isotonic NaCl solution containing 10^{-7} M nonradioactive $FeCl_3$. This preparation was centrifuged and the supernate removed. The supernate of successive washings was found to be free of ^{59}Fe and therefore, single washings were employed in all experiments. Finally, the cells were hemolyzed in 2.0 ml of water and centrifuged for 3 min. Radioactivity of the supernate after hemolysis and centrifugation was counted with a Nuclear-Chicago DS5 well-scintillation detector, attached to a 181A decade scaler (Nuclear-Chicago Corp., Des Plaines, Ill.).

Cell Iron Fractionation, Method 2 After washing, 5.0 ml of packed reticulocytes were suspended in 95.0 ml of isotonic Tris-Cl solution. A 1.0 ml aliquot was introduced into each of a number of test tubes which were then centrifuged for 1 min. Each supernate was replaced with 5.0 ml of the standard medium (containing ^{59}Fe) at 37°C for appropriate time intervals. The washing, centrifugation, and counting were as described under Method I.

Pulse Labeling After washing, 5.0 ml of packed reticulocytes received preliminary treatment, as under Method 2, but with the standard medium modified as follows: either (*a*) Na^+ was omitted, or (*b*) K^+ was omitted. Isotonicity was maintained with Tris-Cl buffer. After agitation in the water bath for 4 min at 37°C the preparation was cooled 20 sec in ice water and then centrifuged in a refrigerated centrifuge for 1 min. The supernate was removed and replaced with 5.0 ml of solution identical in composition except that iron was in the nonradioactive form. This was followed by incubation at 37°C for appropriate time intervals. Finally, the cell culture was centrifuged for 3 min, hemolyzed, and assayed for ^{59}Fe as under Method 1.

RESULTS

The effect of ouabain (10^{-4} M) and a medium lacking Na^+ and K^+ on ^{59}Fe uptake (as a per cent of maximal control uptake) during 3 hr incubation is shown in Fig. 1. The cells were treated as outlined under Method 1. After 3 hr exposure to ouabain, the ^{59}Fe in the soluble supernate was about 20% less than in the control. The action of ouabain was not demonstrated until after 60–90 min. In eight experiments inhibition and latency were demonstrated. When Na^+ and K^+ were omitted from the incubating fluid, considerably less iron ($\approx$38%) moved into or remained in the cell interior.

In order to test the effect of ouabain on iron uptake and the relation between ouabain inhibition and Na^+ concentration in the incubation medium, cells (prepared by Method 2) were incubated for 4 hr in a medium containing 10^{-4} M ouabain and ^{59}Fe and various concentrations of Na^+, and the rate of iron uptake compared to that in a medium containing no ouabain (K^+ absent

in both cases). The uptake of iron was found to increase with greater concentrations of Na^+ in the medium as shown in Fig. 2. Ouabain inhibited the total iron uptake only slightly even at the higher Na^+ concentrations. As the Na^+ concentration was increased above 40 meq/liter, the ^{59}Fe in the supernate increased linearly. In the presence of ouabain, a linear increase in uptake with

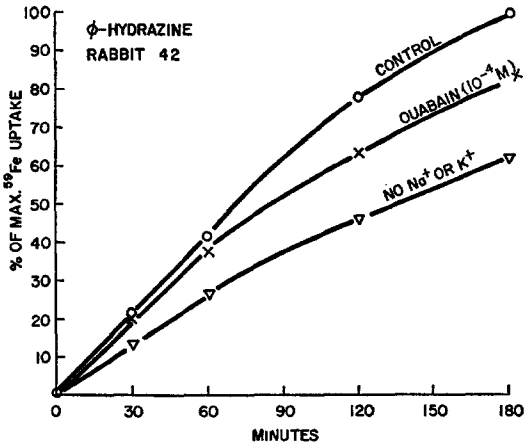

FIGURE 1. Supernatant ^{59}Fe (Method 1), when reticulocytes were incubated without Na^+ and K^+, and with ouabain (Na^+ and K^+ present).

