

# EFFECTS OF PRIMAQUINE AND OTHER RELATED COMPOUNDS ON THE RED BLOOD CELL MEMBRANE. I. $\text{Na}^+$ AND $\text{K}^+$ PERMEABILITY IN NORMAL HUMAN CELLS \*

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The important investigations of Carson, Dern and Beutler and their co-workers (1-8) have identified an inherent deficiency of erythrocyte glucose-6-phosphate dehydrogenase as the biochemical basis for the hemolytic anemia induced in "sensitive" individuals by administration of primaquine and certain related compounds. The relationship between this enzyme deficiency and the mechanism of the hemolytic response remains to be defined. The hemolysis certainly involves disruptive changes in the red cell membrane. The possibility of a direct action of primaquine on the membrane must be seriously considered. As Rothstein (9) has pointed out, it is important to examine the effects of toxic agents on the membranes of the cells as well as their effects on intracellular metabolism, since any administered agent will, of course, first contact the cell membrane.

In normal red blood cells, the studies of Beutler, Dern and Alving (5) indicate that hemolysis is produced *in vitro* only by concentrations of primaquine greatly in excess of pharmacologic blood levels. A prehemolytic loss of  $\text{K}^+$  is, however, induced by subhemolytic concentrations of these agents. It was the purpose of the present investigations to study these *in vitro* prelytic abnormalities in red cell permeability.

It is accepted that the event of hemolysis occurring within a population of red cells represents an "all or none" response of individual cells, although the degree of hemoglobin loss by the individual cell varies with the lytic stimulus (10).

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In the case of prelytic phenomena involving changes in erythrocyte permeability, manifested by loss of  $\text{K}^+$ , red blood cells may respond in a heterogeneous fashion, as discussed by Ponder and Cox (11, 12). Such a heterogeneous response suggests the possibility of an all or none response on the part of some of the red cells as an explanation for a portion of the prelytic loss of  $\text{K}^+$ . Very significant contributions to the problem of individual red cell behavior were made by Passow and Tillman (13) in their studies of the prelytic loss of  $\text{K}^+$  from human red blood cells exposed to  $\text{Pb}^{++}$  and by Eckel (14, 15) who has studied the exchangeable  $\text{K}^+$  compartments of red cells treated with sodium fluoride. The latter investigator demonstrated a rapidly exchangeable  $\text{K}^+$  compartment as well as a more slowly exchangeable  $\text{K}^+$  compartment within the red cell sample and suggested that the rapidly exchangeable compartment represented complete  $\text{K}^+$  permeability in some of the cells. Passow, Rothstein and Loewenstein (16), working with yeast cells, have also demonstrated that the loss of  $\text{K}^+$  following exposure to a variety of basic redox dyes occurs as an all or none event for individual cells. In the present investigation, the prelytic loss of  $\text{K}^+$  and gain of  $\text{Na}^+$  induced by primaquine, methylene blue, and acetylphenylhydrazine was studied by analyzing dose-response and time-response relationships as well as osmotic fragility changes at varying times and drug concentrations.

## METHODS

The blood samples from hematologically normal adult male and female donors were defibrinated with glass beads. The blood was centrifuged at  $1,750 \times G$  for 10 minutes and the buffy coat removed by aspiration. The red cells were resuspended in their own serum. This suspension will hereafter be referred to as whole blood.

The whole blood was incubated in Warburg vessels

with varying concentrations of primaquine diphosphate,<sup>1</sup> methylene blue, or acetylphenylhydrazine. Concentrations of each agent, 10 times the final desired concentration, were prepared in isotonic sodium chloride solution, adjusted to pH 7.4 or 7.8 with dilute NaOH and added to 9 vol of the whole blood. The hematocrits of the experimental preparations were adjusted to between 35 and 40 per cent. Concentrations of primaquine are expressed in moles per liter of whole blood, since the primaquine was rapidly distributed into both the red cell fraction and the serum.

Serum samples were removed at varying time intervals and the loss of  $K^+$  was calculated as outlined by Ponder (17) and expressed as a percentage of the total  $K^+$  content of the cells.  $K^+$  and  $Na^+$  determinations were made with a Beckman flame photometer. With the exception of a few experiments in which hemolysis ranged from 1 to 8 per cent, the great majority of the observations were made on samples in which no hemolysis had occurred.

