Na_2HPO_4 without an increase in the volume of water.

Throughout the experiments, the rats were maintained exclusively on Purina Laboratory Chow (Ralston Purina Co. of Canada) and tap water. The cardiac necroses and the convulsive motor disturbances typical of digitoxin poisoning were assessed in terms of an arbitrary scale of + to +++ (*5*). For histologic studies, specimens were fixed in Susa solution saturated with picric acid and stained with the periodic acid–Schiff technique.

The first manifestations of digitoxin poisoning were the characteristic motor disturbances which became evident within 24 hours after initiation of the experiment in the rats receiving the higher dose of the glycoside (groups 1 and 2). During this early stage, the motor disturbances were almost as severe in the rats given spironolactone as in the corresponding controls. However, in the course of the next 2 days, the rats treated with spironolactone recovered despite continued treatment with digitoxin, whereas the controls became increasingly more affected, so that, toward the end of the experiment, they were almost continuously in convulsions and unable to stand up. The rats receiving the smaller dose (groups 3 and 4) were much less affected; here, convulsions did not appear in treated animals at any time and in the controls only toward the 3rd day.

Cardiac necrosis was sharply limited to the apex of the heart and easily visible to the naked eye as a disk-shaped whitish area (Fig. 1). Histologically, in the affected region, there was massive necrosis of muscle fibers with histiocytic infiltration, but without any evidence of vascular occlusion. The intensity of the cardiac necrosis was greater in rats receiving a high dose of digitoxin than in those receiving a low dose, but spironolactone completely prevented the development of lesions in both groups. Mortality of those receiving high doses was only diminished by treatment with spironolactone, but was totally abolished in those treated with a low dose.

Under the conditions of these experiments, spironolactone is a potent antidote for digitoxin; yet this effect cannot be ascribed with certainty to a competitive antagonism between the cardiac glycoside and the steroid inhibitor. The fact that both spironolactone and digitoxin are steroids possessing a lactone side chain merely suggests such an interpretation.

According to current opinion, the competitive antagonism between spironolactone and mineralocorticoids is limited to their effect upon potassium elimination by the renal tubules. In this respect the action of spironolactone can be duplicated by amiloride, an agent that inhibits renal elimination of potassium, but does not act by competitive inhibition of mineralocorticoids. However, amiloride is much more potent than spironolactone as a potassium-sparing agent and in protecting the rat against the cardiac necrosis produced by fluorocortisol in combination with bisodium phosphate (*6*); yet, even at optimum potassium-sparing doses, amiloride fails to protect the rat against the manifestations of digitoxin poisoning. The same is true of KCl given at the near-toxic dose (1 m*M*) twice daily by stomach tube.

Although additional treatment with phosphate and fat aggravates the cardiac lesions produced by digitoxin, it has no effect on the associated motor disturbances. Thus, these two manifestations of digitalis intoxication are separable, yet both can be prevented by spironolactone. Furthermore, if, under otherwise identical conditions, digitoxin alone is given subcutaneously and spironolactone is given by mouth, the motor disturbances are also prevented; hence, apparently, the prophylactic effect is not due to a direct chemical interaction between the two compounds in the gastrointestinal tract nor to an inhibition of digitoxin absorption from the intestine.

These findings suggest that spironolactone interferes specifically with digitoxin actions not only in the heart but also at various receptor sites and that its protective effect is not due merely to the restriction of potassium elimination. The mechanism of this interaction cannot yet be appraised, and indeed, we have no proof that the digitalis antagonizing effect would occur under clinical conditions. However, since digitalis and spironolactone are frequently administered conjointly to cardiac patients, we wanted to call attention to the possible influence of antimineralocorticoid diuretics upon digitalization.

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Membrane Alterations in Hemolysis: Internalization of Plasmalemma Induced by Primaquine

Abstract. *Incubation of normal human erythrocytes with primaquine, a derivative of 8-aminoquinoline, results in internalization of the cell membrane and the formation of intracellular vacuoles. These changes are similar to those observed in other types of cells in pinocytosis. The reduction in surface cell membrane which accompanies internalization of plasmalemma may be generally significant in the destruction of red cells.*