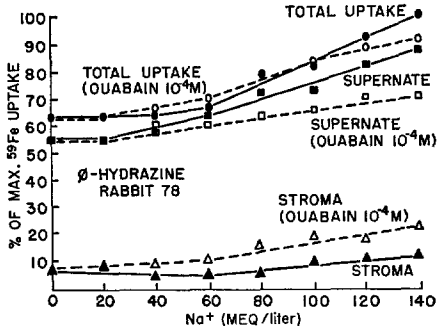

FIGURE 2. Total, supernatant and stroma ^{59}Fe (Method 2), after reticulocytes had been incubated for 4 hr as a function of Na^+ concentration, with and without ouabain (solid symbols, without ouabain; open symbols, with ouabain).

increased Na^+ concentration also occurred, but the slope of the curve is significantly decreased. The distribution between the supernate and the stroma was much greater in the control cells than in the cells exposed to ouabain. Thus ouabain seemed to hinder the ^{59}Fe movement from the cell stroma to the supernate. This inhibition increased as the Na^+ concentration in the incubation medium was increased. Although the amount of iron which was on the stroma increased as the Na ion concentration increased, there was a much greater increase in the amount found in the supernate. Since ouabain inhibits

the movement from stroma to supernate, this implies that it is this step which is Na-K ATPase–dependent.

The effect of increasing Na^+ concentration on iron in the supernate is shown in Fig. 3. The procedure is that of Method 2. K^+ was held constant at 5 mM

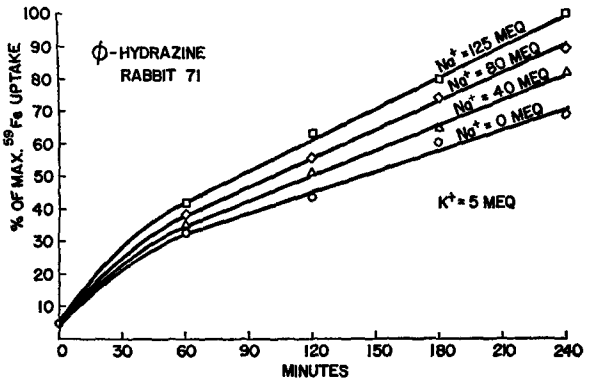

FIGURE 3. Supernatant ^{59}Fe (Method 2), at 60 min intervals, when reticulocytes had been incubated for 4 hr with increasing Na^+ concentrations.

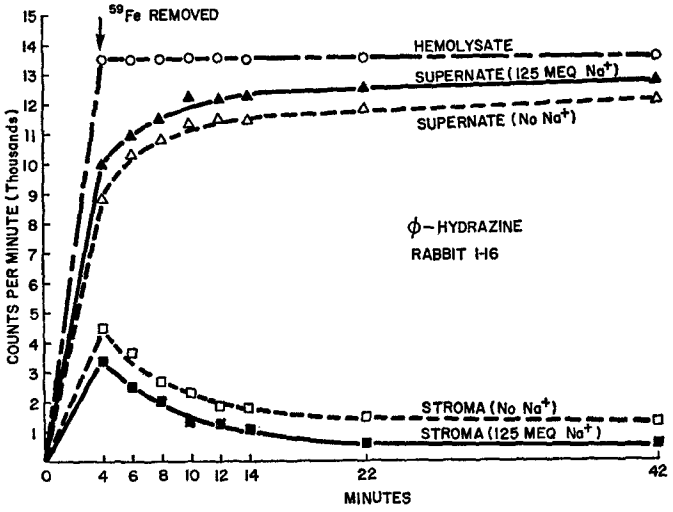

FIGURE 4. ^{59}Fe pulse labeling of reticulocytes with and without 125 meq Na^+ (K^+-free).

and cells were incubated for 4 hr at 37°C. ^{59}Fe moved into the cell interior as incubation proceeded and the rate of accumulation was greatest in the 1st hr. ^{59}Fe concentration was directly correlated with increasing Na^+ concentration. However, it was found that the greater part of the iron movement into the soluble fraction was independent of Na^+.

