Characteristics of the Membrane Defect in the Hereditary Stomatocytosis Syndrome

By James S. Wiley, J. Clive Ellory, Marc A. Shuman, Calvin C. Shaller, and Richard A. Cooper

Cation permeability and lipid composition have been studied in the red cells of five patients with various features of the hereditary stomatocytosis syndrome. Hemolysis was compensated in four patients, and only one patient was anemic. Cell \( \text{Na}^+ \) was increased an average of 3 \( \mu \text{eq} \) per ml cells and cell \( \text{K}^+ \) decreased 14 \( \mu \text{eq} \) per ml cells. Both active and passive fluxes of \( \text{Na}^+ \) and \( \text{K}^+ \) were increased by two to six times normal. Tritiated ouabain binding was increased an average of 2.5-fold, suggesting a proportionally greater number of cation pumps per cell. The coupling ratio of active \( \text{Na}^+ \cdot \text{K}^+ \) fluxes was normal (3:2). Calcium permeability was increased compatible with the degree of reticulocytosis, and cell \( \text{Ca}^{2+} \) content was normal. The lowered sum of \( \text{Na}^+ \) plus \( \text{K}^+ \) was associated with a high MCHC and low cell water. When examined in wet preparations, red cells assumed either a bowl-shaped or an irregular contour, and they appeared as target cells on dry smears. Only when cell water was increased in hypotonic media were stomatocytes seen on smear. The total lipid content of red cells was increased in four patients, although it was normal in one. The mole ratio of cholesterol to phospholipid was always normal; however, phospholipid analysis showed an increased proportion of phosphatidyl choline. The abnormal cells were osmotically resistant due to both an increased membrane surface area and a low total cation content. These patients show two hallmarks of hereditary stomatocytosis: bowl-shaped red cells observed on wet preparations and a marked increase in \( \text{Na}^+ \) and \( \text{K}^+ \) permeability. The heterogeneity of this syndrome in our patients and in others reported with hereditary stomatocytosis appears to result from (1) variability in the increase in surface area which results from an excess of membrane lipid content, particularly phosphatidylcholine, and (2) a variability in cell water content which may be either decreased or increased as a result of changes in the sum of \( \text{Na}^+ \) plus \( \text{K}^+ \) ions.

The syndrome of hereditary hemolytic anemia and stomatocytic red cells was first described by Lock et al. in 1964,1 and subsequent reports have indicated a wide diversity in the severity of the hemolysis, both between different families and among affected members of the same family.2-4 Laboratory investigations have shown an association of stomatocytosis with abnormal cell \( \text{Na}^+ \) and \( \text{K}^+ \) content, and a consistent finding has been an increased \( \text{Na}^+ \) permeability of these cells. Active pumping of cations, measured either by \( \text{K}^+ \) influx or \( \text{Na}^+ \) efflux, was also increased in hereditary stomatocytes.5 This finding would be predicted in patients with high cell \( \text{Na}^+ \), since when \( \text{Na}^+ \) rises above its usual value of 8 \( \mu \text{eq} \) per ml cells, the active cation pump is stimulated.6
In several patients, however, the active Na⁺ efflux was considered to be disproportionately high for the observed level of internal Na⁺.²,⁴

The lipid content of hereditary stomatocytes is variable and has been reported to be either increased or normal in different families.²,⁴,⁵ An unusual alteration in red cell lipid composition has been described in a family with a hereditary nonspherocytic hemolytic process in whom target cells and occasional stomatocytes were present.⁷ In this family, red cell phospholipids were increased, mainly due to an elevated phosphatidyl choline. Moreover, permeability to monovalent cations was increased, since isotopic and net fluxes of Na⁺ and K⁺ were raised some threefold above normal.⁸ The cation abnormalities of this syndrome suggest some similarity to hereditary stomatocytosis, since in both there is an increased Na⁺ permeability and a decreased content of cell K⁺. However, the phospholipid composition of hereditary stomatocytes has not been reported in detail, so that comparison with the phosphatidyl choline-rich cells is not possible. Nevertheless, the total cell lipid has been reported to be increased in several families with hereditary stomatocytosis by an amount comparable with the lipid increase in high phosphatidyl choline hemolytic anemia.²,⁵

In the present study, membrane lipid and cation permeability have been measured in red cells from five patients with a syndrome related to hereditary stomatocytosis. The results confirm the heterogeneity within this syndrome, since there was variability in both lipid and cation composition. However, red cell Na⁺ flux was elevated in all cases. These observations have been compared with the spectrum of hereditary stomatocytosis which has been previously reported.

CASE REPORTS

JC is a 47-yr-old black male who first was noted to be anemic in 1946 following pneumonia. This anemia was treated with transfusion, and, when he was admitted in 1966 for repair of an abdominal hernia, his hemoglobin fell to 10.8 g/dl. In 1968 he was admitted with iron-deficiency anemia and peptic ulceration. He was transfused, and hemoglobin rose from 4.1 to 9.5 g/dl. Reticulocytes varied from 7% to 12%, and haptoglobin was 18 mg/dl. Partial gastrectomy and cholecystectomy were performed in 1969 because of melena. Because of persistent post-operative fever, a laparotomy was performed, during which an enlarged spleen weighing 380 g was removed. In 1972 iron-deficiency anemia and steatorrhea were noted, and his hemoglobin rose on iron therapy to 11–12 g/dl. Reticulocytes have ranged from 2.1% to 4.7%. White cell and platelet counts were normal. Serum haptoglobin was 33 mg/dl; bilirubin, 1.1 mg/dl (0.1 mg/dl conjugated); direct and indirect Coombs’ tests were negative. Hemoglobin electrophoresis on cellulose acetate was normal. Sugar water test for paroxysmal nocturnal hemoglobinuria, heat stability test for unstable hemoglobin, and brilliant cresyl blue incubation for Heinz bodies were also negative. Serum B₁₂, calcium, phosphorus, BUN, glucose, electrolytes, and liver function tests were normal.

DC is the 11-yr-old daughter of patient JC. The spleen was palpated 5 cm below the left costal margin at the age of 3. Hemoglobin was 12.2 g/dl and reticulocytes, 8%. White cell and platelet counts were normal. Hemoglobin electrophoresis showed no abnormalities, and hemoglobin A₂ and F were 2.1% and 2.0%, respectively. Examination of a bone marrow aspirate was normal. Serum bilirubin was 1.2 mg/dl (0.2 mg/dl conjugated). Liver function tests were normal. Direct and indirect Coombs’ tests were negative. She has complained of recurrent episodes of abdominal pain, either central or left upper quadrant in location, which persist for several days on end. Liver function tests were normal. Hemoglobin has remained 11–12 g/dl and reticulocytes, 3%–9%.
TC is the 8-yr-old daughter of patient JC who suffers intermittent episodes of abdominal pain. No splenomegaly can be palpated. Hemoglobin is 13.6 g/dl and reticulocytes, 3.9%.

NS is a 48-yr-old male of German, Irish, and English descent who has suffered intermittent jaundice for the past 20 yrs. Fifteen years previously he underwent cholecystectomy, and tests showed a serum bilirubin of 10 mg/dl (mostly unconjugated), normal liver function tests, normal hemoglobin, and 10% reticulocytes. His spleen was 8 cm below the left costal margin. Peripheral blood smear showed target cells, occasional stomatocytes, and polychromasia. Hemoglobin electrophoresis and screening tests for glycolytic enzyme deficiencies were normal. Brilliant cresyl blue preparations were negative, and the isopropanol and heat stability test for unstable hemoglobins were normal. Coombs' test and sugar water test were negative. Hemoglobins A2 and F were 2.3% and 3.5%, respectively. Autohemolysis was increased to 28%, corrected to 1.1% with glucose supplementation. No family members were available for study.