Osmotic fragility determinations were performed according to the method of Parpart and colleagues (18). With severely damaged cells, some hemolysis occurred in 0.9 per cent NaCl, although there had been none in the whole blood. An additional tube containing 1.0 per cent NaCl was included in the fragility test to mimic the additional osmotic effect of the serum protein, and no additional hemolysis above that found in the serum itself was observed at this concentration of NaCl. Since estimation of per cent hemolysis on the basis of plasma hemoglobin measurements may introduce an error related to the amount of hemoglobin retained by individual hemolyzed or ghost cells, a more specific visual estimate of the percentage of hemolyzed cells was carried out in a manner somewhat similar to the original osmotic fragility studies of Whitby and Hynes (19), who used red blood cell counts. Stained cell preparations were made by placing 50  $\mu$ l of the experimental cell suspensions into 10 ml of the 0.70 per cent NaCl- $PO_4$  buffer (pH 7.4) described by Parpart and co-workers (18) and allowing the injured cells to hemolyze during 20 minutes of incubation at room temperature. The lytic stimulus of 0.70 per cent NaCl was sufficient to lyse those cells which had a marked alteration in permeability, yet did not fragment the lysed cells beyond recognition, as did more dilute NaCl solutions. The cell suspension then was centrifuged, the supernatant aspirated and 1 ml of Dacie's solution (20) added. Coverslip smears then were prepared from the cell suspension in Dacie's solution. This solution was found to prevent destruction of the injured cells on contact with the glass slide. The smears were stained with Wright's stain and the percentage of ghosts in each preparation was estimated. Each estimate was based on microscopic counts of at least 500 cells from each of the 2 coverslips. The standard deviation of the percent-

<sup>1</sup> The primaquine diphosphate was generously supplied by Dr. George D. Wessinger, Sterling-Winthrop Institute, Division of Sterling Drug, Inc., Rensselaer, N. Y.

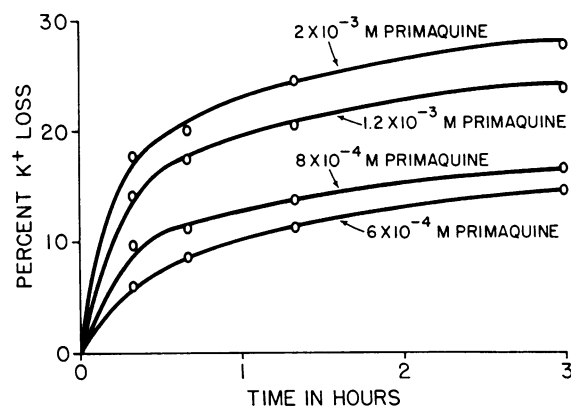


FIG. 1. TIME COURSE OF LOSS OF  $K^+$  FROM NORMAL ERYTHROCYTES INDUCED BY VARYING CONCENTRATIONS OF PRIMAQUINE. Each point is the mean of duplicate determinations and represents the loss of  $K^+$  expressed as a percentage of the total cellular  $K^+$ .

age of ghost cells was  $\pm 3.6$  per cent, based on the pooled variance.

Primaquine-treated cells were stained for Heinz bodies, but the appearance and number of the latter correlated poorly with loss of  $K^+$  and generally appeared at higher concentrations of primaquine than those concentrations producing loss of  $K^+$ . Consequently, no detailed study of Heinz bodies was conducted.

Lactic acid was measured by the method of Barker and Summerson (21) and reduced glutathione by the method of Grunert and Phillips (22) as modified by Beutler (7). Primaquine in concentrations below  $1 \times 10^{-3}$  M was measured by the method of Brodie, Udenfriend and Taggart (23) while above  $1 \times 10^{-3}$  M, the color of a metaphosphoric acid extract of serum or red blood cells was measured directly at 540  $m\mu$  in a Bausch and Lomb Spectronic 20 spectrophotometer. Percentage hemolysis was calculated by the method of Young, Izzo, Altman and Swisher (24).

## RESULTS

*Time course.* The time course of the loss of  $K^+$  from primaquine-treated red cells, illustrated in Figure 1, consisted of two distinct phases. The initial loss which occurred over 30 minutes was relatively rapid and proceeded at a rate related to the concentration of primaquine in the system. After the first 30 minutes, the loss of  $K^+$  proceeded more slowly for the remainder of the experiment (2.5 hours) at the same rate for all concentrations of primaquine tested. Measurements of the serum primaquine concentration made over 3 hours of incubation, starting with a concentration of  $3 \times 10^{-3}$  M, revealed a rapid uptake of the drug by

