A QUANTITATIVE METHOD FOR THE DETERMINATION AND CHARTING OF THE ERYTHROCYTE HYPOTONIC FRAGILITY

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THE DETERMINATION of the degree of hemolysis of the red blood cells in hypotonic salt solutions ('hypotonic fragility') has continued to be of considerable interest since Duncan Johann, in 1867, and Malassez, in 1873, instituted the method of using salt solutions to detect the varying resistance of red cells to hemolysis. Since then, many new technics have been described and much controversial data regarding hypotonic fragility and its interpretation have been amassed. It is only in recent years that full recognition of the importance of this test as a measurement of red cell thickness has been realized.

It is the purpose of this paper to present a new technic for the measurement of hypotonic fragility, based first on the photoelectric determination of the degree of hemolysis and second, upon calculation of the differences in the degree of hemolysis occurring in successive dilutions of hypotonic salt solution. The graphic depiction of these differences, or hemolytic increments, has proved of great value in assessing the red cell population according to its thickness variation, (thickness in relation to diameter), thus indirectly allowing evaluation of the probable type of hemolytic process present.

I. REVIEW OF LITERATURE

So many methods for determining the hypotonic fragility have been described that a thorough review of the literature is extremely difficult. In 1867, Duncan Johann reported an increased fragility in salt solutions of the red blood cells of patients with chlorosis. He did not elaborate on his observations and apparently made no attempts to standardize his method.

Malassez, while attempting to devise a practical method for the counting of red blood cells, observed that cells from different individuals become hemolyzed at different time intervals in the diluting fluid used (a mixture of gum arabic with equal parts of solutions of sodium sulfate and sodium chloride). Malassez then described a quantitative method for the determination of red cell hemolysis consisting of the use of the 'Potain mixer,' (the original red blood cell counting pipet) and in the performance of red cell counts at different time intervals, thus determining the number of residual nonhemolyzed red cells. The time element was of paramount importance. Urcelay, Mallassez's pupil, used a sodium sulfate solution with a specific gravity of 1.010. He established a curve based on the number of nonhemolyzed erythrocytes at different time intervals and concluded that in
normal blood two groups of red cells were present: a smaller one of easily hemolyzed red cells and a larger one of cells which were more resistant to hemolysis.

Chanel used three different dilutions of sodium sulfate in water, substituting the factor of dilution for the time factor and performing red blood cell counts to determine the number of nonhemolyzed red blood cells. Simmel and Whitby and Hynes improved upon his technic. Maragliano (1885 et seq.) studied the resistance of erythrocytes to the action of heat, compression, exsiccation and human serum.

Hamburger, (1887 et seq.) originated the most widely used method, which was modified a number of times by himself and later by others. He used test tubes containing solutions of sodium chloride which varied in strength from 0.30 to 0.60 per cent, adding defibrinated blood which was measured by drops. The interpretation of the test was based on the discoloration of the supernatant fluid by free hemoglobin. Ribierre wrote a comprehensive critical review in 1903 summing up the progress made in the preceding thirty years. He modified Hamburger's technic by standardizing many details.

In the ensuing years, many more modifications of Hamburger's methods were introduced (Mosso, Viola, Widal, Abrami and Bruel, Ponder, Wiseman and Bierbaum, Lepeschkin, Daland and Worthley, Beebe and Hanley, Creed, Waugh and Asherman, Dacie and Vaughan, Hunter, Berk, Parpart). Waugh and Chase in 1928 described a method which combined the principle of counting unhemolyzed red cells in the hypotonic solutions-red cell mixture with the principle of measuring the amount of hemoglobin in the supernatant solution.