Data obtained from the pulse-labeling study are shown in Fig. 4. The experiment was devised to follow iron movement in the stroma and supernate

as influenced by the omission of Na^+ and the presence of 125 mM Na^+ (K^+ absent in both). The plotted curves indicate early progressive movements of ^{59}Fe from the membrane to the cell interior with Na^+ present. The absence of sodium inhibits the movement of a small fraction of the ^{59}Fe from the stroma to the supernate. This fractions remains attached to the stroma during further

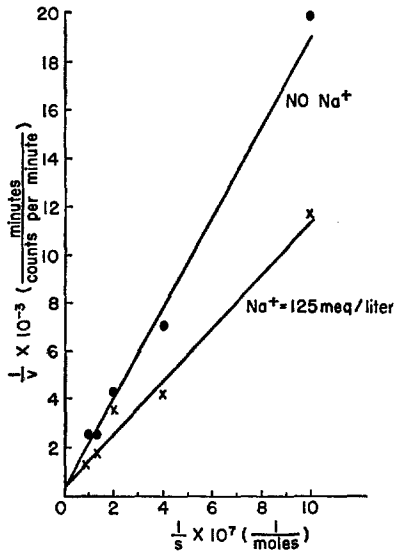

FIGURE 5. Lineweaver-Burk plots obtained from supernatant ^{59}Fe counts when reticulocytes were incubated for 0–6 min with and without Na^+ (K^+-free).

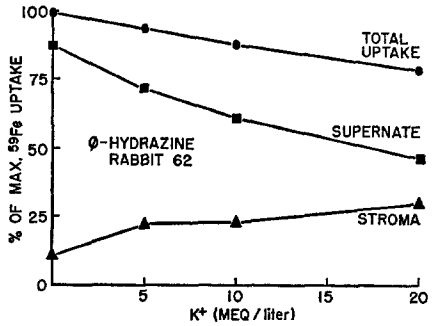

FIGURE 6. Total, supernatant and stroma ^{59}Fe (Method 2), after reticulocytes had been incubated for 1 hr as a function of K^+ concentration.

incubation with nonradioactive iron. This agrees with the observed effect of ouabain.

^{59}Fe in the supernate was measured at various concentrations of iron, in the presence of 125 mM Na^+ or in the absence of Na^+. The experimental treatment was basically that described under Method 2, except that the medium was free of K^+ and samples were taken from 0–6 min. The data are shown in Fig. 5 as a Lineweaver-Burk plot from which the iron concentrations needed to give a

half-maximal rate of uptake can be estimated. This apparent K_m is $\sim 3 \times 10^{-7}$ moles/liter with Na^+ present and $\sim 5 \times 10^{-7}$ moles/liter in the absence of Na^+. Thus, there is a difference in the apparent K_m in the presence and absence of sodium. The V_{max} appears to be the same both in the presence and absence of Na^+. It should be pointed out that many assumptions are inherent in the use of Michaelis-Menten kinetics for such a multistage process.

The uptake of ^{59}Fe for the total cell, hemolysate, and stroma are shown in Fig. 6 as a function of K^+ concentration in the medium (Na^+ not present). Method 2 treatment was followed and the cells were incubated for 2 hr. The total cell uptake of ^{59}Fe decreased when K^+ was increased stepwise to 20 meq/liter. Potassium had an inhibitory influence even at low concentrations.