TABLE I  
Determination of red cell  $\text{Na}^+$  and  $\text{K}^+$  \*

|                                      | Time in<br>Minutes | $\text{K}^+$ Loss<br>mM/1 RBC | $\text{Na}^+$ Gain<br>mM/1 RBC |
|--------------------------------------|--------------------|-------------------------------|--------------------------------|
| $6 \times 10^{-4}$ M<br>Primaquine   | 20                 | 4.1                           | 3.2                            |
|                                      | 40                 | 5.9                           | 5.2                            |
|                                      | 80                 | 7.8                           | ---                            |
|                                      | 180                | 10.1                          | 9.0                            |
| $8 \times 10^{-4}$ M<br>Primaquine   | 20                 | 6.7                           | 3.0                            |
|                                      | 40                 | 7.8                           | 8.0                            |
|                                      | 80                 | 9.4                           | ---                            |
|                                      | 180                | 10.6                          | 9.0                            |
| $1.2 \times 10^{-3}$ M<br>Primaquine | 20                 | 9.7                           | 8.0                            |
|                                      | 40                 | 12.0                          | 9.5                            |
|                                      | 80                 | 14.1                          | 11.5                           |
|                                      | 180                | 16.5                          | 19.5                           |
| $2.0 \times 10^{-3}$ M<br>Primaquine | 20                 | 11.9                          | 9.2                            |
|                                      | 40                 | 13.3                          | 12.5                           |
|                                      | 80                 | 16.4                          | 15.5                           |
|                                      | 180                | 18.8                          | ----                           |

\* These values represent simultaneous measurements of the  $\text{Na}^+$  and  $\text{K}^+$  content of red cells incubated with varying concentrations of primaquine for the time intervals indicated. Each value is the mean of duplicate determinations.

cells during the first 20 minutes with only a slight further additional uptake during the rest of 2 hours' incubation. The time course of primaquine uptake was that of  $\text{K}^+$  loss. After 30 minutes of incubation, the concentration of drug in the cells was twice that in the medium. The ratio of the cellular to plasma concentrations increased to 2.52 in 1 hour and 2.61 in 2 hours. The uptake of drug was slightly less at pH 7.4 than at pH 7.8. In all cases, the drug was concentrated by the cells to some extent, perhaps because of binding to cellular ligands.

$\text{Na}^+$ . A comparison of the amounts of  $\text{Na}^+$  gained by the red cells with the amounts of  $\text{K}^+$  lost was based on simultaneous determinations of red cell  $\text{Na}^+$  content and serum  $\text{K}^+$ . These results are summarized in Table I. Allowing for the relatively large error (5 to 15 per cent) in determinations of red cell  $\text{Na}^+$  introduced by intracellular trapped plasma, it appears that there is a 1:1 ex-

change of  $\text{Na}^+$  for  $\text{K}^+$ , as the latter leaks out of the cells.

*Concentration of primaquine.* Studies of the relationship between loss of  $\text{K}^+$  and concentration of primaquine were carried out over the concentration range ( $2 \times 10^{-4}$  M to  $4 \times 10^{-3}$  M) in which primaquine induced a prelytic loss of  $\text{K}^+$  by the red cells. A few studies were performed, employing higher concentrations, that induced hemolysis. In Figure 2, the loss of  $\text{K}^+$  and amount of hemolysis are plotted against the  $\log_{10}$  of the primaquine concentration. Hemolysis occurred at concentrations of drug in excess of  $5 \times 10^{-3}$  M when the red cells were suspended in serum; when the red cells were suspended in 0.154 M NaCl solution, hemolysis appeared above  $1 \times 10^{-3}$  M. These latter findings conform with those presented by Beutler and colleagues (5). With primaquine concentrations above  $2 \times 10^{-2}$  M in the test system, useful information could not be obtained be-

cause of the formation of a gel of the cell suspension, preventing separation of the serum from the cells. With drug concentrations producing less than 70 per cent hemolysis, however, the amount of hemolysis was related to the  $\log_{10}$  of the primaquine concentration in a normal distribution function. The loss of  $K^+$  appeared at much lower concentrations of drug than those producing hemolysis, although the two curves of Figure 2 overlap to some extent. At a drug concentration of  $5 \times 10^{-3}$  M, no hemolysis was produced, but more than 50 per cent of the  $K^+$  was lost. The slope of the curve relating loss of  $K^+$  and drug concentration was relatively shallow compared with the curve relating hemolysis and drug concentration. Nevertheless, over the range of primaquine concentrations that could be investigated, the slope of the curve describing loss of  $K^+$  resembles the lower half of a log-probability relationship. This relationship is similar to that seen in curves describing a log dose mortality relationship (25). The prelytic volume of the cells treated by primaquine ranged from 1.1 to 1.2 times the control volume. These prelytic red cell volumes were measured by hematocrit determinations as described by Ponder (26).

