Autohemolysis and Other Changes Resulting from the Incubation in Vitro of Red Cells from Patients with Congenital Hemolytic Anemia

By J. G. Selwyn, M.D. (Cantab.) and J. V. Dacie, M.D., M.R.C.P. (Lond.)

The incubation at 37 C. of sterile defibrinated blood from cases of hereditary spherocytosis results in a more rapid increase in osmotic fragility of the red cells\(^2\), \(^2\) and a more rapid rate of autohemolysis\(^6\), \(^1\) than normal. The cause of these unusually rapid changes lies in the red cells and not in the serum, as replacement of the patient's serum by saline or by serum from a normal person does not retard the changes.\(^3\), \(^1\) Moreover, artificial concentration of the blood causes a further increase in both fragility and hemolysis.\(^1\) The beneficial results of splenectomy in hereditary spherocytosis indicate without any doubt that the patient's spleen is of major importance in the causation of hemolysis in vivo. However, the exact mechanism of hemolysis is still not known. Histologic examination shows that a remarkable degree of splenic congestion is a constant and characteristic feature, and it appears highly probable that the blood in the spleen is exposed to the effects of unusually long periods of stagnation and concentration. That congestion with blood may lead to stagnation was suggested by experiments in vitro\(^4\) in which it was found that it took longer than normal to free spleens from patients with hereditary spherocytosis from blood by mean of perfusion in vitro. In vivo, Watson and Paine\(^5\) obtained evidence in favor of the concentration of blood in the spleens of patients with hereditary spherocytosis by causing the spleens to contract by injecting adrenalin during operations for splenectomy. They observed that the increase in packed cell volume of the splenic vein blood was accompanied by the appearance of cells with an increased osmotic fragility and a decreased cell diameter. Dacie\(^1\) found that the cells with the greatest fragility (giving the tail of many fragility curves) gradually disappeared from the general circulation after splenectomy. More recently Emerson, et al.,\(^9\) and Young, et al.,\(^23\) using the differential agglutination technic, showed that from a mixture of spherocytic and normal cells the spleen selectively trapped and retained the spherocytes. All these observations give support to the early suggestion of Ham and Castle\(^1\) that the spherocytes are trapped, concentrated, and retained in the spleen and there undergo the rapid fragility and autohemolytic changes that are observed when blood is incubated in vitro. Ham and Castle also observed that these red cell changes were retarded if the serum of the incubated blood was replaced every two hours by fresh serum. They thought that the rapid increase in osmotic fragility and in the rate of autohemolysis observed in hereditary spherocytosis were due to metabolic processes which, by increasing the osmotically active constituents of the red cells, caused progressive swelling of the already spherocytic cells and therefore premature bursting.

Certain aspects of the metabolism of normal red cells have a bearing on the
problem. Maizels\textsuperscript{16-18} has shown that the low sodium content of human red cells is maintained by a process which derives its energy from the breakdown of glucose. This process actively expels sodium from the cell, overcoming the passive diffusion of sodium into the cell from the high concentration of sodium in the plasma. The high cellular potassium content is thought to be compensatory, keeping the cell cation concentration at the required level. Maizels believed that the sodium-expelling mechanism was probably situated on the inside of the cell membrane. His work, following on that of Harris,\textsuperscript{14} has shown that when normal red cells are stored at +4 C., or incubated at 37 C. in the absence of glucose, their sodium content progressively increases due to unopposed diffusion of sodium into the cells and that the cell potassium falls due to diffusion into the plasma. Maizels\textsuperscript{15} found that the gain in sodium exceeded the loss in potassium, that water entered with the excess sodium, and that the cells increased in volume, the tonicity of the cell contents thereby remaining constant. He also found that if cells were incubated in the presence of glucose, these changes were largely prevented, but if fluoride (which inhibits glycolysis) was present as well, the cation changes took place as before.

In this paper we describe studies on the red cell changes occurring during incubation, carried out in an endeavor to throw further light on the phenomenon of autohemolysis. Red cells from normal persons, cases of hereditary spherocytosis before and after splenectomy, and from cases of nonspherocytic congenital hemolytic anemia have been studied with regard to changes in cell volume, osmotic fragility, autohemolysis, and cell sodium and potassium contents.

It was thought that a comparison of the cation-controlling mechanisms of normal cells and of congenitally defective spherocytes and other types of cells might reveal differences in accord with the gross changes observed on incubation. The results to be presented indicate that congenital spherocytes are not defective in their cation-controlling mechanism, and that in any case a progressive degeneration of the cell and/or its membrane rather than a progressive increase in cell volume is the cause of the increased fragility and hemolysis during incubation. Glucose was found to have a markedly retarding effect on the hemolysis of spherocytes during incubation, but it had only a slight effect on certain other types of congenitally defective cells and no effect at all on the cells of one type which were apparently unable to metabolize glucose normally.

Methods

Defibrinated blood, collected with sterile precautions in 100 ml. conical flasks,\textsuperscript{2} was used throughout this investigation, but oxalated blood (Heller and Paul mixture) was used for cell counts. 3 ml. volumes of the defibrinated blood were transferred to four sterile screw-capped 5 ml. bottles and incubated at 37 C. Two bottles were incubated for 24 hours, after which time the contents of both were mixed together and tested. Two other bottles were incubated for a total of 48 hours, their contents being gently mixed after the first 24 hours. Duplicate bottles were used so that if bacteria were found in any bottle, the bottle could be discarded without loss of the whole sample. Infection in fact very seldom took place.

Osmotic Fragility

A standard method\textsuperscript{6} was used, employing saline solutions buffered to pH 7.4. The blood was diluted 1 in 100 and hemolysis estimated photoelectrically. As a few of the cells of
certain incubated bloods were hemolysed even in 0.85 per cent saline, a solution of 1.2 per cent saline was used as a standard for 0 per cent hemolysis.

Changes in Cell Volume

Packed cell volumes (P.C.V.) were estimated in Wintrobe hematocrit tubes centrifuged for 30 minutes at 3000 rpm in a centrifuge of radius 15 cm., the readings obtained being corrected for trapped intercellular serum according to the results of Chaplin and Mollison.\(^1\) Corrections for the amount of autohemolysis that had occurred during incubation allowed the calculation of the change in mean cell volume (M.C.V.) of the surviving cells. This was expressed as a percentage of the initial volume. P.C.V. readings corrected for trapped serum are described as the true P.C.V.

Autohemolysis

This was estimated by comparing photoelectrically dilutions in N/150 NH\(_4\)OH of whole blood (1 in 100 or 1 in 200) and of serum from incubated blood (1 in 25 or 1 in 50). Appropriate dilutions of fresh serum were used as blank solutions. The percentage hemolysis is then given by the formula:

\[
\frac{R_T \times \left(\frac{100 - \text{PCV}_T}{100}\right)}{R_0 \times 4} \times 100 = \frac{R_T \times (100 - \text{PCV}_T)}{R_0 \times 4}
\]

\(R_T\) = reading in colorimeter of the diluted serum at time T (i.e. 24 or 48 hours).