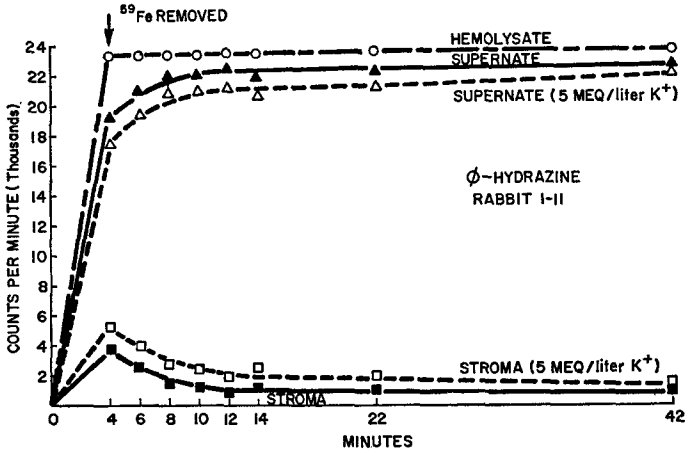

FIGURE 7. ^{59}Fe pulse labeling of reticulocytes with and without 5 meq K^+ (125 meq Na^+ present).

Data from pulse-labeling experiments with either 5 mM K^+ or no K^+ (125 mM Na^+ present in both) are presented in Fig. 7. The presence of potassium inhibits the movement of ^{59}Fe from the stroma to the supernate. During further incubation with nonradioactive iron, this small fraction of ^{59}Fe remains attached to the stroma if potassium is present. This also agrees with the effect of ouabain and the absence of sodium ions.

Experiments were run to determine the effect of temperature on ^{59}Fe movement. In these experiments, duplicate sets of cells, treated as under Method 2, were incubated in standard medium at various temperatures for 30 min. When ^{59}Fe movement at 5°, 15°, 25°, and 35°C is presented in the form of an Arrhenius plot (Fig. 8), the movement of ^{59}Fe into the supernate is seen to be greatly reduced as the temperature is decreased. The Q_{10} between 5° and 15°C or between 25° and 35°C was 1.4 to 2, whereas the Q_{10} between

15° and 25°C was 3.5 to 4. These values are well above that for simple aqueous diffusion and suggest, but by no means prove, that movement of iron into the reticulocyte involves the formation and breaking of chemical bonds. The apparent activation energy between 15° and 25°C was approximately 12,000 cal/°K/mole. These results have been verified in six experiments.

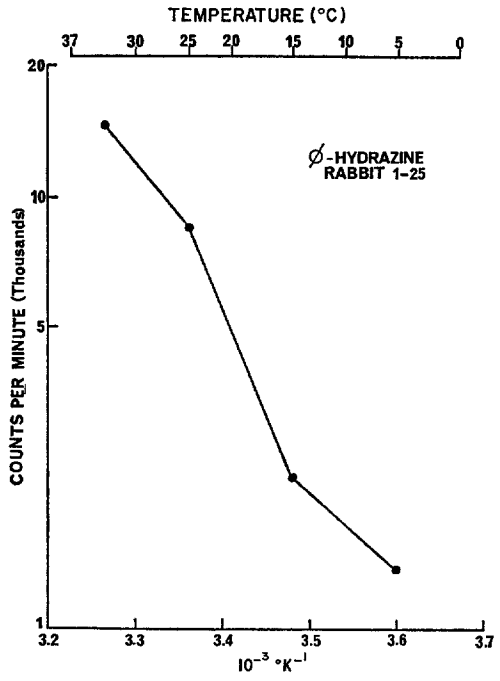

FIGURE 8. Arrhenius plot of ^{59}Fe movement into rabbit reticulocytes in the presence of Na^+ at 35°, 25°, 15°, and 5°C.

DISCUSSION

Post et al. (14) recently reported additional evidence that ouabain is specific for the active sodium pump, but does not affect the passive movement of Na and/or K ions. The effect of ouabain in suppressing part of the iron movement from the stroma to the supernate, but with little if any effect on total uptake, implies that it is the movement from the stroma to the supernate and not the movement from the media to the stroma which is Na-K ATPase–dependent. The reticulocytes present in the cell suspension are known to take up the ^{59}Fe, since rabbit erythrocytes do not take up a significant amount of iron under experimental conditions where the iron is bound to serum proteins as in these experiments (15, 16).