*Osmotic fragility.* Osmotic fragility studies were carried out in order to determine whether

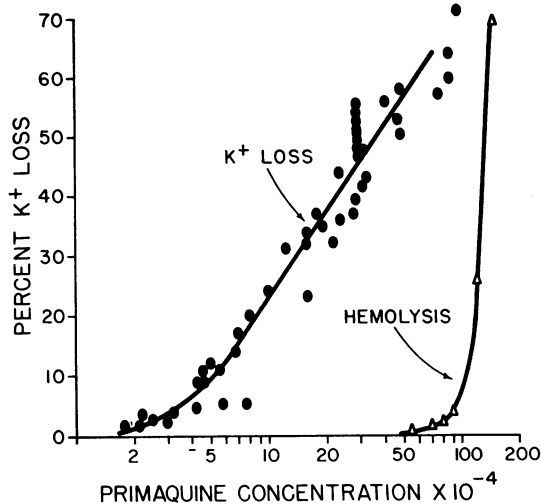


FIG. 2. RELATIONSHIP BETWEEN PERCENTAGE LOSS OF  $K^+$  AND CONCENTRATION OF PRIMAQUINE. The abscissa represents the  $\log_{10}$  of the primaquine concentration in moles per liter of whole blood and the ordinate indicates the percentage loss of  $K^+$  by red cells after 2 hours of incubation with primaquine.

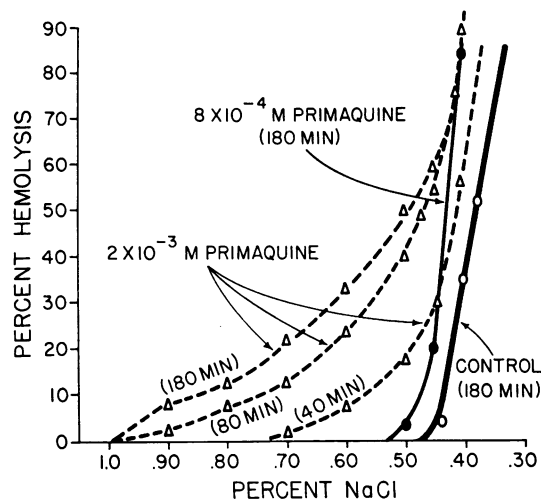


FIG. 3. OSMOTIC FRAGILITY STUDIES. The graph illustrates a representative set of results obtained, utilizing aliquots of the same red cell sample. The osmotic fragility studies were conducted on a control sample incubated without primaquine, on a sample incubated with  $8 \times 10^{-4}$  M primaquine and on three samples incubated with  $2 \times 10^{-3}$  M primaquine, for the time intervals indicated.

changes in fragility could be correlated with alterations in red cell permeability, as manifested by loss of  $K^+$ . Figure 3 is a diagram of osmotic fragility tests obtained after exposing aliquots of a whole blood sample to  $8 \times 10^{-4}$  M primaquine for 180 minutes or to  $2 \times 10^{-3}$  M primaquine for 40, 80 and 180 minutes. Figure 3 also includes an osmotic fragility curve obtained after incubation for 180 minutes of a control aliquot from the same blood sample in the absence of primaquine. In neither of these concentrations of drug was any hemolysis produced in the whole blood. The osmotic fragility patterns produced by these two concentrations of drug clearly illustrate two types of induced changes in the erythrocytes. At the lower drug concentration,  $8 \times 10^{-4}$  M, increased fragility of the whole cell population is evident and the entire curve is shifted symmetrically to the left. At the higher drug concentration, however, in addition to the symmetrical shift of the upper portion of the curve there is an evident upward shift of the curve from the baseline, increasing with time. This change in osmotic fragility is indicative of a profound change in the membrane of some of the cells, which makes them readily susceptible to osmotic lysis.