The following are the most important features which have been modified by different authors:

1. The type of anticoagulant used: at first, defibrinated blood or fresh blood without an anticoagulant was used; later, oxalated, and still later, heparinized blood came to be preferred.
2. The use of whole blood or washed red blood cells.
3. The amount of blood and hypotonic solutions employed.
4. The chemical composition, the number and strengths of the hypotonic solutions chosen. Although most workers used solutions of sodium chloride, others selected solutions of other salts, such as sodium sulfate. Simmel used six different salts, attempting to create a mixture similar to that existing in the blood. Other investigators used serum from the same patient diluted in different proportions with distilled water.
5. The method by which the hypotonic solutions and blood were measured, many authors still using the "drop" as the unit for either or both solutions and blood, others using carefully standardized pipets.
6. The method for determining the beginning and the end of hemolysis and the percentage of hemolyzed red blood cells in various dilutions of hypotonic solutions. Gallerani used the Fleischl hemometer to determine the percentage of hemolyzed erythrocytes. In an attempt to obtain more accurate results, Ponder devised differ-
ent methods to determine the degree of hemolysis: he first used a radiometer, later selenium and potassium photoelectric cells, and still later a Stufenphotometer. Waugh and Asherman were apparently the first to use a photoelectric colorimeter for the measurement of the amount of hemoglobin present in the supernatant fluid in each tube.

7. The method of reporting results and, by some authors, of plotting them in graphic form. Many of the methods require the simultaneous performance of a control test from a known normal subject.

With various changes in method, many different results have been obtained so that the data of any one author for normal and pathologic conditions are seldom comparable to those of another. In most instances, all one can say is that the hypotonic resistance of the red cells is normal, increased or decreased.

II. Method

A. Photoelectric determination of the degree of hemolysis

Our method is a modification of the photoelectric determination of erythrocyte fragility, as first described by Waugh and Asherman in 1938, and later modified by Hunter in 1940. The Evelyn technic for the determination of oxyhemoglobin is used. Since it is known that hemolytic systems are affected by small changes in the pH, temperature, oxygen and carbon dioxide contents, and by the age and the concentration of the red cells, we have attempted some standardization of these variants.

The stock solutions are prepared from freshly distilled water and chemically pure sodium chloride, using the analytic balance. Buffer salts are purposely omitted because the sodium or potassium ions present may affect the tendency of the erythrocytes to hemolyze. Solutions are prepared at percentage intervals of 0.04 between the concentration of 0.04 per cent and 0.20 per cent and between those of 0.52 per cent and 0.80 per cent sodium chloride. Between the concentrations of 0.20 per cent and 0.52 per cent the percentage interval between solutions is 0.02 per cent. The solutions are kept in glass stoppered bottles and the pH of sample solutions checked at two week intervals by the Beckman pH meter. The pH of the distilled water and the solutions was found to range between the two extremes of 5.5 and 7.0. When a variation from these two extremes occurred new solutions were prepared. The saline concentration of the solutions is checked by the silver nitrate method to insure its correctness. Solutions are made up in 500 cc. volume every four to six weeks, more frequently in the summer than in the winter, to compensate for greater evaporation, and are kept at room temperature. A series of 29 conical centrifuge tubes is set up in consecutive order, from 0 (i.e., distilled water) to 0.80 per cent NaCl. Using the same pipet, 10 cc. of each solution is transferred into the appropriately labeled centrifuge tubes. Errors resulting from dilution or concentration of solutions are minimal since these are pipetted always in the same order (see below) and a difference from one solution to the next is too small to result in a significant error.

From 2.5 to 5 cc. of blood are collected without stasis from a suitable vein, and
sufficient heparin is used to prevent coagulation for 4 hours.* The vial of blood is gently rotated to insure complete oxygenation. Exactly 0.02 cc. of whole unwashed blood is delivered into each solution of NaCl by means of a standardized and calibrated Sahli 20 cu.mm. hemoglobin pipet. After the blood has been pipetted into the salt solution, the pipet is filled once again with blood, which is discarded, and then again filled with blood for the next tube of salt solution.