DB is a 62-yr-old Jewish woman who has suffered chronic hemolysis and episodic jaundice all her life. In 1951 her hematocrit was 38%, reticulocytes were 6%, 8%, and serum bilirubin was 3.0 mg/dl (0.4 mg/dl conjugated). During followup her hemoglobin has ranged between 14.0 and 16.7 g/dl and her reticulocytes between 3.5% and 13.2%. In 1954, a 51chromium red cell survival showed a half-life of 14 days. Splenomegaly was noted in 1957, and the spleen tip remains palpable below the left costal margin. Hemoglobin electrophoresis was normal, and fetal hemoglobin was 2.8%. Brilliant cresyl blue preparations and heat stability test for unstable hemoglobin were normal. Coombs' test was negative. Measurement of glucose-6-phosphate dehydrogenase, pyruvate kinase, hexokinase, and red cell glycolytic rate were not abnormally low. Autohemolysis was 17%, correcting to 3% with glucose supplementation. Liver function tests were normal. No family members could be studied, but an uncle suffered recurrent jaundice all his life.

MATERIALS AND METHODS

Hematologic Studies

Red cell indices were obtained on a Coulter Model S Counter, except for the mean corpuscular hemoglobin concentration which was estimated from manual determinations of hemoglobin and hematocrit. Reticulocytes, autohemolysis, and osmotic fragility measurements were performed by standard techniques using fresh blood. During washing of red cells to remove buffy coat, the top 1-2 mm of red cells were also removed with each of four washes, a procedure which reduced the percentage of reticulocytes by a factor of 0.7-0.8. Reticulocyte values shown in Table 1 were measured for the washed cells used in the in vitro measurements. Morphology of the abnormal cells was examined in a wet preparation of fresh blood suitably diluted in isotonic saline plus 20 mM imidazole Cl, pH 7.40, and human serum albumin (0.1 g/dl). When red cells were diluted in autologous heparinized plasma, the plasma was buffered with 0.02 volumes of 0.4 M imidazole Cl, pH 7.40. Only cells which were moving freely in suspension were assessed for their wet morphology. Two normal controls each showed a bowl-cell count less than 1% in a wet preparation. Hemoglobin A2 was measured after electrophoresis on cellulose acetate (pH 8.6), and fetal hemoglobin was quantitated as alkali-resistant hemoglobin.

Preparation of Cell Suspensions

For cation studies, venous blood was collected into heparin and the red cells washed three times in a medium of 145 mM NaCl, 5 mM KCl, and 20 mM imidazole Cl, pH 7.5. For measurement of net Na+ and K+ movements, the cells were suspended in the same medium containing 10 mM glucose and were incubated at 37°C. When ouabain was present, it was added to give a final concentration of 0.1 mM.

Cell Na+, K+, and Water Concentration

Fresh red cells were rapidly washed four times in cold 110 mM MgCl2 to remove extracellular Na+ ions. This procedure took approximately 12 min. The packed cells were hemolyzed in 0.01 N NH4OH and the Na+ and K+ concentrations measured by flame photometry. Cell
Table 1. Hematologic Parameters in Affected Family Members

<table>
<thead>
<tr>
<th></th>
<th>Age</th>
<th>Hb  (g/dl)</th>
<th>PCV (%)</th>
<th>MCV (fl)</th>
<th>MCH (pg)</th>
<th>MCHC (%)</th>
<th>Reticulocyte (%)</th>
<th>Abnormal Cells (%)</th>
<th>HbF (%)</th>
<th>HbA2 (%)</th>
<th>Median Osmotic Fragility (g NaCl/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>JC</td>
<td>47 (M)</td>
<td>11.8</td>
<td>31.6</td>
<td>92</td>
<td>34.4</td>
<td>37.3</td>
<td>3.3</td>
<td>40</td>
<td>0.1</td>
<td>2.6</td>
<td>0.29</td>
</tr>
<tr>
<td>DC</td>
<td>12 (F)</td>
<td>12.2</td>
<td>32.1</td>
<td>85</td>
<td>32.1</td>
<td>38.0</td>
<td>6.4</td>
<td>20</td>
<td>2.0</td>
<td>2.1</td>
<td>0.36</td>
</tr>
<tr>
<td>TC</td>
<td>7 (F)</td>
<td>13.6</td>
<td>35.8</td>
<td>84</td>
<td>31.8</td>
<td>38.0</td>
<td>3.9</td>
<td>20</td>
<td>1.1</td>
<td>3.3</td>
<td>0.35</td>
</tr>
<tr>
<td>HS</td>
<td>48 (M)</td>
<td>14.1</td>
<td>38.6</td>
<td>99</td>
<td>36.1</td>
<td>36.3</td>
<td>6.5</td>
<td>50</td>
<td>3.5</td>
<td>2.3</td>
<td>0.33</td>
</tr>
<tr>
<td>DB</td>
<td>62 (F)</td>
<td>15.6</td>
<td>43.6</td>
<td>95</td>
<td>33.9</td>
<td>35.7</td>
<td>7.8</td>
<td>20</td>
<td>2.8</td>
<td>—</td>
<td>0.39</td>
</tr>
<tr>
<td>Normals</td>
<td>82-98</td>
<td>27-33</td>
<td>30-36</td>
<td>0.5-1.5</td>
<td>&lt;1.0</td>
<td>&lt;1.0</td>
<td>1.8-3.3</td>
<td>0.40-0.44</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Values for MCHC were estimated from manual determinations of hemoglobin and hematocrit.
†Measured after cells were washed thrice to remove buffy coat.
‡Estimated from examination of a wet preparation of red cells diluted in buffered saline, pH 7.40, plus human serum albumin. Both bowl forms and cells with a convoluted margin were counted.
§Measured as alkali-resistant hemoglobin.
||Patient splenectomized.

Water was measured by drying a sample of packed cells to constant weight at 95°C. Cells were packed at 38,000 g for 1 hr at 4°C, and the trapped extracellular space was shown to be less than 1% by use of 14C-sucrose.

**Cation Fluxes**

Sodium influx was measured from the uptake of radioactivity by cells incubated in media containing 22NaCl, plus 10 mM glucose and 50 μM ouabain. Sodium efflux, K⁺ influx, and K⁺ efflux were measured as described previously.