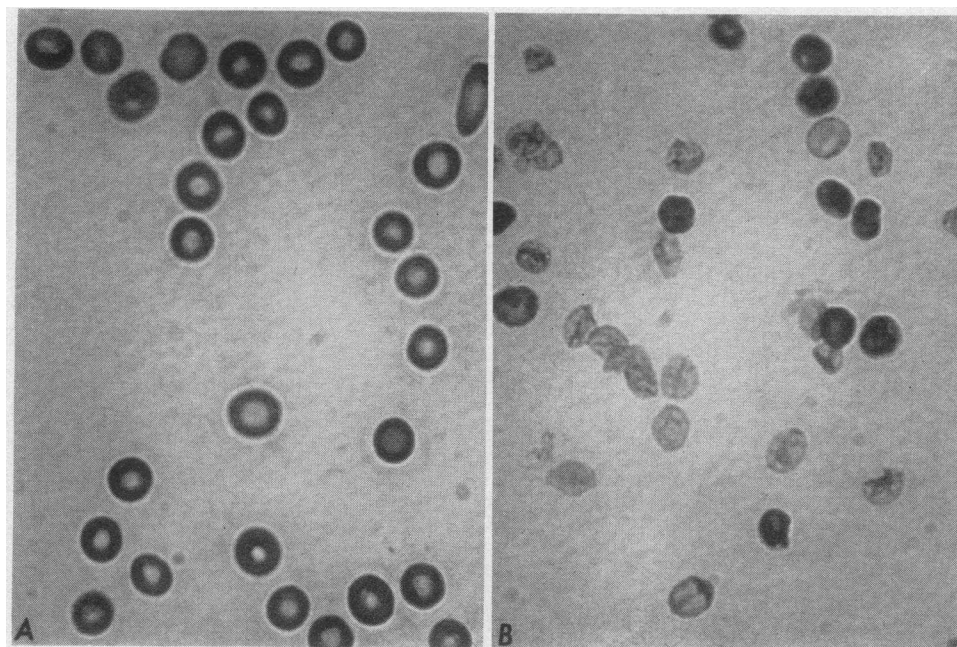


FIG. 4. ERYTHROCYTE GHOSTS PREPARED BY OSMOTIC LYSIS OF CELLS PREVIOUSLY TREATED WITH PRIMAQUINE. The red cells were hemolyzed in 0.70 per cent NaCl after incubation with the drug; 4A is a photograph of a control sample incubated without primaquine and then exposed to 0.70 per cent NaCl; 4B demonstrates a sample exposed to  $3 \times 10^{-3}$  M primaquine with 50 per cent loss of  $K^+$  and production of 50 per cent ghosts after exposure to 0.70 per cent NaCl.

*Osmotic fragility studies employing staining of erythrocyte ghosts.* Although the osmotic fragility studies suggested that some of the  $K^+$  was lost from the primaquine-treated red cells in an

“all or none” manner, more direct confirmation of this point was sought by incubating red cells with primaquine, placing them in 0.70 per cent NaCl to produce lysis of those cells with damaged membranes, and staining the entire sample to visualize the hemolyzed ghost cells. Figure 4 illustrates two of these stained red cell ghost preparations; Figure 4A was prepared from a control sample of blood without primaquine which was incubated and treated in the same fashion as was a primaquine-treated sample with 50 per cent ghosts, shown in 4B.

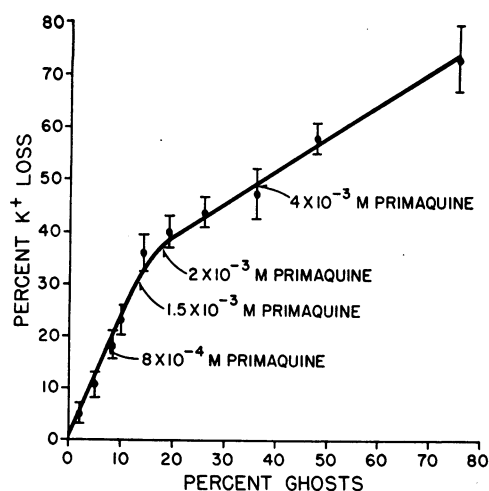


FIG. 5. RELATIONSHIP OF PERCENTAGE LOSS OF  $K^+$  INDUCED BY PRIMAQUINE TO PERCENTAGE GHOSTS PRODUCED BY SUBSEQUENT EXPOSURE TO HYPOTONIC SODIUM CHLORIDE. The standard deviation about the mean value for each point is indicated on the graph.

Figure 5 represents a graph of the percentage of ghost cells versus the percentage loss of  $K^+$ . Although increased cation permeability is associated with increased osmotic fragility, if loss of  $K^+$  occurred only as an all or none phenomenon in individual cells, one might expect a linear 1:1 relationship between loss of  $K^+$  and the proportion of ghost cells. A simple relationship of this type was not found. Rather, the curve of Figure 5 appears to be a composite of two lines of different slopes. At lower concentrations of primaquine, the percentage loss of  $K^+$  induced was greater than the percentage of cells hemolyzed by hypotonic

saline. Thus the K<sup>+</sup> released was greater than that which could have come from only those cells with markedly abnormal membranes.<sup>2</sup> The upper part of the curve, however, shows a loss of K<sup>+</sup> more nearly directly proportional to the percentage of ghosts in the sample. These data suggest a mixed response of a population of primaquine-treated erythrocytes—some loss of K<sup>+</sup> occurs from all of the cells, and a complete release of K<sup>+</sup> occurs in other cells, with the all or none response predominant at higher concentrations of primaquine. Such an analysis of the relationship of the loss of K<sup>+</sup> and lysis of cells is consistent with the two types of osmotic fragility response shown in Figure 3. This interpretation is further supported by the fact that the inflection point of the curve of Figure 5 at 30 per cent loss of K<sup>+</sup> occurs at a concentration of  $1.5 \times 10^{-3}$  M primaquine, a concentration of drug between those which produce the two types of osmotic fragility response.