This is done in order to avoid hemolysis or dilution of the blood sample which might result from the fact that the pipet had become "contaminated" by the salt solution. It is advisable, when pipetting both the solution and the blood, to do so always in the same order so that whatever error results from lowering or raising the saline concentration will always be in the same direction in all tests. Accordingly, we have pipetted the salt solutions by proceeding from the lower to the higher concentrations to avoid raising the saline concentrations, and we have pipetted blood by proceeding from the tubes in which no (or lesser) hemolysis is expected to occur to tubes in which greater hemolysis probably will take place.

The tubes are inverted several times, left for one hour at room temperature, and then centrifuged at 1500 r.p.m. for ten minutes. The supernatant fluid from each test tube is carefully decanted and read in the Evelyn colorimeter for hemoglobin content, using filter No. 540. Two perfectly matched absorption cells are used for all the readings. The first cell contains the supernatant fluid from the 0.80 per cent tube, which is used as the blank, since no hemolysis usually occurs in this dilution, and the second cell is used to match the consecutive dilutions against the blank. If there is any reason to suspect that hemolysis will take place, or has taken place at 0.80 per cent NaCl solution, a higher concentration of salt solution, i.e., 0.85 per cent NaCl solution or 0.90 per cent, may be used for the blank.

Bile pigments, or any other plasma constituents which might alter the light absorption, will not interfere with the reading since they are present in the same amount in all the other tubes. In any event, the use of the appropriate filter automatically eliminates the possible error deriving from the presence of chromogens other than hemoglobin.

Several of the technics thus far devised include a "correction" for anemia, which involves the use of a larger amount of blood or removal of a certain amount of plasma whenever anemia is present in order to include the same volume of red cells. In our experience, fragility curves in both normal and abnormal subjects, using different amounts of blood, yielded curves with irregular variations which were within the limit of experimental error. Alterations of erythrocyte fragility were by no means proportional to the degree of severity of the anemia. Bloods with the same degree of anemia from patients with different conditions showed distinctly different types of curves. For these reasons, and since the results of our tests are reported in terms of percentage of the total amount of red blood cells hemolyzed, "correction" for anemia did not appear necessary and was, therefore, not carried out.

* One drop of Roche Organon Heparin ("Liquaemin") as delivered from a No. 20 needle is used for each sample of blood.
The use of washed red blood cells rather than of whole, fresh, unmodified blood has been a highly controversial issue since Widal, Abrami and Brulé showed that increased hemolysis to hypotonic salt solutions could be demonstrated in some patients whose unwashed erythrocytes yielded a normal type of fragility. Several investigations later indicated that washing the cholesterol off the red blood cells surface, as with a solution of sucrose, would result in an increase of erythrocyte fragility, whereas the removal of lecithin with Brinkman's solution would result in an increased resistance. The cholesterol lecithin ratio was considered as very important in this regard.

The above results could not be duplicated by other workers, who were unable to find any alteration in red cell fragility by washing erythrocytes with physiologic solutions (Saslow). Ponder and Saslow established that washing rabbits' red cells with sodium chloride or glucose solutions isotonic with rabbits' plasma did not alter the volume of red blood cells. They stated that the concentration of such solutions should be of 1.12 per cent NaCl.

Ponder studied the inhibitory effect of blood serum on hemolysis, but these investigations were performed on fragility to hemolytic substances like saponin, bile salts and sodium hydroxide, where an interaction between the hemolytic agent and various plasma components could be expected. These results therefore could not be applied to hypotonic salt fragility.

In our laboratory, parallel fragilities performed on whole blood and on washed red blood cells showed somewhat less resistance in the latter, perhaps because of sodium chloride which was used for washing may be regarded as a slightly hypotonic solution. We finally concluded that the use of whole unmodified blood was preferable to that of "washed" blood since it eliminated one step of doubtful value and since "washing" might actually injure the red cells, thus making them hypotonically fragile. In certain cases of hemolytic anemia, greater erythrocyte fragility might possibly be demonstrated with washed than with unwashed red cells. This fact was first stressed by Widal, Abrami and Brulé. As yet we have been unable to confirm their results, although further studies on this point are in progress.

Variation in the fragility of the same subject from day to day was found, but this was never of sufficient magnitude to give a significant difference in the interpretation of the results. This confirms the findings of Whitby and Hynes in 1935.