\(R_0\) = true packed cell volume at time T.

Cell Sodium and Potassium Estimations

These were made using the flame photometer designed by Domingo and Klyne.\(^7\) Serum potassium concentrations were estimated on dilutions of 1 in 20, 1 in 40, or 1 in 100 in 140 mEq Na/liter solution (to mask variations in serum sodium). Serum sodium concentrations were estimated on 1 in 100 dilutions in 5 mEq K/liter solution (to mask serum potassium variations). The initial cell potassium content was calculated from the serum and whole blood potassium concentrations and the true P.C.V. For the whole blood estimation, 1 ml. of blood was partially hemolysed by mixing with 2 ml. of distilled water, centrifuged after a few minutes, the supernatant kept, and another 2 ml. of water added to the cell deposit. Finally the whole was mixed together to give a 1 in 5 dilution of hemolysed blood from which a 1 in 100 dilution was made in 140 mEq Na/liter solution. The initial cell-sodium content was measured by centrifuging blood as for P.C.V. estimations, removing all the serum and the buffy coat, carefully washing traces of serum away from the sides of the tube and the top of the red cell column with 5 per cent glucose in distilled sodium-free water, and then removing any dregs of the solution together with the top 2 to 3 mm. of the cell column. The remaining cell column, free from any supernatant fluid, was mixed to distribute the trapped serum evenly, the proportion present being calculated from the cell-column height,\(^2\) and 1 ml. pipetted off to make a 1 in 20 hemolysed dilution in distilled sodium-free water. The result was then corrected for the trapped serum sodium. The accuracy of the above estimations was determined by running a series of tests in duplicate. The results were:

| Test            | Range of Values (mEq/liter) | Confidence Limits (±%)
|-----------------|-----------------------------|------------------------
| Serum potassium | 3.8–5.6                     | ± 6%                   
| Serum sodium    | 113–164                     | ± 2%                   
| Cell potassium  | 91–125                      | ± 4%                   
| Cell sodium     | 7.3–12.2                    | ± 5%                   

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The changes in cell sodium and potassium during incubation were calculated from the changes in the serum concentrations and in the true P.C.V., allowing for the cations released by hemolysis. Firstly, the sodium and potassium contents per liter cells were calculated with reference to the initial cell volume to give the absolute gain or loss per liter fresh cells, and secondly, the sodium and potassium concentrations per liter cell water at 0, 24, and 48 hours were calculated with regard to changes in cell volume and their causal changes in cell water content. In fresh sera, sodium and potassium concentrations per liter serum water were calculated by taking the serum water to be 93 per cent by volume (deviations from this for normal sera are almost certainly less than ±1.5 per cent\(^1\)); in incubated sera, allowance was made for alterations in the serum water due to changes in the true P.C.V. and the release of hemoglobin by hemolysis (density of hemoglobin = 1.35\(^\circ\)).

**Cell Water Content**

This was measured by drying at 105\(^\circ\)C. a weighed 1 ml. sample of centrifuged and supernatant-free fresh cells, prepared as for cell sodium estimations. The changes in cell water during incubation were calculated from the cell volume changes.

**Percentage Cation Efficiency**

The various bloods that have been tested have had true packed cell volumes varying from 16.5 per cent to 48 per cent; consequently the individual results of cation changes cannot be compared directly. For example, if during incubation the cell sodium content in a normal and an anemic blood rises by equal amounts, the serum sodium concentration will fall less in the anemic blood than in the normal blood and so sodium will continue to diffuse more rapidly into the cells of the anemic blood than into the cells of the normal blood. Hence at the end of the experiment the cells of the anemic blood will have a higher sodium content than the cells of the normal blood simply as a result of the anemia. Similarly, the rate of diffusion of potassium out of incubated red cells will be affected by the initial cell potassium level and by the rate of increase in serum concentration which depends in turn on the relative volume of serum. In order to compare results the percentage efficiency

![Diagram](https://example.com/diagram.png)

Fig. 1.—The calculation of a cell-sodium efficiency (see text).
for sodium and potassium was calculated for each blood. Red cells can be regarded as 100 per cent efficient if their cation contents remain unchanged during the period of incubation, the active expulsion of sodium from the cells counter-balancing the passive diffusion of sodium into them and the potassium content also remaining unchanged. A measure of the efficiency is given by: Difference between the extracellular and intracellular cation concentrations per liter water × Time. (The cation concentration per liter water is the effective concentration as, especially in the red cell, protein occupies a significant proportion of available space.) One hundred per cent efficient values were calculated for both sodium and potassium and compared with the values calculated from the observed changes in cell and serum cation concentrations per liter water. For example, in a theoretic case (fig. 1), the area AB × AF or, as AB = 1, the value AF represents the efficiency of the cells in maintaining the cell and serum sodium concentrations at their initial levels for one day, and 2AF the efficiency for two days; these values thus being the 100 per cent efficiency values for 24 and 48 hours respectively. The areas ADGF (given by \( \frac{AF + DG}{2} \)) and ADEHGF (given by \( \frac{AF + DG + DH + EH}{2} \)) represent the observed efficiency for one and two days respectively in an actual experiment; hence the percentage sodium efficiency for these cells would be \( \frac{AF + DG}{2} \times 100 \) AF and \( \left( \frac{AF + DG + DH + EH}{2} \right) \times \frac{100}{2AF} \) at 24 and 48 hours respectively. In some experiments the areas to be estimated are of different shape but the calculation is similar in principle.

**Experiments on the Effect of Glucose**

The initial glucose levels (nonfasting) in the normal bloods ranged from 90 to 129 mg./100 ml. blood; and experiments showed a glucose disappearance rate of 15 to 20 mg./hour, which agrees with other estimates. Similar results were obtained on the abnormal bloods and it is a safe assumption that the glucose contents of all bloods had disappeared or fallen to very low levels by about 8 hours.

Further experiments were carried out to study the effect on the red cell changes of the continued presence of glucose throughout the 48 hours of incubation. A duplicate set of four 5 ml. bottles was prepared by adding to each bottle 0.15 ml. of sterile 10 per cent glucose and then 3 ml. of defibrinated blood. This raised the glucose concentrations to about 500 mg./100 ml. blood (range: 480 to 600; mean 516 mg. per cent). After 48 hours' incubation the concentrations ranged from 149 to 347 mg./100 ml. blood. The glucose estimations were done by a micromethod and the glucose used calculated in Gm. per liter red cells, using the true P.C.V. and ignoring the presence of leukocytes.

It was found experimentally that the maximum glucose concentration in the red cells was not attained until the blood had stood at room temperature for about 1 hour. Consequently all samples were allowed to stand for this time before any tests were done. It was noted with all types of red cells that the P.C.V. did not show the expected fall after the addition of glucose solution and it was calculated that the cells had increased in volume by 2 to 3 per cent as a result of the increased glucose concentration. The glucose addition also slightly increased the osmotic fragility of the cells, presumably as a result of this increase in cell volume and also because the internal osmotic pressure of such cells would be higher than normal.