The destruction of erythrocytes in physiological or pathological processes is often related to alterations in the integrity of the membrane (*1*). Thus, mechanisms of hemolysis in normal extracellular fluid include changes in effective cation permeability, changes in macromolecular permeability, or fragmentation of the membrane (symmetrical lipid loss). In the latter case, the loss of membrane lipid is accompanied by a decrease in the surface area and an increase in spheroidicity which results in a change in the cell from a deformable body to a more rigid one. Fragmentation may occur repeatedly without loss of cell contents to the surrounding medium (*2*). Such an occurrence attests to the ability of the membrane to repair itself. We now present

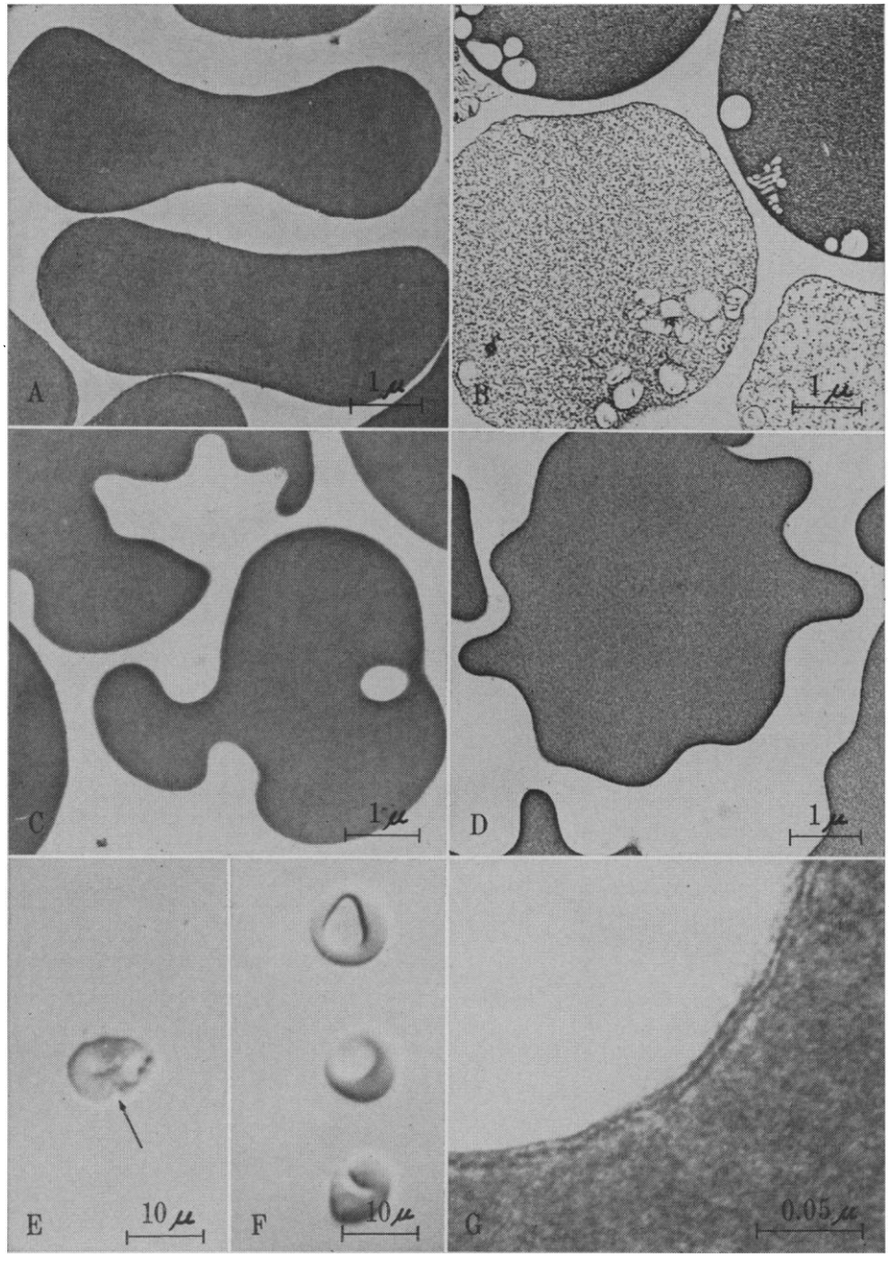

Fig. 1. (A) Electron micrograph of control red blood cells showing characteristic biconcave shape. Cells were fixed in suspension in 1.25 percent glutaraldehyde, collected on Millipore filters, washed in 0.067*M* cacodylate buffer overnight, and treated in 1 percent osmium tetroxide buffered with *s*-collidine. Cells were stained en bloc with uranyl acetate, dehydrated, and embedded in Epon (× 10,400). (B) Cells treated with 1.0 m*M* primaquine for 2 hours. All cells contain peripheral vacuolization. Several degrees of hemolysis are shown. In the upper right there are two cells with normal internal density; in the lower left the cells show intermediate loss of hemoglobin, and in the lower right a more extreme loss with flocculent internal contents. Specimen preparation as in (A) (× 10,400). (C) Cells treated with 1.0 m*M* primaquine and 0.1 m*M* palmitic acid for 2 hours. The shape of the cells with several large broad-based invaginations is irregular. A single peripheral vacuole is seen at the right. Specimen preparation as in (A) (× 10,400). (D) Cells treated with 0.1 m*M* palmitic acid for 2 hours. There are shallow indentations along the cell periphery. Peripheral vacuoles are absent. Fixation and preparation as in (A) (× 10,400). (E) Light micrograph taken with Nomarski optics of cell treated with 1.0 m*M* primaquine for 2 hours. Note the irregularity in contour (arrow) with large and small vacuoles (× 1,100). (F) Light micrograph with Nomarski optics of cells incubated for 2 hours with 1.0 m*M* primaquine and 0.1 m*M* palmitic acid. Note the appearance of the large invaginations (× 1,100). (G) Wall of peripheral vacuole in cells treated with 1.0 m*M* primaquine for 2 hours. The vacuole is surrounded by a typical unit membrane identical with plasma membrane. Specimen preparations as in (A) (× 300,000).