**3H-ouabain Binding to Red Cells**

The binding of ouabain to red cells was measured essentially as described by Hoffman. 3H-ouabain, 5 Ci/mM in ethanol-benzene solution, New England Nuclear Lot No. 184-196, was evaporated to dryness under a stream of nitrogen and redissolved in 20-fold excess of unlabeled ouabain (Sigma Chemical Co., St. Louis, Mo.) to give a stock solution of 10 μM ouabain in water of an activity of 12.5 μCi/ml. The exact concentration of ouabain was confirmed by spectrophotometric analysis using a molar extinction coefficient of 1.54 × 10⁴ at the absorption peak of 220 nm. This stock solution was stable at 4°C for several months. To obtain binding of ouabain, red cells were incubated at 20% hematocrit in a medium of 150 mM NaCl, 1 mM KCl, 20 mM imidazole Cl, pH 7.5, 10 mM glucose, plus 20 nM 3H-ouabain. Parallel incubations were performed in identical media with or without addition of 3H-ouabain or with 3H-ouabain plus excess (0.1 mM) unlabeled ouabain. Under the latter condition, there was no significant radioactivity bound to the cells in any of the experiments, suggesting that nonspecific binding of contaminants did not contribute to the 3H-ouabain-binding results. Each flask was sampled at two time intervals to determine both the 3H binding and the degree of inhibition of the pump as judged from the K⁺ influx. The time intervals were either 30 and 60 min or 45 and 90 min and were chosen so that the cells incubated with 20 nM ouabain showed 40%-80% inhibition of the pump. All samples were washed three times in cold 150 mM NaCl to remove unbound radioactivity. To measure the bound 3H-ouabain, duplicate 0.5-ml portions of the washed packed cells were added to 15 ml Aquafluor (New England Nuclear); the vial was vortexed immediately and allowed to stand 48 hr in the dark. Bray's solution was unsuitable for removing 3H-ouabain bound to red cells and gave binding values of 65%-75% of those obtained with Aquafluor. The tritium was then counted (Packard Tri-Carb Liquid Scintillation Counter Model 3375) at a constant quench factor as judged by constant values for the artificial external standard (absolute efficiency was 30%). To convert the bound 3H-ouabain from counts per minute per milliliter cells into molecules per cell, the specific activity of the 3H-ouabain was measured, as was the number of red cells per milliliter of the packed cells by counting a suitable dilution (1:62,500) in a Coulter Model ZB Counter. The degree of inhibition of the pump was assessed by the K⁺ influx which was measured on a sample of the washed cells incubated in a medium of 7 mM 42KCl, 145 mM NaCl, 20 mM imidazole Cl, pH 7.5, plus...
10 mM glucose. Active K⁺ influx was taken as the component of the total flux inhibited by 0.1 mM ouabain, and the fractional inhibition of the active component was calculated for the cells incubated with 20 nM ³H-ouabain (this was usually in the range of 40%-80% inhibition). For normal cells, a linear relation was obtained between the fractional inhibition of active K⁺ influx and the number of ³H-ouabain molecules bound per cell. It was assumed that this linear relation between ouabain binding and pump inhibition was valid over the whole range of pump inhibitions, so that the total number of ouabain-binding sites per cell would equal the number of ³H-ouabain molecules bound per cell when there was 100% inhibition of active K⁺ influx.

**Lipid Studies**

For measurement of their lipid content, red cells were extracted with 80 volumes of isopropanol and chloroform for measurement of cholesterollipid phosphorus. Nonlipid phosphorus was removed from the extracts by thrice washing with 0.05 M KCl (video volume). Total phospholipid of red cells was taken to equal lipid phosphorus multiplied by 25. For phospholipid analysis, 0.01 volume of the antioxidant 2,3 diert-butyl, 4 methyl phenol (1% BHT in methanol) was added, and phospholipids were separated by thin-layer chromatography on precoated Silica Gel-G TLC plates (E. Merck, A.G., Darmstadt, Germany) using a chloroform: methanol:glacial acetic acid:water (25: 15:4:2) solvent. Red cells were enumerated in the Coulter Model ZB electronic counter.

**Red Cell Calcium Permeability and Content**

Calcium influx was measured from the uptake of ⁴⁵Ca by cells which had been preincubated so as to deplete ATP and thereby inhibit the activity of the outwardly-directed Ca²⁺ pump. Red cells were washed in a medium of 145 mM NaCl, 5 mM KCl, and 20 mM imidazole Cl, pH 7.5, and preincubated for 90 min in the same medium plus 1 mM sodium iodoacetate and 5 mM inosine. Iodoacetate blocks ATP formation by inhibiting glyceraldehyde-3-phosphate dehydrogenase, while inosine causes ATP consumption in the phosphofructokinase reaction. It was demonstrated, using the assay for Ca²⁺ efflux described below, that no measurable outward Ca²⁺ pumping could be detected in these ATP-depleted cells. The cells were then washed twice and added to prewarmed media of 145 mM NaCl, 5 mM KCl, 20 mM imidazole Cl, pH 7.5, plus 1.5 mM ⁴⁵CaCl₂ (1 μCi/ml). Samples were taken after 15 min, 1, 2, 3, and 4 hr, and was washed four times in cold 150 mM NaCl plus 1 mM NaEGTA (sodium ethyleneglycol tetraacetate). Each cell pellet was hemolyzed in 0.01 N NH₄OH, and part of the hemolysate was taken for measurement of hemoglobin concentration as the cyanmethemoglobin derivative. The remainder of the hemolysate was deproteinized with 6% (w/v) perchloric acid plus 1 mM NaEGTA, and ⁴⁵Ca²⁺ in the supernatant was measured by liquid scintillation counting. To convert the uptake of radioactive calcium from nanomole per micromole Hb into nanomole per milliliter cells, the MCHC (micromoles Hb per milliliter cells) was measured in each experiment.

Calcium efflux was measured in cells which were preloaded with Ca²⁺ by use of an anion, salicylate, which adsorbs strongly to fixed positive charges in the membrane and greatly increases transmembrane cation movements. Washed red cells were added to a medium of 150 mM Na salicylate, 5 mM imidazole Cl, pH 7.5, plus 2 mM ⁴⁵CaCl₂ (2 μCi/ml) at 0°C and incubated for 3 hr. The cells were then washed five times at 0°C in a medium of 145 mM NaCl, 5 mM KCl, plus 20 mM imidazole Cl, pH 7.5, which reduced red cell salicylate concentration below 0.5 mM. After the fifth wash, 1 ml of packed cells was added to 10 ml of the same medium plus 10 mM glucose which was prewarmed to 37°C. Samples were taken at 0.5, 1, 2, 3, 4 min into tubes standing in an icebath; the tubes were spun at 0°C and a sample of the supernatant taken for liquid scintillation counting of the radioactivity present outside the cells (N). A portion of the whole cell suspension was also taken for counting to determine the radioactivity initially present inside the cells (N₀). The rate constant (k) for Ca²⁺ efflux was derived from the slope of the line between 0.5 min and 4 min when log (1 – N₀/N) was plotted against time.

Total cell calcium content was measured by atomic absorption spectroscopy of a dry-ashed sample of red cells. Red cells were freed of white cells by thrice washing in saline and then washed four times at 0°C in 150 mM NaCl plus 1 mM NaEGTA. The packed cells were then hemolyzed.
in 0.01 N NH₄OH, and a sample of the hemolysate was added to a silica crucible (Vitreosil 5-ml High Form Silica Crucibles, Thermal American Co., Montville, N.J.), evaporated to dryness under an infrared lamp, and combusted at 500°C overnight in a muffle furnace. The residue was extracted with dilute acid by adding 3 ml of 0.1 N HCl plus 10 mM SrCl₂ to the crucible and shaking for 2 hr at room temperature. The contents of the crucible were decanted into polypropylene tubes, and the iron oxide was allowed to settle. Calcium in the supernatant was analyzed on a Varian Techtron 1200 Atomic Absorption Spectrophotometer at 422.6 nm using acetylene fuel plus compressed air.

Recovery studies of Ca²⁺ added to crucibles and dry ashed at 500°C established that no significant amounts of Ca²⁺ were retained by the silica crucibles. 45CaCl₂ (1.25, 2.5, 3.75, 5, and 74 nmoles) was added to crucibles, 5 ml of a red cell hemolysate added, the contents dry ashed and extracted in dilute acid as above. The recovery of 45Ca was estimated by liquid scintillation counting and was 81%, 85%, 85%, 95%, and 101%, respectively, for each of the above amounts of added Ca²⁺.