**Experiments on the Glucose Metabolism of Red Cells**

Platelet-free and leukocyte-free blood was prepared by two successive centrifugations of defibrinated blood, discarding each time the buffy coat with subjacent red cells, and adjusting the final P.C.V. to about 40 per cent. (The total leukocyte count was never more than 500/cu. mm.) The glucose concentration was raised to about 150 mg./100 ml. blood by adding 5 per cent glucose. Then 2 to 3 ml. of blood in a 5 ml. bottle was rotated at 12 rpm at 37 C. All the bloods had a pH of 8.0 to 8.2, the optimum for red cell glycolysis being about pH 8.0. After 30 minutes' equilibration, samples for glucose estimation were taken every
hour for 5 hours and the rate of glucose breakdown was calculated in \( \text{mg. per cu. mm. blood per hour} \) from the slope of the straight line best fitted to all the points. Careful red cell and reticulocyte counts were done by standard methods.

**pH Determinations**

These were made with a Cambridge glass electrode pH meter on samples of blood immediately after their removal from the bottles in which they were incubated.

**RESULTS**

The results are described in three main sections: (1) changes in red cell volume and in cell cation content; (2) autohemolysis and changes in osmotic fragility; (3) glucose metabolism of red cells. They are arranged in this way for the purpose of clarity, as when the results as a whole were reviewed, the results described in section 1 appeared to be inter-related, as did those in section 2. In table 1 are given the summarized data obtained from twelve normal subjects and nineteen cases of congenital hemolytic anemia and hemolytic traits, and in table 2 are given the results obtained on the same bloods with added glucose.

**Changes in Red-Cell Volume and in Cell Cation Content**

**Normal blood.** Initially the normal red cells had cation concentrations of 8 to 12 mEq. sodium and 100 to 114 mEq. potassium per liter cells and a water content of 70.5 to 72 per cent by volume.* These values agree well with Maizels' results\(^5\) of 10 to 15 mEq., 101 to 110 mEq., and 69 to 72 per cent, respectively. During the first 24 hours' of incubation the cells underwent an average increase in mean cell volume of 28 per cent, but during the second 24 hours they decreased in volume to an average of 7 per cent above the initial volume. The cell sodium content increased progressively; at 24 and 48 hours it had risen by an average of 47 and 53 mEq. per liter cells, respectively. The cell potassium content fell progressively by an average of 10 and 55 mEq. per liter cells during the 24 and 48 hours, respectively. These mean changes for normal cells are shown graphically in figure 2, and it is apparent that the M.C.V. changes followed the changes in the total sodium + potassium content. After 24 hours' incubation, the cells had gained more sodium than they had lost potassium with the result that the total cation content had increased by 31 per cent. Water entered the cells to maintain the osmotic pressure and increased the cell volume. During the next 24 hours the potassium loss exceeded the sodium gain and the total cation content decreased to its initial value; the cells lost water and decreased in size.

When all the results of percentage changes in M.C.V. were plotted against the percentage changes in \( \text{Na} + \text{K} \) content per liter cells as in figure 3, the points were shown statistically to have a straight line relationship, there being no significant difference between the slopes of the 24 and 48 hour regression lines \((p = 0.6 \text{ to } 0.5)\). The 24 hour line passes almost through the origin and, having a slope of 1.05 which is not significantly different from 1, shows that the M.C.V. changes were proportional to the total \( \text{Na} + \text{K} \) changes. The 48 hour line does

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* Defibrination of blood with its consequent aeration produces blood of about pH 8.0. The red cells shrink slightly (about 5 per cent) and their cation content increases by a similar amount.\(^17\) A pH of 8.0 is not outside the range (6.6 to 8.2) in which the cation-controlling mechanism can function.\(^18\)
| Case number | Type of red cell | Observation | True P.C.V. (%) | M.C.H.C. (%) | % change in M.C.V. | % haemolytic | Osmotic fragility | % NaCl giving 1% haemolysis | Cell cation concentration (mEq/liter cells) | Total % change in Na+K content | Cation concentration (mEq/liter water) | Percentage cation efficiency |
|-------------|-----------------|-------------|----------------|--------------|------------------|--------------|-----------------|----------------------------|---------------------------------|-----------------------------------|---------------------------------|
| 1-12        | Normal          | Max.        | 43.5 ± 36       | 2.0          | +40             | +11          | 0.0             | 0.5            | 3.5               | 0.52               | 0.73               | 0.85               |
|             |                 | Min.        | 41.5 ± 32       | 0.2          | +13             | -3           | 0.0             | 0.4             | 0.47              | 0.60               | 0.74               |
|             |                 | Mean        | 43.5 ± 34       | 1.0          | +28             | +7           | 0.2             | 1.5             | 0.49              | 0.64               | 0.81               |
| 13-22       | Pre-apl. bcr. sph. | Max.        | 37.5 ± 37       | 1.7          | +6              | +10          | 0.4             | 7.5             | 0.50              | 0.83               |
|             |                 | Min.        | 20.5 ± 34       | 1.7          | +6              | +10          | 0.4             | 7.5             | 0.50              | 0.83               |
|             |                 | Mean        | 30.5 ± 37       | 1.7          | +6              | +10          | 0.4             | 7.5             | 0.50              | 0.83               |
| 14, 19-21   | Pre-apl. bcr. sph. | Mean        | 28.5 ± 37.5     | 3.7          | +22             | +1           | 0.9             | 0.68            | 0.71               |
|             |                 | Mean        | 38.5 ± 35.5     | 3.7          | +29             | -1           | 0.5             | 0.60            | 0.51               |
| 22          | Pre-S. Id. A.H.A. | 28.5 ± 34.5   | 47             | +11           | -10            | 6.4          | 0.80            | 18.1            |

**Table 1.** Red Cell Changes During Incubation of Whole Blood
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<tr>
<th></th>
<th>Hered. ellip.</th>
<th>Hered. ellip. with H.A.</th>
<th>Mean (2 obs.)</th>
<th>Mean (2 obs.)</th>
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<td>0 0.2 1.6 0.48 0.63 10 30 50 102 89 38 112 +27 ± 19 14 50 80 144 80 58 117 42.1 89 70 75 53</td>
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</table>

* Calculated from observed P.C.V.
† Mean of four cases.
‡ Percentage hemolysis in 0.85 per cent NaCl.
§ Pre- and post-spl. her. sph. = pre- and postsplenectomy hereditary spherocytosis; pre. S. Id. A.H.A. = pre-splenectomy idiopathic acquired hemolytic anemia; congen. non-sph. types 1 and 2 = congenital nonspherocytic hemolytic anemia, types 1 and 2; hered. ellip. = hereditary elliptocytosis; H.A. = hemolytic anemia.
### Table 2.—Red Cell Changes During Incubation of Whole Blood with Added Glucose

<table>
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<tr>
<th>Case number</th>
<th>Type of red cell$^\dagger$</th>
<th>Observation</th>
<th>Glucose used (Gm. liter red cells)*</th>
<th>% change in M.C.V.</th>
<th>% auto-hemolysis</th>
<th>Osmotic fragility % NaCl giving 1% hemolysis</th>
<th>Cell cation concentration (mEq/liter cells)</th>
<th>Cation concentration (mEq/liter water)</th>
<th>Percentage cation efficiency</th>
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* W.B.C. ignored. $^\dagger$ Mean of four cases. $^\ddagger$ Percentage hemolysis in 0.85 per cent NaCl. $^\S$ Red cell types as in table 1.
Fig. 2.—Mean autohemolysis, and P.C.V. and cell cation changes during the incubation of blood from normal subjects. The P.C.V. values have been corrected for the amount of autohemolysis.