*Metabolic observations.* Metabolic disturbances which might account for K<sup>+</sup> loss could not be found in primaquine-treated red cells. Lactic acid production during 2 hours of incubation of nor-

mal erythrocytes and red cells incubated with varying concentrations of primaquine was compared. Normal red cells produced  $17.0 \pm 6.0$  (SD) mg lactic acid per 100 ml RBC per 2 hours, while red cells incubated with  $1 \times 10^{-3}$  M primaquine produced  $14.9 \pm 1.1$  (SD) mg per 100 ml and cells exposed to  $3 \times 10^{-3}$  M primaquine produced  $17.7 \pm 5.3$  (SD) mg per 100 ml during the same period. There are no significant differences among these results.

The reduced glutathione content of red blood cells from six normal individuals remained at normal levels after the cells were incubated with concentrations of primaquine that caused large losses of K<sup>+</sup>. The mean pre-incubation value for reduced glutathione was  $62.9 \pm 19.7$  mg per 100 ml of normal red blood cells. Aliquots of the same red cell samples after 2 hours' incubation at 37° C in the absence of primaquine were found to contain  $70.6 \pm 16$  mg per 100 ml of reduced glutathione. After 2 hours' incubation with  $1 \times 10^{-3}$  M primaquine, a value of  $72.7 \pm 13.0$  mg per 100 ml of reduced glutathione was obtained, and after incubation with  $3 \times 10^{-3}$  M primaquine,  $61.7 \pm 10.7$  mg per 100 ml of reduced glutathione was present.

Methemoglobin formation was measured in red cell samples after 2 hours of incubation and found to equal 2 per cent of the total hemoglobin present in untreated samples. The same value was found for red cell samples treated with  $2, 5$  or  $10 \times 10^{-4}$  M concentrations of primaquine, concentrations which result in significant loss of K<sup>+</sup>. Treatment with a concentration of  $2 \times 10^{-3}$  M primaquine, which induces a 50 per cent loss of K<sup>+</sup>, resulted in an increase in amounts of methemoglobin to 8 to 12 per cent of the total hemoglobin after 2 hours of incubation.

*pH.* The loss of K<sup>+</sup> induced by treatment of erythrocytes with  $3 \times 10^{-3}$  M primaquine was greater at pH 7.8 than at pH 7.4; 51.3 per cent  $\pm 6.7$  (N = 15) of the cellular K<sup>+</sup> is lost at the higher pH compared with a loss of 38.4 per cent  $\pm 7.7$  (N = 6) of the K<sup>+</sup> at pH 7.4.

*Effect of serum.* Primaquine treatment of the erythrocytes in normal serum at pH 7.8 was found to augment significantly the loss of K<sup>+</sup> compared with red cells treated in 0.154 M NaCl solution. Primaquine at concentrations of  $3 \times 10^{-3}$  M in serum resulted in the loss of 51.3 per cent of the cellular K<sup>+</sup>; the same concentration of drug in

<sup>2</sup> Although the initial portion of Figure 5, indicating percentage loss of K<sup>+</sup> in excess of the number of destroyed cells, conceivably could be explained by the breakdown of a small number of cells having much more than normal content of K<sup>+</sup>, rather than some loss of K<sup>+</sup> by all cells, this is extremely unlikely on a theoretical basis. Utilizing the recent values for mean corpuscular volume obtained through study of individual erythrocytes by Houchin, Munn and Parnell (27), it can be shown that 99.99 per cent of normal erythrocytes examined by them had a volume less than 1.24 times the mean volume (3 SD from the mean). Turning to the data presented in Figure 5, however, it is apparent that if there were no loss of K<sup>+</sup> by all cells, 10 per cent of the cells would have to lose 22 per cent of the total K<sup>+</sup> content of the red cell sample. This would require that the K<sup>+</sup> content of the 10 per cent of hemolyzed cells was approximately 300 mmoles per L of RBC water. Calculation of the obligate increase in cell water for this theoretical situation, taking into account Ponder's correction for the deviation in behavior of the normal red cell from that of a perfect osmometer (28), would indicate a 1.8-fold increase in cell water or 1.26-fold increase in cell volume. As shown above, however, less than 0.01 per cent of the cells has a volume 1.26 times the mean volume, since 99.99 per cent have a volume less than 1.24 times the mean. This demonstrates that some of the loss of K<sup>+</sup> indicated by the first portion of the curve in Figure 5 represented a loss greater than that accounted for by individual cell breakdown.