Statistics

Mean values ± 1 SD are shown unless otherwise noted. Regression lines and correlation coefficients were calculated by the method of least squares and differences between sample means analyzed by a t test.

RESULTS

Morphology

Red cells with an irregular convoluted margin as well as bowl-shaped red cells were seen on examination of wet preparations of blood from all affected patients (Fig. 1). The proportion of abnormal forms varied from 50% of red cells from NS to 20% of red cells from DC, TC, or DB. Two variations of bowl forms were observed, depending on the plane of focus and the orientation of the red cell membrane.
the cell: one form which appeared symmetrically bowl shaped and a second form in which the mouth of the bowl was distorted to produce a narrow slit or stoma* (Fig. 1). The predominant abnormal cell was one with a convoluted margin when patients' blood was diluted in autologous heparinized plasma, whereas both bowl forms and convoluted margin cells were seen in blood diluted with buffered saline plus human serum albumin. Examination of a dry, Wright-stained smear showed many target cells with few stomatocytic forms (Fig. 2A). A small number of shrunken and crenated cells were present. Occasional cells were seen in which the periphery appeared to be teased into a fine filament. When this occurred at opposite poles of the cell it produced a clam-like appearance. Polychromasia was also present.

If the abnormal red cells were swollen by exposure to hypotonic saline, then a subsequent dry smear showed stomatocytes (Fig. 2B). Fresh blood was diluted in 10 volumes of 0.4–0.45 g NaCl per dl plus 0.1 g/dl human serum albumin and 10 mM imidazole Cl, pH 7.4.† After 5 min, a thick dry smear was made and stained with Wright-Giemsa. Stomatocytes were seen in blood from all the patients (Fig. 2B) but were not seen in normal blood treated identically.

Routine Hematology Studies

The hematologic parameters are shown in Table 1. Only patient JC was anemic, while his daughters (DC and TC) and the other two patients showed a fully compensated hemolytic state. All patients had evidence of increased red cell production, with reticulocyte counts between 3% and 8%. When the MCHC of red cells was estimated from manual determinations of hemoglobin and hematocrit, elevated values between 36 and 38 g/dl were repeatedly obtained for hereditary stomatocytes. The MCHC derived from Coulter S indices were slightly lower than the manually determined MCHC, but the high MCHC of hereditary stomatocytes was still evident. Corresponding to the higher MCHC of these abnormal cells, their water content was reduced by approximately 5%, with values ranging between 620 and 627 mg/g compared with a normal mean of 658 ± 6 mg/g (Table 3).

Previous studies have shown elevations in the percentage of fetal hemoglobin in hereditary stomatocytic red cells. Fetal hemoglobin was also elevated in four of the five patients studied (Table 1), although the increase was slight and did not exceed 3.5% (normal, less than 1.0%).

Red Cell Lipids

Previous reports indicated that hereditary stomatocytes often contained excessive amounts of lipid, so the cholesterol and phospholipid content of these cells was measured. Because patient JC had been splenectomized, his red cell lipids were compared with a group of postsplenectomy controls. Red cell lipids from the four patients who had not undergone splenectomy were compared with a group of normal subjects (Table 2). In four out of the five patients

---

*The term stomatocyte has been reserved for cells showing a narrow slit or stoma on dry morphology.
†The human serum albumin was acid and lowered the final pH to 6.9–7.2.
the cholesterol plus phospholipid were increased by amounts ranging from 11% to 18%, and the cholesterol:phospholipid molar ratio was identical to normal controls. However, cells from DB had a normal lipid content. The individual classes of phospholipids present in the red cells were analyzed in both patients and normals. The proportion of phosphatidyl choline was 37%-40% (mean, 37.6%) which was significantly increased above the normal mean of 33.8% ($p < 0.02$).

Red cell osmotic fragility was decreased in all patients. Patient JC, who had been splenectomized, had the most osmotically resistant cells, with a median corpuscular fragility of 0.29 g per dl NaCl. The other four patients showed median corpuscular fragilities of 0.33, 0.35, 0.36, and 0.39 g per dl NaCl which were all below the normal range (0.40-0.44 g per dl NaCl). A linear relation existed between the median osmotic fragility and the amount of cholesterol or phospholipid per cell (Fig. 3), suggesting that the excess lipid contributed directly to the surface area of these cells. The regression line, however, did not pass through the normal mean, which indicated that a second factor contrib-
STOMATOCYTOSIS SYNDROME

Fig. 3. Relation of osmotic fragility to red cell lipids in patients with hereditary stomatocytosis. The regression line showed a correlation coefficient \( r = 0.95 \). Mean for normals ±1 SD is shown in the shaded rectangle.

uated to the osmotic resistance of these cells. This factor appeared to be the lower cell content of Na\(^+\) plus K\(^+\) described below.

**Intracellular Cation Content**

Four patients had a cell Na\(^+\) which was within the normal range of 5.9–11.7 μeq per ml cells, while one patient (NS) had an elevated cell Na\(^+\) of 15.1 μeq per ml cells (Table 3). The mean cell Na\(^+\) for the five hereditary stomatocytes was 11.0 ± 2.3 μeq per ml cells, which represented a small but significant increment above the normal mean of 7.9 ± 1.5 μeq per ml cells (\( p < 0.01 \)). Even if patient NS was excluded from the comparison, the mean cell Na\(^+\) of the remaining patients was significantly above the normal mean (\( p < 0.01 \)). In contrast to the slight increment in cell Na\(^+\), the cell K\(^+\) was 10–20 μeq per ml cells less than normal in all five patients, and the mean K\(^+\) of 85.0 ± 3.8 μeq per ml cells was significantly below normal (\( p < 0.001 \); Table 3). The K\(^+\) deficit was also reflected in the total content of monovalent cations, Na\(^+\) plus K\(^+\), which was below normal (\( p < 0.001 \)) whether expressed per unit volume of cells or per unit number of cells.

**Sodium Fluxes**

Fluxes of both Na\(^+\) and K\(^+\) were measured in the presence and absence of ouabain, and the inhibition of the total flux by ouabain was taken as the active component (Table 3). Sodium influx was increased in all patients, and it ranged from 6.2 to 12.2 μeq per ml cells per hr compared with a normal of 2.0 ± 0.2
Table 2. Red Cell Lipids in Hereditary Stomatocytosis

<table>
<thead>
<tr>
<th>Cholesterol (µg per 10⁶ cells)</th>
<th>Phospholipid (µg per 10⁶ cells)</th>
<th>Molar Ratio Cholesterol : Phospholipid</th>
<th>Phospholipid Classes (% of total)</th>
<th>Total Lipid (% of Control)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Lysolecithin</td>
<td>Sphingomyelin</td>
<td>Phosphatidyl-Choline</td>
</tr>
<tr>
<td>JC</td>
<td>18.7</td>
<td>42.2</td>
<td>0.89</td>
<td>2</td>
</tr>
<tr>
<td>DC</td>
<td>15.3</td>
<td>33.4</td>
<td>0.92</td>
<td>0</td>
</tr>
<tr>
<td>TC</td>
<td>15.8</td>
<td>33.9</td>
<td>0.93</td>
<td>1</td>
</tr>
<tr>
<td>NS</td>
<td>15.4</td>
<td>36.4</td>
<td>0.85</td>
<td>2</td>
</tr>
<tr>
<td>DB</td>
<td>13.0</td>
<td>29.6</td>
<td>0.88</td>
<td>3</td>
</tr>
<tr>
<td>Normal Subjects</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>± SD</td>
<td>0.5</td>
<td>1.3</td>
<td>0.9</td>
<td>0.9</td>
</tr>
<tr>
<td>Controls</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Postspenectomy</td>
<td>16.5</td>
<td>35.5</td>
<td>0.93</td>
<td></td>
</tr>
<tr>
<td>± SD</td>
<td>1.2</td>
<td>3.2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are the mean of two measurements on separate days for each patient. Normal means are for 21 subjects before splenectomy and 14 controls after splenectomy. Patient JC is splenectomized.