Fig. 3.—The relationship between the percentage changes in M.C.V. and those in the total cell Na + K content during the incubation of blood from normal subjects (0 = 24 hour values; + = 48 hour values). For the regression line of the 24 hour values (——), \( p = 0.02 \) to 0.01 and slope = 1.05; for that of the 48 hour values (----), \( p = 0.02 \) to 0.01 and slope = 1.36. The mean 24 hour points (△) and mean 48 hour points (▲) for cases 27 and 28 (type 1 nonspherocytic red cells) are also shown.
not pass through the origin, the M.C.V. equalling the initial value when the
cation content was -10 per cent.

The pH of the fresh defibrinated bloods ranged from 8.1 to 8.2; after 24 hours' 
incubation and the conversion of glucose to lactic acid, it varied from 7.7 to 7.8 
and after 48 hours, from 7.7 to 7.9. The pH determinations on the abnormal 
bloods did not differ significantly from these figures.

Normal blood with added glucose. The continued presence of glucose greatly 
affected the red-cell changes as shown in figure 2. There was only a small po-
tassium loss; this was exceeded by the sodium gain so that the total Na + K 
content rose slowly during the whole 48 hours with a parallel increase in cell 
volume. The regression line for the 24- and 48-hour values of the percentage 
increase in M.C.V. and in Na + K content was a straight line which passed 
through the origin and had a slope of 0.92 (not significantly different from 1; 
\( p = 0.4 \) to 0.3). The smaller cation changes gave higher figures for the per-
centage cation efficiencies than those obtained without added glucose; in fact, 
in four cases the 24 hour sodium efficiencies were 100 per cent. This improved 
maintenance in the cell cation composition in the continued presence of glucose 
was to be expected in accordance with the previously mentioned work 
of Maizels.\(^{16-18}\)

The continued breakdown of glucose to lactic acid naturally caused the pH 
to fall lower than before; at 24 hours the pH ranged from 7.3 to 7.4 and at 48 
hours from 6.9 to 7.1. Again the pH values obtained with abnormal bloods did 
not differ significantly from these ranges.

![Graph showing autohemolysis and P.C.V. and cell cation changes during the incubation of blood from cases of hereditary spherocytosis before splenectomy. The P.C.V. values have been corrected for the amount of autohemolysis.](image-url)
Hereditary spherocytosis. The patients have been grouped into two categories: ten patients were studied before splenectomy; four were studied both before and after splenectomy, the latter observations being made from two to six weeks after the operation.

Before splenectomy. The composition of presplenectomy hereditary spherocytes was found to differ from that of normal cells: the mean corpuscular hemoglobin concentration (M.C.H.C.) was higher (34 to 41 per cent), the water content was lower (65 to 69 per cent), and the total Na + K content lower (87 to 106 mEq./liter cells) due to a lower potassium concentration (75 to 96 mEq./liter cells). These figures agree well with Maizels’ results\textsuperscript{15} of M.C.H.C. 31 to 39 per cent (corrected for new Haldane standard of 14.8 Gm./100 ml. = 100 per cent), water 65 to 69 per cent, and potassium 72 to 92 mEq./liter cells. Maizels pointed out that the hemoglobin and water contents of red cells varied inversely and that the ionic content was dependent on the water content.

The cell sodium increases were more varied than with normal cells but the mean increase was similar and the mean sodium efficiency was almost identical. The cell potassium losses were greater than with normal cells; in eight of the fifteen observations the cell potassium concentration per liter water had fallen to a lower level than the serum concentration at the end of 48 hours’ incubation. The large potassium losses were partly due to the bloods being anemic, because when the potassium efficiencies were calculated, the mean 24 hour efficiency did not differ significantly from the normal (p = 0.2 to 0.1) and the 48 hour efficiency was just significantly lower than normal (p = 0.05 to 0.02).

The mean results for the ten presplenectomy cases have been plotted in figure 4, and again it is apparent that, as for normal cells, the changes in M.C.V. follow those in the total Na + K content. The individual results at 24 and 48 hours are plotted in figure 5 and they were found statistically to have straight line relationships. The 24 hour line is not significantly different from that for normal cells (p for slope and for mean = 0.4 to 0.3), but it is of interest that the line does not pass through the origin, being similar in this respect to the line for normal cells at 48 hours. Furthermore, the spherocyte 48 hour line is significantly different from that of normal cells (for slope, p = 0.01 to 0.001, and for mean, p = <0.001), there being in many instances small M.C.V. changes but large cation losses.

After splenectomy. The results obtained with the four cases studied before and after splenectomy showed that as a result of the operation, the M.C.H.C. fell from 37.5 to 35.5 per cent, and the total cell Na + K content rose from 102 to 111 mEq./liter cells in association with a rise in cell water from 67 to 69 per cent. Similar changes were noted by Maizels.\textsuperscript{15} The M.C.V. and cation changes observed during incubation of blood taken after splenectomy were not significantly different from the changes before splenectomy.

Hereditary spherocytes with added glucose. As with normal cells the continued presence of glucose improved the cation efficiencies of hereditary spherocytes. The sodium efficiencies were increased by a normal amount, the mean 48 hour value being not significantly different from that with normal cells and glucose (p = 0.2 to 0.1). The cell potassium losses were improved, but they were still large and caused the M.C.V. to fall slightly during the second 24 hours (figure 4).
instead of rising steadily as with normal cells. These losses were again partly
but not wholly due to anemia as the mean 48 hour efficiency was significantly
lower than the normal ($p = 0.01$ to $0.001$). As before, the M.C.V. and total
Na + K changes had straight line relationships. Both the 24 and 48 hour regression
lines had slopes which were not significantly different from 1 (1.2 and 1.6
respectively) and both passed to the left of the origin as did the 48 hour line for
normal cells without added glucose (figure 3).

Observations made after splenectomy yielded similar results.

**Idiopathic acquired hemolytic anemia.** One case of acquired hemolytic anemia
with warm autoantibodies and marked spherocytosis was studied. The initial
cell cation composition was similar to that in hereditary spherocytosis; the
potassium content was low and the cation efficiencies were normal. The cell
volume changes followed those in the total cation content. With added glucose
the sodium efficiency was only moderately improved and the potassium efficiency
was unaltered. The cell volume changes were also unaltered.