evidence that erythrocytes may undergo invaginations of the cell membrane which then appear to pinch off and seal, forming intracellular vacuoles. This internalization of the cell membrane is reminiscent of pinocytosis observed in other cell types. We have observed this process after the treatment of normal human erythrocytes with the 8-aminoquinoline derivative primaquine. It seems likely that the hemolysis of normal erythrocytes induced by this drug in vitro follows from the reduction in surface area of the cell which must accompany the internalization of the cell membrane. This view is substantiated by the finding that simultaneous treatment of red cells with palmitic acid, which prevents the hemolysis induced by primaquine (*3*), also decreases the extent of the internalization of the erythrocyte membrane.

Washed normal human red blood cells were incubated in Krebs-Ringer phosphate buffer (*p*H 7.4) with 200 mg of glucose per 100 ml in a shaking incubator at 37°C. The volume of the incubation mixture was 2.0 ml, and the hematocrit was 30 percent. Control tubes contained no other additions; experimental tubes contained 1.0 m*M* primaquine diphosphate, 1.0 m*M* primaquine diphosphate plus 0.1 m*M* palmitic acid, or 0.1 m*M* palmitic acid alone. The experiments were terminated at 2 hours, and the percentage of hemoglobin loss was determined by measuring the hemoglobin released into the medium (*4*). Samples for morphologic study were also taken. Details of the morphologic techniques are given in the figure legends.

Incubation of normal human red cells with high concentrations of primaquine in the presence of glucose results in rapid hemolysis (*5*). In our study, 15 percent of the hemoglobin was lost to the medium in primaquine-treated cells at the end of 2 hours. Control cells or cells treated with fatty acid plus primaquine or with fatty acid alone showed no detectable hemolysis during this period.

Control cells were typical biconcave discs (Fig. 1A) surrounded by a trilaminar unit membrane. Internally the cells were homogeneous and of high electron density. In contrast, cells treated with primaquine were spheroidal and contained numerous intracellular peripheral vesicles (Fig. 1B). These vesicles were sometimes in clusters with areas of confluence. The trilaminar membrane lining the vesicles (Fig. 1G) was identical with the surface membrane. Some