saline resulted in the mean loss of only 35.7 per cent. The mean difference in loss of  $K^+$  between 12 paired samples was 14.2 per cent  $\pm$  8.4 (2 SD). This difference is highly significant. A similar difference, although less marked, was also observed in tests carried out at pH 7.4. Heat inactivation of the serum at 56° C for 30 minutes did not alter this effect nor did the addition of ethylenediamine tetraacetate in concentrations from  $1 \times 10^{-4}$  M to  $9 \times 10^{-3}$  M to the serum. The addition of physiological concentrations of  $Ca^{++}$ ,  $Mg^{++}$ , or  $Zn^{++}$ , alone and in combination, to the samples treated in NaCl solution did not result in increased loss of  $K^+$ .

*Other agents.* The effects of other agents, including methylene blue and acetylphenylhydrazine, on the loss of erythrocyte  $K^+$  was evaluated. These agents were studied in systems at a pH of 7.8. Methylene blue induced 0 to 100 per cent loss of  $K^+$  over a concentration range extending from  $4 \times 10^{-4}$  M to  $6 \times 10^{-3}$  M, when the red cells were suspended in serum. Acetylphenylhydrazine in serum produced a similar effect at concentrations between  $3 \times 10^{-4}$  and  $2.4 \times 10^{-2}$  M. Both of these agents, when studied *in vitro* like primaquine, demonstrated a dual relationship between loss of  $K^+$  and osmotic fragility, and both also exerted their effects on loss of  $K^+$  more readily in serum than in NaCl solution.

#### DISCUSSION

Based on the time-response and osmotic fragility data presented, the *in vitro* prelytic loss of  $K^+$  induced by primaquine, methylene blue and acetylphenylhydrazine is composed of two fractions. A smaller loss of  $K^+$ , occurring at lower concentrations of primaquine, appears to affect all cells uniformly. In addition, depending on the drug concentration, there may be an all or none loss of  $K^+$  involving just a portion of the red cell population. The all or none type of response found is consistent with the observations of Passow and co-workers (16) who found that methylene blue induced a loss of  $K^+$  from yeast cells as an "all or none" phenomenon. In addition, the finding of two types of loss of  $K^+$  by red cells agrees with similar findings by Passow and Tillman (13), who studied the effects of  $Pb^{++}$  on red cells, and by Eckel (14, 15) who studied fluoride-poisoned red cells.

The osmotic fragility tests on the red blood cells incubated with primaquine indicated a decreased resistance to osmotic lysis in contradistinction to the work of Passow and Tillman (13) which showed an increased resistance to osmotic lysis of human red cells treated with  $Pb^{++}$ , although such cells had also lost  $K^+$  in a fashion analogous to the cells treated with primaquine. One explanation for this apparent discrepancy may be found in the work of Vincent and Blackburn (29, 30) who also studied  $Pb^{++}$ -induced loss of  $K^+$  from red blood cells. Vincent found that an initial loss of  $K^+$  from the cells was not accompanied by a simultaneous inward movement of  $Na^+$ ; rather, movement of  $Na^+$  followed some time later. This observation suggests that the observed decreased resistance to osmotic lysis of red cells treated with primaquine was related to a general increase in cation permeability, as illustrated by the entry of  $Na^+$  concomitant with loss of  $K^+$ . By contrast, entry of  $Na^+$  appears to be delayed in cells treated with  $Pb^{++}$ , making them shrink and become osmotically resistant. Although differing in direction of shift, the pattern of alteration in osmotic fragility induced by primaquine is compatible with the patterns found by Passow and Tillman (13) and Eckel (14, 15), which these authors interpreted as evidence of an "all or none" response by segments of a red cell population. In addition, the finding in the present investigation of a symmetrical shift of the entire osmotic fragility curve with time also correlates well with the observations of these other investigators; the symmetrical shift in osmotic fragility can be interpreted similarly to represent some increased permeability of all of the remaining cells.

Although lactate production and reduced glutathione content can be considered only gross measures of glycolytic integrity, the very rapid appearance of the all or none loss of  $K^+$ , coupled with the lack of effect of primaquine on lactate production or reduced glutathione content, suggests that *in vitro* effects of primaquine may be physicochemical effects on the membrane rather than interference with metabolically determined  $K^+$  gradients. Additional evidence for such a direct effect on the red cell membrane lies in the fact that the critical hemolytic volume of red cells exposed to primaquine is only 110 to 120 per cent of their normal volume, indicating a distinct decrease in

the ability of treated red cells to resist hemolysis in the face of swelling, as pointed out by Ponder (26).