**Hereditary elliptocytosis.** The patient described as case 24 was an adult with
elliptocytosis and signs of hemolytic anemia (table 1). Her father, case 23, was
found to have symptomless elliptocytosis. The father's red cells had a normal
cation content and behaved normally on incubation; the low sodium efficiency
is not thought to be significantly abnormal. On the other hand the daughter's
red cells had a low initial potassium content, and a low potassium efficiency which
resulted in a small but continuous fall in the M.C.V. The addition of glucose
improved the cation efficiencies by normal amounts, the potassium remaining
low and the sodium rising to within the normal limits from a slightly low level.

Case 26, described as case 11 by Dacie, et al., is a 4 year old boy who had a
severe hemolytic anemia of obscure origin in infancy. Splenectomy caused a
marked clinical improvement. This patient's blood was studied after splenec-
tomy. The blood film showed numerous fragments of red cells and microsphero-
cytes. Case 25 is the 6 year old brother of case 26; his red cells are moderately
elliptic in shape, as are the cells of the boys' mother. The cell volume changes on
incubation of the bloods of both these boys were normal.

**Congenital nonspherocytic hemolytic anemia, type 1.** This type of atypical
congenital hemolytic anemia is represented by two boys, cases 27 and 29, each
having a mildly affected mother, cases 28 and 30. Case 27 was described as case 5
by Dacie, et al. Their red cells varied slightly in size with occasional macrocytes
and oval cells; the blood of both the affected mothers showed similar but less
marked changes. In both cases 27 and 28 the cell cation contents and efficiencies
were normal and there was a normal relationship between the M.C.V. and cation
changes (fig. 3). The cell potassium losses were slightly greater than normal,
causing the M.C.V. values at 48 hours to be slightly lower than normal, but these
losses were due to the presence of anemia. The M.C.V. changes were also normal
in cases 29 and 30. Glucose had a normal effect on the cell volume and cation
changes.

The blood of the father of case 29 was normal in every way; the father of case
27 could not be examined.

**Congenital nonspherocytic hemolytic anemia, type 2.** This second type of atypical
congenital hemolytic anemia is mainly distinguished from the first by a striking
difference in behavior of the red cells during incubation. Two cases and their relatives have been studied; case 31 is a 30 year old woman and case 32 a 13 year old girl (cases 1 and 4 of Dacie, et al.). Both had their spleens removed in early childhood with no effect upon the hemolytic anemia. Blood films showed marked macrocytosis and very high reticulocytosis (table 1).

The results obtained with the red cells of case 31 were strikingly abnormal. The fresh cells had a low normal M.C.H.C. of 32 per cent with a high water content of 73.6 per cent by volume, and hence a high cation content of 132 with a high potassium content of 121 mEq./liter cells. These differences from normal probably reflect the high proportion of reticulocytes which owe much of their larger volume to water. During incubation (fig. 6) the sodium gain by these cells and the sodium efficiency were normal, but the potassium loss was very large and led to a low efficiency even when corrected for the influence of anemia. Although the total Na + K content decreased greatly, the M.C.V. had not altered at 24 hours and at 48 hours it had only decreased by a little (fig. 5). With added glucose it was surprising to find that the M.C.V. changes and the cation efficiencies were not altered at all.

The blood of the father of case 31 could not be examined; that of her mother is normal in every way.

In case 32 the hemolytic process in vivo was less severe than in case 31. The changes in vitro were also found to be less remarkable, but they were still markedly abnormal. The fresh red cells had a high total cation content with a high water content of 73 per cent, but in contrast to case 31 the M.C.V. changes and cation efficiencies on incubation were within the normal range. Glucose, however, did not improve the cation efficiencies.

Case 33, the mother of case 32, had a persistent and unexplained mild anemia

![Figure 5](https://www.bloodjournal.org)
and reticulocytosis, but her red cell morphology, composition, and behavior on incubation both with and without added glucose were normal. The father's blood was normal in every way.

**Autohemolysis and Changes in Osmotic Fragility**

Normal blood. After 24 hours' incubation normal red cells had undergone an average autohemolysis of 0.2 per cent; at 48 hours autohemolysis averaged 1.5 per cent. This progressive lysis was not associated with a progressive increase in cell volume but, as described above, with an initial increase followed by a decrease in cell volume to near the initial value. On the other hand, the progressive lysis can be compared with the progressive increase that was observed in the osmotic fragility of the most fragile cells. The mean NaCl concentration causing 1 per cent lysis rose by 0.15 per cent and 0.17 per cent during the first and second 24 hour periods respectively. (Individual 24 hour fragility results are shown in figure 7 together with the autohemolysis at 48 hours.) The median cell fragility (M.C.F.) reflected the M.C.V. changes in that its 24 to 48 hour increase was always less than the 0 to 24 hour increase and often the 48 hour value was less than the 24 hour value; but the M.C.F. values were not correlated with autohemolysis.

Normal blood with added glucose. The preservation of red cells by glucose in vitro, first described in 1916 by Rous and Turner, was manifest once again in a marked reduction of the autohemolysis by the addition of glucose (fig. 8). In six of the eleven experiments there was no lysis at all during the 48 hours' incubation.

**Figure 6.** Mean autohemolysis, and P.C.V. and cell cation changes during the incubation of blood from case 31 (type 2 nonspherocytic congenital hemolytic anemia). The P.C.V. values have been corrected for the amount of autohemolysis.
The addition of glucose to the fresh blood slightly increased the red cell fragility (see Methods), the mean NaCl concentration causing 1 per cent lysis rising from 0.49 per cent to 0.52 per cent. After 24 hours' incubation, the mean NaCl concentration causing 1 per cent lysis was 0.67 per cent, the increase (0.03 per cent) above the figure for normal cells without added glucose (0.64 per cent) being the same as that resulting from the addition of glucose to fresh blood. At 48 hours this increase was 0.02 per cent NaCl. Hence it is clear that the added glucose had no effect on the increase in fragility due to incubation.

Hereditary spherocytosis. Before splenectomy. In the case of hereditary spherocytosis it was even more obvious than with normal cells that autohemolysis was not due to progressive swelling of the cells. The 24 hour figures for autohemolysis and M.C.V. changes were not very different from the normal, but at 48 hours when the average M.C.V. of the surviving cells had returned to its initial value, the average lysis was about twelve times the normal. As with normal cells the lysis can be compared with the progressive increase in fragility of the most fragile cells. After 24 hours' incubation, the mean NaCl concentration causing 1 per cent lysis had risen from 0.60 per cent to more than 0.85 per cent (fig. 7), this latter solution causing a mean of 4 per cent lysis. As with normal cells also,
the shape of the fragility curves seemed unimportant, although the M.C.F. again reflected the M.C.V. changes.

After splenectomy. As in previous reports, splenectomy resulted in the disappearance of the most fragile cells, the mean NaCl concentration using 1 per cent lysis of fresh cells falling from 0.68 per cent to 0.60 per cent (the M.C.F. increased slightly from 0.474 per cent to 0.477 per cent NaCl). In blood incubated for 24 hours, the most fragile cells were only slightly less fragile than those in the bloods taken before splenectomy. However, this was associated with a decrease in the mean 48 hour autohemolysis from 19 per cent to 9 per cent.