Table 3. Cation Concentrations and Isotopic Fluxes in Hereditary Stomatocytosis

<table>
<thead>
<tr>
<th>No⁺ (µeq per ml cells)</th>
<th>K⁺ (µeq per ml cells)</th>
<th>Na⁺ + K⁺ (µeq per ml cells)</th>
<th>Cell water (mg/g)</th>
<th>Na⁺ Influx* (µeq per ml cells per hr)</th>
<th>Na⁺ Efflux* (µeq per ml cells per hr)</th>
<th>K⁺ Influx* (µeq per ml cells per hr)</th>
<th>K⁺ Efflux* (µeq per ml cells per hr)</th>
<th>Ouabain-binding S1est (molecules per cell ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>JC</td>
<td>10.5</td>
<td>86.1</td>
<td>96.6</td>
<td>627</td>
<td>9.0</td>
<td>8.0</td>
<td>1.9</td>
<td>5.4</td>
</tr>
<tr>
<td>DC</td>
<td>9.8</td>
<td>89.0</td>
<td>98.8</td>
<td>620</td>
<td>6.6</td>
<td>5.75</td>
<td>1.85</td>
<td>4.5</td>
</tr>
<tr>
<td>TC</td>
<td>9.7</td>
<td>87.8</td>
<td>97.5</td>
<td>—</td>
<td>6.25</td>
<td>6.15</td>
<td>1.75</td>
<td>6.5</td>
</tr>
<tr>
<td>NS</td>
<td>15.1</td>
<td>79.5</td>
<td>94.6</td>
<td>622</td>
<td>12.2</td>
<td>9.7</td>
<td>5.3</td>
<td>8.0</td>
</tr>
<tr>
<td>DB</td>
<td>10.2</td>
<td>82.9</td>
<td>93.1</td>
<td>625</td>
<td>6.2</td>
<td>4.4</td>
<td>1.2</td>
<td>4.6</td>
</tr>
<tr>
<td>Normals</td>
<td>7.9</td>
<td>99.0</td>
<td>106.9</td>
<td>6581</td>
<td>0.2</td>
<td>2.0</td>
<td>0.8</td>
<td>1.5</td>
</tr>
<tr>
<td>(SD)</td>
<td>1.5</td>
<td>5.2</td>
<td>5.0</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
</tr>
</tbody>
</table>

*All fluxes were measured on cells incubated in 145 mM NaCl, 5 mM KCI, 20 mM imidazole CI, pH 7.5. However, for K⁺ influx, the external K⁺ varied from 5.8 to 7.8 mM.

**NaCl and KCl were the isotopes employed.

†Calculated for 100% inhibition of active K⁺ influx. Values for patients are the mean of quadruplicate estimations in a single experiment, while the normal mean was obtained on six separate donors.

‡Mean cell water of 658 mg per g wet weight cells is equivalent to 72% water (v/v).
\( \mu \text{eq per ml cells per hr.} \) The influx was reproducible since values of 6.7 and 6.5 \( \mu \text{eq per ml cells per hr} \) were obtained at intervals several months apart for DC, while values of 9.1 and 8.9 \( \mu \text{eq per ml cells per hr} \) were also obtained for JC on different days. The highest Na\(^+\) influx was observed in NS who also was the only patient with a cell Na\(^+\) above the normal range. Sodium influx was measured over a range of external Na\(^+\) concentrations from 10 to 145 mM Na\(^+\) ion. An increased Na\(^+\) permeability of hereditary stomatocytes was apparent over this entire range.

Sodium efflux was also increased in hereditary stomatocytes as total efflux ranged from 9.7 to 4.4 \( \mu \text{eq per ml cells per hr} \) for patients compared with 2.9 ± 0.25 \( \mu \text{eq per ml cells per hr} \) for normal cells. Both the active (ouabain-sensitive) and passive (ouabain-insensitive) components of Na\(^+\) efflux were increased two-to fivefold in the abnormal cells (Table 3), despite an intracellular Na\(^+\) concentration which was slightly increased by a mean of 3 \( \mu \text{eq per ml cells} \).

**Potassium Fluxes**

Potassium influx and efflux were increased in hereditary stomatocytes by an amount comparable with the elevation in Na\(^+\) fluxes (Table 3). Both the active (ouabain-sensitive) and passive (ouabain-insensitive) components of K\(^+\) influx were raised in approximately the same proportion, so that active K\(^+\) influx accounted for 76%-86% of the total K\(^+\) flux in both normal and abnormal red cells. Potassium efflux measured in two patients was elevated three-to fourfold above normal.

The kinetic properties of the active cation pump with respect to external K\(^+\) ions were studied in one patient (JC). When the active (ouabain-sensitive) K\(^+\) influx was measured at various external K\(^+\) concentrations (K\(_{o}\)), a saturating curve was obtained which had the same form in both the normal and abnormal cells. Kinetic analysis showed that external K\(^+\) ions produced half-maximal activation of the active cation pump at a concentration (K\(_{m}\)) of 1.8 mM in a high Na\(^+\) medium and at a K\(_{m}\) of 0.25 mM in a choline chloride (Na\(^+\)-free) medium. This difference between the apparent K\(_{m}\) for K\(^+\) activation of the pump in Na\(^+\)-rich and Na\(^+\)-free media agreed closely with the values obtained by Garrahan and Glynn and probably arose from an inhibitory action of Na\(^+\) ions at the site where K\(^+\) ions activate the pump. Hereditary stomatocytic cells showed exactly the same apparent K\(_{m}\) for K\(^+\) ions as for normal cells, although the maximum velocity of active K\(^+\) influx was greater in stomatocytic cells. One explanation for this increased maximum velocity of the cation pump was provided by measurement of ouabain binding.

**\(^3\)H-Ouabain Binding**

In four patients with hereditary stomatocytosis, the mean number of ouabain-binding sites per red cell was approximately 2.5-fold normal (Table 3). Assuming the same stoichiometry for ouabain binding to pumps in the two types of cells, it can be concluded that hereditary stomatocytes have more cation pump sites than normal. The rate at which \(^3\)H-ouabain bound to hereditary stomatocytic cells was also slightly increased above normal, although the reason for this difference was not clear.
Washed red cells from JC were incubated with glucose (10 mM) or glucose plus ouabain (0.1 mM) for 3 and 6 hr at 37°C. Cation concentrations in μeq per ml cells.