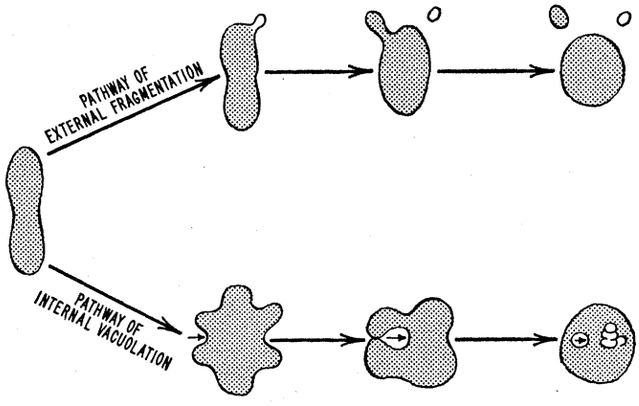

Fig. 2. Representation of two mechanisms leading from a normal biconcave disc to a sphere with reduced surface area. Along the top is shown the mechanism of fragmentation described by Weed (*1*) which involves budding of small protrusions from the disc and from early sphere stages, leading ultimately to spheres and to small fragmented particles. Along the bottom is shown the mechanism proposed for chemical hemolysis in which the biconcave disc becomes a crenated sphere with large broad-based invaginations. This is followed by an irregular sphere with internalization of the surface membrane in the form of vacuoles ultimately leading to a sphere with numerous small peripheral vacuoles. The arrow indicates the progression of a single point of the cell membrane.

cells in primaquine-treated preparations were partially hemolyzed, as evidenced by a decrease in density and increased flocculence of the internal matrix. In other cells plasmalemmal continuity was interrupted and hemoglobin was extruded into the extracellular space. Primaquine-treated preparations also showed numerous red cell ghosts with clear interiors and evidence of some dense material adhering to the internal aspect of the plasma membrane. Numerous peripheral vesicles remained in the ghosts. Examination of primaquine-treated cells with Nomarski optics revealed changes in contour, including large irregular invaginations and small circular lucent areas (Fig. 1E) corresponding to the changes seen in sectioned preparations. Cells treated with primaquine in the presence of fatty acid were spheroidal with broad-based, deep invaginations; they contained only occasional vesicles (Fig. 1, C and F). Cells treated with fatty acid alone were also spheroidal but had many shallow indentations around their circumferences (Fig. 1D).

The action of primaquine in erythrocytes which lack the enzyme glucose-6-phosphate dehydrogenase is associated with the generation of hydrogen peroxide (*6*) and the failure of these genetically abnormal cells to adequately detoxify intracellular peroxide (*4, 7*). Normal erythrocytes, which we studied, may also undergo a primaquine-induced hemolysis in vitro which is apparently unrelated to the formation of peroxide or to its detoxification (*8*). Such hemolysis requires large concentrations of the drug (1.0 m*M* or greater), is preceded by a leak of potassium ions from the cells (*9*), and is accompanied by a stimulation in the incorporation of long chain fatty acids into membrane phospholipids (*10*). Our experiments also show that hemolysis induced in normal red cells by primaquine is associated with characteristic morphologic changes involving the plasma membrane. The most striking of these is the appearance of numerous intracellular vesicles which appear to arise from the constriction and pinching off of broad-based invaginations. These invaginations result in a decrease in surface area, thus reducing the critical hemolytic volume of the cell. The consequences of membrane internalization are therefore similar to those produced by fragmentation in that both processes result in a decrease in cell surface membrane. On the basis of light microscopy, Ponder (*11*) proposed that decreased surface area was a consequence of "wrinkling of the surface ultrastructure." Our microscopic observations support and extend his views.

If membrane loss takes place by internalization, it is necessary to assume that the vesicles formed do not communicate with the surface. If they represented invaginations which could unfold, an effective reduction in surface would not occur. However, connections between the vesicles and the surface were never encountered though many thousands of cells were examined; moreover, the vesicles persisted even in hemolyzed ghosts.

We visualize the events which lead to internalization of the red cell membrane and subsequent hemolysis as diagrammed in Fig. 2. It is probable that shallow indentations of the membrane during treatment with primaquine lead, eventually, to deep invaginations and pinching off internally of portions of the surface membrane. This process effectively removes a part of the surface membrane, permitting the cell to reach critical hemolytic volume more rapidly. We view the appearance of cells treated with fatty acid alone as representative of an early stage in hemolysis by internalization since, at higher concentrations or over longer periods of time, fatty acids will cause hemolysis. Although the mechanisms by which fatty acid stabilizes primaquine-treated cells are not known, one may view the appearance of cells treated with primaquine plus fatty acid as intermediate between cells treated with fatty acid alone and those treated with primaquine. Glauert *et al.* (*12*) have reported a somewhat similar temporal sequence of changes after treatment of cells with vitamin A. Also, Penniston and Green (*13*) have reported morphological changes induced by adenosine triphosphate in erythrocyte ghosts resembling those seen in pinocytosis. We suggest that the internalization of membranes may be a biologically significant process leading to red cell destruction.

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