Hereditary spherocytosis with added glucose. As with normal cells the initial M.C.V. and osmotic fragility were slightly increased by the addition of glucose. The autohemolysis was again markedly reduced, the mean falling from 17.5 per cent to 3.5 per cent (fig. 8). The 24 hour fragility curves showed a striking alteration; although the M.C.F. was usually slightly higher as a result of the added glucose, the fragility of the most fragile cells was in all cases lower than that in the blood without added glucose (tables 1 and 2), except in one case when it was equal in both bloods. Hence, allowing for the fragility increase due to the added glucose, the real decrease in fragility is probably more than this observed decrease. Thus the addition of glucose to spherocytes materially reduces both the autohemolysis and the increase in fragility on incubation, but neither is restored to normal.

Observations made after splenectomy yielded similar results.

Idiopathic acquired hemolytic anemia. The blood of case 22 showed a markedly

Fig. 8.—The effect of added glucose on the 48 hour autohemolysis of different types of blood. (In the absence of any effect, points would fall on the diagonal line.) Key as for figure 7. Note trebled scale for normal range of autohemolysis.
increased autohemolysis which was associated with 18 per cent lysis of the cells in 0.85 per cent NaCl after 24 hours' incubation. But in contrast to hereditary spherocytosis both these features were not decreased by the addition of glucose (figs. 7 and 8).

**Hereditary elliptocytosis.** The rates of autohemolysis in cases 23, 24 and 25 were normal, as were the initial osmotic fragilities and the fragilities after incubation of the bloods for 24 hours. As with normal blood the addition of glucose did not affect the fragility changes. In contrast to the normal, however, the addition of glucose did not reduce autohemolysis to the normal level (fig. 8).

The initial osmotic fragility of the cells of case 26 was increased above normal, microspherocytes being present. On incubation of the blood there was a markedly increased autohemolysis, and after 24 hours' incubation the most fragile cells were lysed in 0.85 per cent NaCl. Glucose significantly reduced the rate of autohemolysis during the second 24 hours of incubation but had no effect on the osmotic fragility at 24 hours.

**Congenital nonspherocytic hemolytic anemia, type 1.** In all four cases the initial and 24 hour osmotic fragilities were normal, as were the rates of autohemolysis (fig. 7). The addition of glucose had a normal effect on the fragilities, but the autohemolysis was not reduced to the normal level of less than 0.2 per cent at 48 hours (fig. 8). The blood of the father of case 29 showed normal rates of autohemolysis both with and without added glucose.

**Congenital nonspherocytic hemolytic anemia, type 2.** Incubation of the blood of case 31 caused a markedly increased autohemolysis and there was a very great increase in osmotic fragility. The osmotic fragility of unincubated blood was slightly reduced but after 24 hours' incubation 0.85 per cent NaCl solution caused a mean of 6 per cent lysis (fig. 7). These changes were taking place while the cells, as mentioned previously, were shrinking slightly and not increasing in volume at all. As with the cell volume and cation changes, glucose did not improve the autohemolysis (fig. 8) or the fragility changes.

Autohemolysis and fragility changes were increased in the blood of case 32; as with case 31, the addition of glucose was without effect. It is of interest to note that in both these cases the degree of autohemolysis was greater at 24 hours than with the hereditary spherocytes.

The bloods of the mother (case 33) and of the father of case 32 behaved normally on incubation both with and without added glucose. It is thought that case 33 has a mild form of the anemia from which her daughter is suffering, but that the present technics are unable to detect the mild red cell defect.

**Experiments on the effect of rotation.** A few experiments were carried out to determine the effect on autohemolysis at 37 C. of continuous rotation of the blood samples at 2 rpm. No marked effect was found. Autohemolysis was moderately reduced with normal cells and slightly reduced with type 2 nonspherocytic cells. The effect on hereditary spherocytes was variable; sometimes the lysis was slightly increased, perhaps reflecting a raised mechanical fragility.

**The Glucose Metabolism of Red Cells**

Mammalian red cells derive their required energy mainly, if not entirely, from the breakdown of glucose into lactic acid according to the Embden-Meyerhof
AUTOHEMOLYSIS DURING INCUBATION

glycolytic scheme. The rate of glucose breakdown is independent of the glucose concentration if this is below 500 mg./100 ml. blood and is the same in aerobic as in anaerobic conditions. The oxygen consumption of mature red cells is minute (about 0.03 cu.mm. O2/mg. dry weight/hour); that of reticulocytes is about 30 to 50 times greater. In the experiments described above the fresh bloods were fully oxygenated by the defibrination and during the 48 hours' incubation the oxygen consumption was dependent on the number of reticulocytes and leukocytes and the amount of glucose available as substrate. The degree of oxygenation does not affect red cell glycolysis and its dependent cation-controlling mechanism; it does, however, affect the utilization of glucose by reticulocytes and leukocytes. These facts probably explain the wide variation that was found in the estimates (table 2) of the amount of glucose used during incubation of the normal and pathologic bloods. The results in hereditary spherocytosis and in nonspherocytic hemolytic anemia (type 1) were comparable with the normal; that for the patient with acquired hemolytic anemia was high in accordance with the high reticulocytosis. However, the figures for the patients with non-spherocytic hemolytic anemia (type 2) were only slightly raised despite the even higher reticulocytosis.

Further experiments were done as described in Methods to obtain more accurate results (table 3). Blood containing a high proportion of reticulocytes was obtained from a case of paroxysmal nocturnal hemoglobinuria and two cases of acquired hemolytic anemia of the autoantibody type. It was thought probable that their red cells and reticulocytes had no inborn defect and that they would possess normal glycolytic powers. The rate of glucose breakdown for these and three normal bloods were determined, each result being expressed as an equation with x = μg. glucose used per million adult red cells per hour and y = μg. glucose used per million reticulocytes per hour (e.g. in case 1: 3.91 x + 0.04 y =

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* P.N.H. = paroxysmal nocturnal hemoglobinuria; A.H.A. = acquired hemolytic anemia.
Each normal blood was paired with one of the abnormal and the three pairs of equations solved for $x$ and $y$. The values for $x$ were 27.2, 30.8 and 31.4 (mean 29.8), and those for $y$ were 175, 190 and 170 (mean 178). This agreement for $x$ and $y$ was considered to confirm the supposition that the cells of the patients with noncongenital hemolytic anemia had normal glycolytic powers. Rates of glucose breakdown per cu.mm. of blood were calculated (table 3), taking $x = 30$ and $y = 180$.

The glucose disappearance in blood from two cases of hereditary spherocytosis and the two cases of type 2 nonspherocytic hemolytic anemia was measured and compared with rates of disappearance calculated in the same way. The results with the spherocytes showed a normal rate of glucose utilization; on the other hand, the cells of cases 31 and 32 used only 25 per cent and 30 per cent respectively of the theoretic total.