**Coupling Ratio of the Active Cation Pump**

Active efflux of Na⁺ is known to be coupled to active K⁺ influx in a ratio of 3:2 for normal red cells. The ratio of active Na⁺ to K⁺ fluxes for normal red cells was found to be 1.31, which agreed with previous reports, while the corresponding ratios for the patients were 1.48, 1.28, 1.21, 0.96, and 0.95, not significantly different from normal. The coupling ratio of the active cation pump was also calculated from uphill net movements of Na⁺ and K⁺. Cells were incubated with glucose either with or without ouabain and analyzed for Na⁺ and K⁺ after 3 or 6 hr at 37°C. With no inhibitor present, the cells maintained high K⁺ and low Na⁺ levels, but in the presence of ouabain they gained Na⁺ and lost K⁺. The difference between Na⁺ concentrations with and without ouabain represented a net movement by the active cation pump, and the difference in K⁺ concentrations also represented the concurrent net K⁺ movement by the pump. These values for net uphill movement of cations in hereditary stomatocytes from JC are shown in Table 4. After 3 hr of incubation, the active pump had moved 19.0 μeq of Na⁺ and 14.5 μeq of K⁺ per ml cells; while after 6 hr the values were 29.0 μeq Na⁺ and 22.5 μeq K⁺ per ml cells. The coupling ratio of the cation pump, calculated from net movements, was 1.30, and this value was not different from the coupling ratio of 1.48 calculated from isotopic fluxes. It was concluded from both the fluxes and from the net movements that the active cation pump in hereditary stomatocytes was normally coupled.

**Calcium Content and Permeability**

Human red cells are extremely impermeable to divalent cations, and the uptake of calcium is 1000-fold less than for Na⁺ ions. Moreover, the calcium pump can rapidly extrude any calcium which has entered the cell, so that the calcium influx can only be measured in ATP-depleted cells which lack the energy source for the calcium pump. Isotopic Ca²⁺ uptake into ATP-depleted red cells from normal donors showed a biphasic curve with a rapid initial uptake of isotope of 2.3 nmole per ml cells in 15 min followed by a slower linear uptake of approximately 0.8 nmole per ml cells every hour up to 4 hr of incubation. After 1 hr of incubation, isotopic calcium entry was 3.0 ± 0.6, and after 4 hr it was 5.4 ± 1.5 nmole per ml cells. Uptake of ⁴⁵Ca²⁺ into ATP-depleted hereditary stomatocytes was accelerated, and both the initial rapid entry and the linear portion of the curve were above normal. By 4 hr of incubation, the calcium uptake varied between 15.5 and 46.0 nmole per ml cells (Fig. 4). Calcium permeability was also increased in ATP-depleted control cells.

<table>
<thead>
<tr>
<th>Table 4. Net Cation Movements in Hereditary Stomatocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
<tr>
<td>Glucose</td>
</tr>
<tr>
<td>Glucose + ouabain</td>
</tr>
<tr>
<td>Active net movement</td>
</tr>
<tr>
<td>Ratio active Na⁺:K⁺ movements</td>
</tr>
</tbody>
</table>

Washed red cells from JC were incubated with glucose (10 mM) or glucose plus ouabain (0.1 mM) for 3 and 6 hr at 37°C. Cation concentrations in μeq per ml cells.
Fig. 4. Calcium uptake by hereditary stomatocytes compared with control red cells containing 2%-20% reticulocytes. Cells were depleted of ATP and incubated in buffered saline media containing 1.5 mM Ca\(^{2+}\) for 4 hr. Mean for normals ± 1 SD is shown in the shaded rectangle.

with a reticulocytosis due to hemantinic therapy or immune hemolytic anemia, and Ca\(^{2+}\) uptake varied from 15 to 38 nmole per ml cells per 4 hr when the reticulocytes were 4%-8%. Three of the patients had a Ca\(^{2+}\) uptake lower than expected for the reticulocytosis, so it was clear that increased Ca\(^{2+}\) permeability was not an invariable feature of hereditary stomatocytosis (Fig. 4). It was of interest that the patient with the highest Na\(^{+}\) influx (NS) had the highest Ca\(^{2+}\) uptake, while the three patients with the lowest Na\(^{+}\) influxes also had the lowest Ca\(^{2+}\) uptakes.

The Ca\(^{2+}\) content of hereditary stomatocytes was found not to be different from that of normal red cells. The five patients had red cell Ca\(^{2+}\) concentrations of 3.7, 2.4, 3.5, 3.0, and 7.3 nmole per ml cells, and the mean value 4.0 ± 1.9 nmole per ml cells was not different from the normal mean, 5.4 ± 2.4 nmole per ml cells.

To examine the activity of the calcium pump, the extrusion of Ca\(^{2+}\) from hereditary stomatocytes and normal cells was compared at 37°C when both kinds of cells had been preincubated to load the cells with 0.2-0.4 μmole per ml of isotopic calcium. Normal red cells extruded almost 80% of their calcium with a half-time of 2.0 min (k = 0.35 min\(^{-1}\)), while the initial 20% was lost so rapidly that its kinetics could not be followed by conventional sampling techniques. Hereditary stomatocytes from JC showed almost identical kinetics, with a major fraction of the isotopic calcium being extruded with a half-time of 1.8 min (k = 0.38 min\(^{-1}\)) and another 15% of the calcium being lost within the first 15 sec. It was concluded that the calcium pump behaved identically in normal and hereditary stomatocytic red cells.
DISCUSSION

Bowl-shaped red cells* are seen in wet preparations of blood in those conditions in which normocytic normochromic target cells occur. Bowl forms are also seen in wet preparations of blood in some hereditary hemolytic anemias with stomatocytes. Thus, in dry preparations, the bowl-shaped cell is distorted as it flattens on the slide and produces either a target cell or a stomatocyte, although it is not clear why sometimes one and not the other cell type is observed.

The five affected patients under study had both bowl-shaped cells and cells with an irregular, undulating margin on wet preparations of blood. Both types were seen when blood was diluted in buffered saline, whereas convoluted margin cells were the only abnormal form in blood diluted in autologous plasma. All patients had low cell water content with target cells on dry smear. Osmotic swelling of these cells gave rise to stomatocytes on smear, so it is likely that the water content of a cell with excess membrane may influence its dried morphology. It is of interest that, in hereditary stomatocytosis with normal cell water, both stomatocytes and some targets occurred on smear, while a case with increased cell water showed only stomatocytes on smear.

Our demonstration that target cells and stomatocytes may differ only in their water content has diagnostic implications. Hereditary hemolytic anemia with target cells may be considered within the spectrum of hereditary stomatocytosis, since both are cells with excessive membrane and marked abnormality of cation fluxes. However, in some patients the net result of altered cation permeability is a decreased total cation content, decreased cell water, increased MCHC, and target appearance in dried smears. In other patients, cation excess results in overhydration, low MCHC, and stomatocytes on smear.

None of our five patients showed evidence of liver disease or hemoglobinopathy which may also be associated with target cells. Moreover, the dominant inheritance of hemolytic anemia in the C family was consistent with previous descriptions of hereditary stomatocytosis. It was of interest that the two affected daughters in the C family suffered recurrent episodes of abdominal pain, either generalized or in the left upper quadrant, which has been described in hereditary stomatocytosis.