Jandl et al. have investigated the possibility that iron uptake in the red cell might be suppressed by inhibition of heme synthesis (2). They found that when incorporation of iron into hemoglobin was almost entirely prevented by 5×10^{-4} M Pb^{++}, whole cell uptake of ^{59}Fe by human reticulocytes was only

slightly diminished. In experiments in this laboratory (15), the inhibitory effect of ouabain on iron movement into cells was not influenced by either lead or puromycin, which inhibits heme biosynthesis. Uptake of ^{59}Fe by reticulocytes after application of 10^{-3} M 2,4-dinitrophenol or 10^{-3} M iodoacetic acid, markedly decreased ^{59}Fe movement in the whole cell (2, 15). A marked temperature dependence of the Na-K ATPase was reported by Wood and Beutler (17). Our present results that show temperature dependence of iron movement appear to parallel their findings for the Na-K ATPase system.

A considerable part of the rate of iron movement into the interior of the reticulocyte in the present experiments was a function of the Na^+ concentration in the medium. When cells were incubated for up to 4 hr in a medium free of Na^+ and K^+, less iron moved into the cell interior. When Na^+ was present in the incubating fluid in a concentration above that normally found in the rabbit blood cell, about 30 mM, ^{59}Fe accumulation was a function of the external Na concentration. The first sign of ouabain restraining action on iron movement, in contrast to the control, appeared above 40 mM Na^+. Also, ouabain displayed no action on iron movement until after 60–90 min. Konsek and Bishop (18) reported that intracellular sodium concentration in erythrocytes increased only after exposure to ouabain for 1–2 hr. The observed latent action of ouabain in reducing iron movement into the cell could be associated with the time necessary for the internal sodium concentration to increase. Since part of the iron movement into the cell is dependent on a higher external sodium concentration than an internal one, only after the sodium gradient is reduced by the action of ouabain would the ^{59}Fe movement be reduced. The inhibitory action of K^+ on iron movement in the reticulocyte is quite evident, as shown in Fig. 7.

From both uptake and pulse-labeling experiments, it can be seen that movement of ^{59}Fe from the cell surface to the cell interior is influenced by sodium more than the movement from the media to the cell surface. Potassium decreases the movement from the cell surface to the cell interior, but only very slightly decreases ^{59}Fe movement from the medium to the cell surface. The implication is that sodium increases movement from the cell surface into the cell whereas potassium decreases this movement. Both sodium and potassium have an opposite but very small effect on iron movement from the medium to the cell surface. These effects agree with the observed effect of ouabain and further support the observation that Na-K ATPase is involved in the movement of a small amount of iron from the stroma to the supernate.

Several features of these studies, especially those pertaining to Na^+ dependency, parallel Crane's findings in investigation of sugar transport in the intestine (19). Crane postulated that a mobile carrier was capable only of equilibrium. The asymmetry required to achieve uphill accumulation of the substance was attributed to a gradient of Na^+ concentration downhill into the

cell, maintained by operation of an outwardly directed, energy-dependent Na^+ pump located in the membrane. Two forms of a carrier were conceived. One was the Na^+-loaded form in which the conformation of the substrate binding site has a higher affinity for the substrate. The other was a K^+-loaded form with reduced affinity for the substrate.

The findings in the present experiments and those reported from this laboratory earlier (5, 15, 16) constitute supporting evidence that the Na-K ATPase influences only a small part of iron transport, and that the Na-K ATPase appears to be important in the movement of iron from the cell membrane (stroma) to the cell interior.

The authors wish to acknowledge the technical assistance of Mrs. Lois Hornbeck.
This work was supported in part by Public Health Service Grant AM09069, and by a National Institutes of Health Predoctoral Fellowship, No. 5-F1-GM-29845, to W. C. Wise.

Received for publication 7 June 1968.

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