**DISCUSSION**

**Cell Volume and Cation Changes During Incubation and Their Relationship to Autohemolysis**

A normal red cell in a sufficiently hypotonic medium swells up to the critical volume of 160 per cent of its initial volume and then hemolyses. The greatest increase in cell volume observed in the present study as a result of the incubation of static blood was 152 per cent of the initial volume. In every case the 48 hour cell volume was less than the 24 hour volume; nevertheless, there was more autohemolysis during the second 24 hour period than in the first. In particular, the average M.C.V. changes at 48 hours for the two types of red cell undergoing marked autohemolysis, the hereditary spherocytes and the nonspherocytic type 2 cells, were 0 per cent and −8 per cent respectively. These observations disprove the theory that in incubated static blood the red cells lyse by progressive swelling. It is true that many types of red cell swell during the first 24 hours due to an increase in osmotically active substances. This is, however, not due to metabolic processes; it is due to the absence of a metabolic process—an absence of glycolysis and the associated expulsion of sodium ions which normally keep the intracellular sodium and water contents constant. Maizels has already demonstrated the ionic changes associated with the swelling of normal red cells; the same mechanisms apply to hereditary spherocytes and many other types of cells.

In most of the experiments in the present study the percentage changes in the M.C.V. during incubation were proportional to those in the total cell Na + K content. The regression lines relating these two changes passed through the origin in three instances: with normal cells at 24 hours and with normal cells with added glucose at 24 and 48 hours. On the other hand, the lines for normal cells at 48 hours and for spherocytes with and without added glucose at 24 and 48 hours showed no change in M.C.V. for a cation change of −10 to −16 per cent, the 48 hour spherocyte line showing in addition very little change in M.C.V. in proportion to the cation changes (fig. 5). It is thought that these discrepancies may be due to a degenerative change of unknown nature affecting the red cell and/or its membrane. With normal red cells this is apparent at 48 hours but is prevented by the presence of added glucose; with hereditary spherocytes it is
apparent at 24 hours and worse at 48 hours but no worse at 48 hours than at 24 hours in the presence of glucose. These features are well correlated with the autohemolysis, especially is case 31 (congenital type 2 non sphero cytic) where large cation decreases caused practically no volume changes and the autohemolysis was markedly increased at both 24 and 48 hours. The cell potassium losses are also well correlated with these phenomena, the moderate degeneration of spherocytes and the marked degeneration of the cells of case 31 being associated with slightly low and markedly low potassium efficiencies respectively. Nevertheless a large potassium loss is not always associated with increased autohemolysis; in case 24 with elliptocytosis and hemolytic anemia there was a low potassium efficiency but normal autohemolysis, and the same was observed with the blood of a girl with Mediterranean anemia.

Osmotic Fragility Changes During Incubation

The osmotic fragility changes during incubation parallel autohemolysis. In every case with an increased rate of autohemolysis, the most fragile cells after 24 hours' incubation had almost, if not entirely, lost their ability to swell in hypotonic media, some cells undergoing lysis in 0.85 per cent NaCl (fig. 7). This, however, was not due to an increase in cell volume. Fixed and stained films of incubated red cells showed that they became crenated spheres. This change occurred slowly with normal cells, but rapidly with the cells undergoing rapid autohemolysis. It thus appears that those cells which undergo lysis in 0.85 per cent NaCl, break up because their membranes have become shrunken and distorted and have lost their expansibility. This structural change is associated with an increased rate of autohemolysis.

The nature of the degenerative changes which affect incubated red cells is as yet undetermined. It is also unknown whether substances in the serum affect in any way the degenerative process. Lytic substances such as lysolecithin have been extracted from incubated serum and even from incubated red cells. In the serum before extraction these substances are probably combined with proteins or lipoids and are present only as weakly lytic complexes. To what extent, if any, the red cell changes described in this paper are influenced by these complexes is not known.

The Effect of Glucose

The continued presence of glucose, of well known benefit in the storage of blood in vitro, enables the expulsion of sodium from normal cells to continue and so improves the cation efficiencies. It is probable that the sodium efficiencies obtained on all the bloods without added glucose simply represent unopposed sodium diffusion into the red cells and do not measure any function of the cells apart from the permeability of their membranes. The improvement in the sodium

* Since this paper was submitted, further experiments have failed to demonstrate that serum incubated at 37 C. for 24 hours has a greater lytic activity than fresh serum. In fact, in experiments with the bloods of three normal persons, four cases of hereditary spherocytosis (two presple nectomy and two post splenectomy), and case 31, there was in all cases less autohemolysis after 48 hours' incubation at 37 C. in the mixture of fresh red cells and incubated serum than in the mixture of fresh red cells and fresh serum.
efficiency on adding glucose is thought to be an index of the control of sodium by the cells. The sodium metabolism of hereditary spherocytes and the cells of nonspherocytic congenital hemolytic anemia, type 1, has been shown to be normal. That of the hereditary elliptocytes (cases 23 and 24) is also considered normal as the slightly low sodium efficiencies were raised to within normal limits by the addition of glucose. In contrast, the sodium efficiency of the antibody-coated spherocytes of case 22 (acquired hemolytic anemia) was lower than normal in the presence of glucose; presumably the cells were irreversibly damaged by the antibody in such a way as to prevent the sodium-expelling mechanism from functioning normally. Similarly, the addition of glucose had no beneficial effect on the increased autohemolysis. The utilization of glucose, however, was apparently not affected.

The addition of glucose markedly reduced the autohemolysis of normal cells and the greatly increased autohemolysis of hereditary spherocytes. The observation by Ham and Castle\textsuperscript{15} that replacement of serum every two hours with fresh serum reduced the lysis of incubated spherocytes is at least partly explained by the glucose content of the fresh serum. Similarly, the increase or reduction of lysis caused by artificially concentrating or diluting blood with serum is explicable by the different amounts of glucose available to the red cells. The reduction of lysis by glucose is not due to a lowering of the pH by lactic acid production although increased acidity plays a part in this; in one experiment with hereditary spherocytes, for instance, the acidification of the blood by adding HCl to the serum reduced the lysis by about 40 per cent in contrast to the reduction of 70 to 95 per cent produced by glucose.

The autohemolysis after 48 hours of incubation of prespleenectomy hereditary spherocytes was markedly reduced by glucose, but at 24 hours the lysis was hardly reduced at all. It seems possible that the 24 hour lysis was at least mainly due to the presence of a few cells which were so degenerate that continued glycolysis was of no benefit.

The Glucose Metabolism of Red Cells

The utilization of glucose by hereditary spherocytcs was found to be normal, which is in accord with the beneficial effects of glucose on the autohemolysis and cation changes of these cells. The beneficial effects of glucose on the hereditary elliptocytes and on the type 1 nonspherocytic cells indicate that these types of cell also utilize glucose normally. In contrast, the type 2 nonspherocytic cells were not benefited at all by the continued presence of glucose and their glucose utilization was markedly reduced. It appears likely that the greatly increased lysis of these cells in vitro, and probably also in vivo, is related to their defective glucose utilization.