Routine tests for the evaluation of hereditary hemolytic anemias are of limited use in the diagnosis of hereditary stomatocytosis and give inconsistent results between different families (Table 5) which may account for the infrequency with which this condition is diagnosed. Osmotic fragility is usually decreased, but in two families it was reported to be increased. All five patients with hereditary stomatocytes in the present study showed a decreased osmotic fragility. Autohemolysis of sterile blood is always elevated, but the increased lysis may or may not be corrected by glucose addition (Table 5). In the patients studied here, autohemolysis was increased and did correct with glucose supplementation. Hemoglobin F was elevated in four of the present patients, although the elevation was not as great as that found in some hereditary stomatocytics by

*This term includes both symmetrical bowls and bowls showing a slit-like orifice.
Table 5. Hematologic, Cation Flux, and Lipid Data Reported for Families With Hereditary Stomatocytosis and Related Syndromes

<table>
<thead>
<tr>
<th></th>
<th>Zarkowsky et al.²</th>
<th>Ohki et al.³</th>
<th>Miller et al.⁴</th>
<th>Honig et al.⁵</th>
<th>Jaffe &amp; Gottfried⁷</th>
<th>Shohet et al.⁸</th>
<th>Glader et al.⁹</th>
<th>This Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemoglobin (g/dl)*</td>
<td>6.2</td>
<td>12.7</td>
<td>10.0</td>
<td>12.2</td>
<td>12.6</td>
<td>10.0</td>
<td>11.8-15.6</td>
<td></td>
</tr>
<tr>
<td>Reticulocytes (%)</td>
<td>20</td>
<td>1.8</td>
<td>18</td>
<td>0.9</td>
<td>12</td>
<td>25</td>
<td>3.3-7.8</td>
<td></td>
</tr>
<tr>
<td>MCV (cu μ)</td>
<td>118</td>
<td>112</td>
<td>99</td>
<td>85</td>
<td>92</td>
<td>106</td>
<td>84-99</td>
<td></td>
</tr>
<tr>
<td>MCHC (g/dl)</td>
<td>23</td>
<td>28</td>
<td>34</td>
<td>36</td>
<td>34</td>
<td>36</td>
<td>35.7-38.0</td>
<td></td>
</tr>
<tr>
<td>Osmotic fragility</td>
<td>High</td>
<td>High</td>
<td>Low</td>
<td>Low</td>
<td>Low</td>
<td>Low</td>
<td>Low</td>
<td></td>
</tr>
<tr>
<td>Autohemolysis</td>
<td>(a) no glucose</td>
<td>Increased</td>
<td>Increased</td>
<td>Increased</td>
<td>Increased</td>
<td>—</td>
<td>Increased</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(b) added glucose</td>
<td>No effect</td>
<td>Corrects</td>
<td>Corrects</td>
<td>Corrects</td>
<td>Corrects</td>
<td>Corrects</td>
<td></td>
</tr>
<tr>
<td>Cell Na⁺ (μeq/ml)</td>
<td>100</td>
<td>64</td>
<td>21</td>
<td>7</td>
<td>22</td>
<td>18</td>
<td>9-15</td>
<td></td>
</tr>
<tr>
<td>Cell K⁺ (μeq/ml)</td>
<td>40</td>
<td>72</td>
<td>87</td>
<td>95</td>
<td>75</td>
<td>59</td>
<td>79-89</td>
<td></td>
</tr>
<tr>
<td>Na⁺ influx (μeq/ml/hr)</td>
<td>107</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>9.5</td>
<td>—</td>
<td>6.2-12.2</td>
<td></td>
</tr>
<tr>
<td>Na⁺ efflux (μeq/ml/hr)</td>
<td>—</td>
<td>6</td>
<td>20</td>
<td>Increased</td>
<td>4.7</td>
<td>—</td>
<td>5.6-15.0</td>
<td></td>
</tr>
<tr>
<td>K⁺ influx (μeq/ml/hr)</td>
<td>31</td>
<td>3.3</td>
<td>1.7</td>
<td>—</td>
<td>4.7</td>
<td>—</td>
<td>5.4-10.0</td>
<td></td>
</tr>
<tr>
<td>Cation pump Na:K coupling ratio</td>
<td>3.2</td>
<td>2.5:1</td>
<td>2.6:1</td>
<td>—</td>
<td>1:1</td>
<td>3.2</td>
<td>3.2</td>
<td></td>
</tr>
<tr>
<td>Total lipid per cell</td>
<td>Increased</td>
<td>Increased</td>
<td>Normal</td>
<td>—</td>
<td>Increased</td>
<td>—</td>
<td>Increased or Normal</td>
<td></td>
</tr>
<tr>
<td>Phosphatidyl choline per cell</td>
<td>—</td>
<td>—</td>
<td>Normal</td>
<td>—</td>
<td>Increased</td>
<td>—</td>
<td>Increased</td>
<td></td>
</tr>
</tbody>
</table>

*Presplenectomy values for the propositus except values from Glader which are postsplenectomy.

†Direct measurements of isotopic flux only are tabulated. It is invalid to calculate a net flux as the difference between opposite isotopic flux and the rate of net cation change.

Miller et al.⁴ However, our patients did not have a thalassemic syndrome since hemoglobin A₂ was always normal, and no patient had microcytic, hypochromic red cells. Indeed, MCHC was clearly increased above normal in three of the patients up to a level of 37-38 g/dl which is usually only seen in hereditary spherocytosis. A high MCHC does not seem to be a frequent finding in hereditary stomatocytosis (Table 5), although it has been observed in several members of the family studied by Miller et al.⁴ If present, a high MCHC may suggest hereditary stomatocytosis if supported by the findings of reduced osmotic fragility and increased autohemolysis. An increased Na⁺ flux, either influx or efflux, appears to be the most consistent abnormality in this disease and is reproducible in the same patient on different days. Since the Na⁺ influx can be performed with ⁴²Na isotope in several hours, this measurement is probably of diagnostic value.

The present results suggest that at least two factors contribute to the osmotic resistance of hereditary stomatocytes: first the increased membrane lipid content and second the reduced content of cations in these cells. It is known that the surface area of red cells varies in direct proportion to the amount of lipid present,³⁵ and four of the five patients in this study had increased amounts of red cell lipid. The second factor leading to osmotic resistance is the reduced content of Na⁺ plus K⁺ ions present in hereditary stomatocytes. The cation content of erythrocytes is a major determinant of cell water, and several studies have confirmed that alterations in Na⁺ plus K⁺ produce proportional changes in cell water and cell volume.³⁶,³⁷ The decrease in Na⁺ plus K⁺ was associated with an increased or high-normal MCHC and a decrease in cell water in our five patients.

Increased Na⁺ fluxes are characteristic of red cells from patients with hereditary stomatocytosis and have been found in every case where this measure-
ment has been made (Table 5). Although reticulocytes have increased Na⁺ influx, the data of Bernstein indicate that passive Na⁺ movements are only increased by 20%-30% in cells with a 5% reticulocytosis. It seems probable that the two- to sixfold increase in Na⁺ turnover of hereditary stomatocytes was not due to the younger mean cell age of these cells. A similar conclusion was also reached in a study of the increased Na⁺ fluxes in hereditary spherocytosis. In the present study, both the influx and efflux of Na⁺ ions were increased several fold, while cell Na⁺ concentration lay within the normal range in four patients. It appears from previous studies that cell Na⁺ concentrations in hereditary stomatocytes rise only if Na⁺ influx is increased by more than fivefold. In the present series, patient NS, with a sixfold increase in Na⁺ influx, was the only patient with a cell Na⁺ above the normal range. The other patients had a three- to fivefold increase in Na⁺ influx, and the increased cation pumping in these cells maintained cell Na⁺ within the normal range. Values for the ouabain-sensitive Na⁺ efflux and K⁺ influx in Table 3 show that stomatocytes have a two- to fivefold increase in active cation pumping. The basis for this increased pumping was investigated by measuring ouabain-binding sites.