Reduced glutathione content of primaquine-treated red cells was investigated because Benesch and Benesch (31), Sheets, Hamilton and DeGowin (32) and Fegler (33) all have called attention to the importance of sulfhydryl (SH) groups for maintenance of red cell integrity, and Eichel (34) and Woodward (35) have found reduced glutathione to constitute approximately 95 per cent of the nonprotein sulfhydryl content of red cells. Passow and colleagues (16) have suggested that the loss of K<sup>+</sup> from yeast, induced by basic aniline dyes, may be related to alteration of SH groups by these oxidizing agents. Beutler and colleagues (6, 7), however, have pointed out that normal red blood cells are able to maintain their reduced glutathione in the face of 500 mg per 100 ml acetylphenylhydrazine, a concentration found to produce leakage of 100 per cent of cellular K<sup>+</sup> in our studies. Therefore, it appears that the loss of K<sup>+</sup> induced in normal red cells by acetylphenylhydrazine and primaquine is not related to any alteration in the total reduced glutathione content of the cells, since the loss of K<sup>+</sup> is produced by concentrations of primaquine that do not measurably affect the total reduced glutathione content of the cells. A critical, but numerically small, alteration by primaquine of SH groups in the membrane itself, however, cannot be excluded.

The reason for the marked enhancement of the primaquine-induced loss of red cell K<sup>+</sup> by an increase in pH from 7.4 to 7.8 is not clear but may be related in part to the moderate increase in uptake of primaquine by the red cells at the higher pH. In addition, as Emerson, Ham and Castle (36, 37) have pointed out, the oxidizing properties of these resonating compounds is enhanced by increased pH, and they would be expected to exert a more marked effect under these circumstances.

One of the most interesting, although unexplained, observations is the enhancing effect of serum on the loss of K<sup>+</sup> induced by all three agents studied. The enhancing effect of serum on aniline-induced hemolysis has been known since it was first described by Browning and Mackie in 1914 (38) and again in 1933 by Ponder (39, 40).

Ponder (41) has pointed out that the aniline dye-red cell system alone differed from other hemolytic systems he had studied in that the action of all other hemolytic agents, except the aniline compounds, is inhibited by the presence of serum. While serum enhanced the loss of K<sup>+</sup> induced by primaquine, methylene blue and acetylphenylhydrazine in the present study, the enhancing effect was unaltered by the addition of chelating agents, heat treatment or the addition of cations to the saline system. These observations seem to exclude activities of complement or divalent cations in this effect. The amount of primaquine taken up by the cells was essentially the same or slightly greater in a saline-containing system, so that the effect of serum is apparently not related to differences in distribution of primaquine.

#### SUMMARY

1. Primaquine diphosphate induces an *in vitro* prehemolytic loss of K<sup>+</sup> from normal human red blood cells over a concentration range from  $2 \times 10^{-4}$  M to  $5 \times 10^{-3}$  M primaquine per L of whole blood. Above  $5 \times 10^{-3}$  M, hemolysis begins to appear.

2. The time course of the prelytic loss of K<sup>+</sup>, the alterations in osmotic fragility and evidence provided by a technique for visual observation of the affected cells, all indicate that in addition to some loss of K<sup>+</sup> by all the cells in a sample in the presence of higher concentrations of primaquine, there is a portion of the red cell population which develops an "all or none" complete permeability to K<sup>+</sup>. The proportion of loss of K<sup>+</sup> due to the latter mechanism is dependent upon the primaquine concentration.

3. Concentrations of primaquine that induce loss of K<sup>+</sup> by red cells do not affect lactic acid production over a 2 hour period of incubation. Similarly, the reduced glutathione content of normal red cells is unaffected by their exposure for 2 hours to concentrations of primaquine that cause significant loss of K<sup>+</sup>. Significant loss of K<sup>+</sup> can also be induced by concentrations of primaquine that produce no methemoglobinemia; at drug concentrations producing 50 per cent loss of K<sup>+</sup>, 8 to 12 per cent methemoglobin can be detected.

4. The presence of serum in the incubation system distinctly enhances the loss of K<sup>+</sup> induced by a given concentration of primaquine. In addition,



the magnitude of the loss of  $K^+$  at a given primaquine concentration varies directly with the pH of the whole blood.

5. Methylene blue and acetylphenylhydrazine induce a similar prelytic loss of  $K^+$ , and like primaquine, these agents exert a greater effect in the presence of serum.

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