Relationship between Changes in Vitro and Hemolysis in Vivo

It is interesting to compare the effects of added glucose in vitro with the effects of splenectomy in vivo. Both cases 31 and 32 (type 2 nonspherocytic) have undergone splenectomy with no benefit to their anemia—glucose did not improve the in vitro lysis; case 27 (type 1 nonspherocytic) was also not improved by splenectomy—glucose only slightly improved the normal rate of autohemoly-
In contrast, the constant and complete relief of anemia following splenectomy in hereditary spherocytosis is matched by a marked effect of glucose in vitro. This led to the idea that in this disease the spherocytes, while stagnating in the congested splenic pulp, might develop cumulative damage leading ultimately to lysis because insufficient glucose was diffusing into them from the blood circulating through the spleen. However, glucose estimations have failed to substantiate this hypothesis; e.g., at the time of the splenectomy of case 21, the glucose concentration in capillary blood was 151, in arm vein blood 143, in splenic vein blood 139, and in splenic pulp blood 136 mg./100 ml. blood. It is possible that the blood obtained may have contained only a small proportion of stagnant blood as opposed to freely circulating blood. By taking further samples from other parts of the spleen, it was found that glucose disappears from the pulp blood at about 100 mg./100 ml. per hour at room temperature. The probably faster rate of consumption in vivo at 37 C. might cause the glucose concentration in stagnant blood to fall to low levels.

The Use of in Vitro Studies in Diagnosis

Finally, the procedures used in this work seem to be useful in diagnostic problems. The 48 hour autohemolysis in sterile defibrinated blood was constantly increased in cases of hereditary spherocytosis, as was the osmotic fragility of the most fragile cells after 24 hours' incubation. In agreement with Young, et al., it was found that mild cases of hereditary spherocytosis with only slightly increased fragility were more readily distinguished from normal subjects using the incubation fragility method. As also noted by Young, et al., it was found that the shape of the incubation fragility curve was unimportant, the point of commencing lysis being the diagnostic criterion. Study of the rate of autohemolysis, however, was found to be of further use; it was one of the main distinctions between two groups of cases of atypical congenital hemolytic anemia, classified in this paper as congenital nonspherocytic hemolytic anemia, types 1 and 2. The increased autohemolysis of type 2 was distinguished from that of hereditary spherocytes by the absence of the beneficial effect of added glucose; similarly, the lysis of type 1 cells could be distinguished from that of normal cells by the smaller effect of glucose. Moreover, these technics can be usefully applied to the differentiation of anemias of obscure origin from those due to congenital red cell defects. For instance, two women showing the slight spherocytosis often found in late pregnancy were recently investigated to exclude congenital hemolytic anemia; autohemolysis and incubation fragility were normal in both cases. Another patient with mild anemia, moderate jaundice, and splenomegaly was similarly investigated. The incubation results were normal, and glucose reduced the 48 hour lysis to 0.1 per cent. It was thought that these figures excluded a hemolytic process; liver biopsy later proved the existence of cirrhosis.

Summary

Observations were made on the changes in volume, osmotic fragility, and cation contents of red cells incubated in serum at 37 C. for 24 and 48 hours. The results show that spontaneous autohemolysis is not due to progressive swelling of the cells, but is probably due to degenerative changes in the cell membranes.
On incubation, normal red cells increase in volume during the first 24 hours due to a gain in sodium and water; the cells lose potassium but at a slower rate than they gain sodium. During the second 24 hours of incubation the loss in potassium exceeds the gain in sodium and the cells shrink to near their original volume. These cation changes and the autohemolysis are greatly reduced if glucose is present throughout the 48 hours of incubation.

Red cells from several different types of congenital hemolytic anemia were also studied; important deviations from the normal pattern were observed. In hereditary spherocytosis the rates of autohemolysis, of increase in osmotic fragility, and of potassium loss are greater than normal. The continued presence of glucose during incubation markedly retarded these changes.

In hereditary elliptocytosis the red cells behaved normally on incubation. In one case of elliptocytosis with hemolytic anemia, autohemolysis was normal but there was an increased potassium loss. In another patient with hemolytic anemia and increased osmotic fragility autohemolysis was greatly increased. In all these cases of elliptocytosis, glucose reduced the autohemolysis moderately but not to a normal degree.

Four cases of congenital nonspherocytic hemolytic anemia were studied. In two patients (type 1) autohemolysis, osmotic fragility and cation changes on incubation were normal; glucose had a normal effect on the fragility and cation changes, but only slightly reduced the autohemolysis. In the two other patients (type 2) autohemolysis, increase in osmotic fragility, and loss of potassium were markedly increased. Glucose did not retard any of these changes and it was found that the cells were unable to utilize glucose at the normal rate.

**SUMMARIO IN INTERLINGUA**

Esseva observate le cambios del volumine, del fragilitate osmotic, e del contito de cationes inn erythrocytas incubate in sero a 37 C. pro 24 e 48 horas. Le resultatos monstra que autohemolyse spontanea es debite non al tumescentia progressiue del cellulas sed probablemente a cambios degenerative in le membraees celular.

Sub incubation, erythrocytas normal augmenta br volumine durannte le prime 24 horas in consequentia de un augmentation de lor contento de sodium e aqua. Le cellulas perde potassium sed a un rapiditate inferior al rata de lor ganio de sodium. Durante le secunde 24 horas le perdita in potassium excede le ganio in sodium, e le cellulas se contrahe a quasi lor volumine original. Iste cambios cationie e le autohemolyse es grandemente reducite si glucosa es presente durante le 48 horas de incubation.

Erythrocytas ab plure diverse typos de congenite anemia hemolytic esseva etiam studiate. Hic importante deviationes del schema normal esseva observate. In spherocytosis hereditari le rataes de autohemolyse, de accrescimento in fragilitate osmotic, e de perdita de potassium es plus grande que normal. Le presentia continue de glucosa durante le incubation retardava iste cambios marcatemente.

In elliptocytosis hereditari le erythrocytas reageva normalmente. In un caso de elliptocytosis con anemia hemolytic, le autohemolyse esseva normal, sed le perdita de potassium esseva augmentate. In un altere patiete con anemia hemolytic e accrescite fragilitate osmotic, le autohemolyse esseva grandemente ac-
crescite. In omne iste casos de elliptocytosis, le addition de glucosa reduceva le autohemolyse moderamente, sed non usque al grado normal.

Quatro casos de congenite anemia hemolytic nonspherocytic esseva studiate. In un prime typo—representate per duo patientes—autohemolyse, fragilitate osmotic, e cambios de cationes sub incubation esseva normal. Le effecto de glucosa super le fragilitate osmotic e le cambios de cationes esseva normal, sed illo reduceva le autohemolyse solo mininimemente. In le secunde typo—representate per le altere duo patientes—autohemolyse, acercimento in fragilitate osmotic, e perdita de potassium esseva marcatamente augmentate. Glucosa non retardava iste cambios e il eseva constatate que le cellulas non poteva utilisar glucosa al rata normal.

REFERENCES

Autohemolysis and Other Changes Resulting from the Incubation in Vitro of Red Cells from Patients with Congenital Hemolytic Anemia

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