The measurement of tritiated ouabain or digoxin binding to human red cells give values of around 250 sites per cell when the pump is maximally inhibited. Hoffman further suggested that the number of pump sites measured by ouabain binding may be an overestimate due to the inclusion of an unknown number of nonspecific sites. Recently, Gardner and Conlon have claimed that the method of Hoffman underestimates the number of sites, and these authors have calculated a value of 1200 sites for a normal cell. The present study employed the technique of tritiated ouabain binding, according to Hoffman, to compare stomatocytic and normal red cells on the same day and under the same experimental conditions. The mean value for six normal persons was 336 ± 34 sites per cell, while the number of sites was increased 2.5-fold above normal in four hereditary stomatocytic patients. Despite the controversy over the absolute number of ouabain-binding sites per cell, the above results are consistent with the relative magnitude of active Na⁺ and K⁺ fluxes and lead to the conclusion that hereditary stomatocytic red cells have an increased density of cation pump sites. A similar result has been reported for hereditary spherocytes, where an increased rate of active cation pumping seems to result from an increased number of pumps rather than from an altered $K_m$ towards the activating cations. Reticulocytes possess increased amounts of cation pump ATP-ase, and the elevated reticulocytes in hereditary stomatocytosis may contribute to the greater ouabain binding by these cells. However, in one study, the active fluxes of Na⁺ and K⁺ differed only slightly between a reticulocyte-rich and reticulocyte-poor fraction of hereditary stomatocytic blood. Further experiments will be necessary to determine the contribution of reticulocytes to the increased ouabain binding of hereditary stomatocytes.

Two functional parameters of the active cation pump have been investigated in hereditary stomatocytes and found to be normal: the Na⁺:K⁺ coupling ratio and the activation kinetics of the pump towards external K⁺ ions. While active cation fluxes are increased in hereditary stomatocytes due to the increased density of cation pumps, the coupling ratio of active Na⁺ efflux to active K⁺
influx did not differ from the normal value of 3:2. Wide variations of this coupling ratio in hereditary stomatocytosis have been reported, ranging from 26:1 in a patient reported by Miller et al. to 3:2 in the patient studied by Zarkowsky et al. In another family with hereditary hemolytic anemia possibly related to stomatocytosis, the Na⁺:K⁺ coupling ratio was 1:1. However, it has been recently shown that the very high ouabain-sensitive Na⁺ efflux in Miller’s patient was due to Na⁺-Na⁺ exchange through the cation pump, and this exchange diffusion did not contribute to true uphill Na⁺ pumping. Flame photometric measurements of Na⁺ and K⁺ movements in the absence and presence of ouabain gave the Na⁺:K⁺ coupling ratio independent of Na⁺:Na⁺ exchange through the pump so that, in the present study, active cation movements were measured by flame photometry as well as by isotopic fluxes to obtain two independent measures of the coupling ratio. There was good agreement in the Na⁺:K⁺ coupling ratio estimated by net movements (1.3:1) or by fluxes (mean of 1.2:1). Since normal cells showed a coupling ratio of 1.3:1, it was concluded that the cation pump was normally coupled. Moreover, kinetic analysis of K⁺ influx both in high-Na⁺ and Na⁺-free media showed that the outward facing site of the cation pump had the same kinetic properties as in normal cells. It is of interest that several kinetic parameters of the active cation pump have been examined in stomatocytic cells by Oski et al. who found them to be normal.

The disparity between the large K⁺ deficit (mean, 14 μeq per ml cells) and the small Na⁺ increment (mean, 3 μeq per ml cells) in hereditary stomatocytes suggested a possible defect in the Ca²⁺ permeability of these cells. Entry of Ca²⁺ into human red cells has been shown to mediate a rapid outward K⁺ movement with little change in the Na⁺ content. Both Ca²⁺ permeability and Ca²⁺ concentration of hereditary stomatocytes were measured to find if leakiness to Ca²⁺ was responsible for the low K⁺ content of these cells. Hereditary stomatocytes had a normal Ca²⁺ content, and their Ca²⁺ influx was not increased out of proportion to the reticulocytosis. Moreover the activity of the outwardly directed Ca²⁺ pump was also normal, so that an abnormality of Ca²⁺ fluxes was not the explanation of the low K⁺ concentration of these cells.

Our studies describe a group of hereditary stomatocytes with homogeneous features similar to those of the families reported by Miller et al. and Honig et al., although the latter family had cup-shaped elliptocytes rather than the more typical stomatocytes. Our patients were also similar to the family with high phosphatidyl choline hemolytic anemia described by Jaffe and Gottfried and by Shohet et al. and also to a family recently reported by Glader et al., although this family did not have stomatocytes. Despite the similarities between the above families, it is clear from Table 5 that there is heterogeneity in hereditary stomatocytosis. Hemolysis ranges from severe in one reported case to mild or even absent in a family whose members maintain normal hemoglobin levels with normal or minimally elevated reticulocytes. Even within the one family there was considerable variability in the rate of hemolysis between different affected members. Reasons for this variability are not clear, although one factor causing hemolysis may be an abnormal cell water content of hereditary stomatocytes (either too high or too low) which may impede their ability.
to deform during passage through the splenic microvasculature. A wide range of cation fluxes and cation composition may lead to the variability in cell water content. In three families there was a reversal of the usual high-K\(^+\), low-Na\(^+\) composition of the red cell, such that cell Na\(^+\) was raised to 50–100 μeq per ml cells, and cell K\(^+\) was reduced to 40–70 μeq per ml cells.\(^{25,48}\) However, in most cases of hereditary stomatocytosis, including the present study, there was only slight elevation of cell Na\(^+\) coupled with a consistent decrease in cell K\(^+\) concentration.\(^{3,4,36,49}\) Water content of hereditary stomatocytes varies according to the sum of monovalent cations, Na\(^+\) + K\(^+\), and may be either increased\(^{25}\) or decreased as in this study. Sodium influx measurements have also shown a heterogeneity in hereditary stomatocytosis, since this flux can vary from three to over 30 times normal (Table 5). Thus, there is a spectrum in the cation composition and permeability of hereditary stomatocytosis which suggests a genetic heterogeneity which may underlie the variability in this disorder. Indeed, such heterogeneity might suggest that the patients tabulated in Table 5 include examples of several syndromes which are only related morphologically. However, the constancy of the permeability defect would argue that these patients fall within the spectrum of a single syndrome.

ACKNOWLEDGMENT

The authors are grateful to Mrs. Elizabeth Armer for excellent technical assistance. We thank Dr. John E. Benzel, of Wilmington, Delaware, for referring patient NS for investigation and Dr. Elias Schwartz of the Childrens Hospital of Philadelphia for measurements of hemoglobin A\(_2\) and F.

REFERENCES

13. Wiley JS: Red cell survival studies in
STOMATOCYTOSIS SYNDROME

355

15. Hoffman JF: The interaction between trinitiated ouabain and the Na-K pump in red blood cells. J Gen Physiol 54:343s, 1969
42. Gardner JD, Conlon TP: The effects of sodium and potassium on ouabain binding by human erythrocytes. J Gen Physiol 60:609, 1972
44. Dutcher PO, Segel GB, Feig SA, Miller...
Characteristics of the membrane defect in the hereditary stomatocytosis syndrome

JS Wiley, JC Ellory, MA Shuman, CC Shaller and RA Cooper