B. Method for plotting the curve of hemolysis

By plotting the per cent of hemolysis along the ordinate, against the decreasing strengths of saline along the abscissa, a sigmoid curve results. From inspection of the fragility curves as obtained by our method and of those of other investigators, it was noted that definite irregularities occurred in the rate of progression of hemolysis from tube to tube no matter how carefully the tests were performed. It was believed that in certain pathologic conditions these irregularities might represent changes in the "thickness population" of the blood, not well demonstrated by the ordinary methods in use for graphic charting.

A method was therefore devised in which the "hemolytic increments," were calculated and charted. This was done by determination of the additional amount of hemolysis occurring in the successive tubes of hypotonic salt solution which
contained progressively diminishing saline concentrations. For example, if no hemolysis occurs at 0.56 per cent sodium chloride, and 10 per cent of the red blood cells hemolyze at 0.52 per cent, and 50 per cent of the red cells hemolyze at 0.50 per cent, the hemolytic increment (i.e., the increase in degree of hemolysis) is 10 in the 0.52 per cent solution and 40 in the 0.50 per cent solution. The hemolytic increments, when plotted, yield curves that are somewhat similar in configuration to those obtained with Price-Jones curves of red cell diameters. They are considerably more graphic and informative than those obtained with the ordinary methods for plotting photoelectric fragility.

The actual figures for drawing the new type of curve are obtained in the following manner:

1. By using conversion tables, change the photolometer readings into grams of oxyhemoglobin liberated.
2. Calculate the per cent of blood hemolyzed in each tube by dividing the hemoglobin reading in grams in the o tube (i.e., the total amount of hemoglobin) by the hemoglobin reading in grams in each tube.
3. Find the hemolytic increment by subtracting the percentage of blood hemolyzed in the first tube from that of the second, the second from the third, etc.
4. Draw a curve, plotting the percentage saline solutions on the abscissa and the hemolytic increments on the ordinate.

By proceeding from the observer's left to his right along the curve, it is seen that by adding the value at any one point to the values of all previous points (left to right) we obtain the total amount of red cells which have been hemolyzed at that concentration of sodium chloride. In other words, the value of the percentage of red blood cells hemolyzed, appearing in the curve at each concentration of sodium chloride expresses only the percentage of red blood cells which was hemolyzed at that particular concentration of sodium chloride and which had not been hemolyzed in the previous solutions.

If practically all cells tend to hemolyze at one point (isohemolysis) a sharply peaked monophasic curve results but if, on the other hand, the cells are hemolyzed over a wide range of hypotonicity (anisohemolysis) a biphasic or multiphasic curve results. The data obtained from determinations in both normal subjects and in those with various types of blood dyscrasias, revealed that certain diseases followed a more or less definite pattern with respect to the shape of the plotted curve of hemolytic increments. It appeared probable that the curves gave a graphic picture of the red blood cell thickness population, at least with the thickness diameter ratio as a principal factor.

In addition to the graphic representation of the hemolytic increments the following features have appeared to us to be of significance: (1) the concentration of the solution which shows beginning hemolysis; (2) the solution in which hemolysis is complete (since in a number of cases, both normal and pathologic, 100 per cent of the red blood cells hemolyze only in distilled water, we have concluded that the solution in which 90 per cent of the red blood cells hemolyze is a more significant index of total hemolysis); (3) the solution in which there is 50 per cent hemolysis, this solution being defined as indicating the "mean corpuscular fragility"; (4) the breadth of the curve; (5) the height of the highest hemolytic increment.
Mediterranean Anemia
Severe Form (Cooley's)

The curves are charted both by the "conventional" method and by that using hemolytic increments. The first curve (normal) is tall, monophasic and narrow-based with most hemolysis occurring between concentrations of 0.52 and 0.44 per cent NaCl. The second curve, (hemolytic anemia) is broader and with more than one peak. Hemolysis begins in physiologic salt solution and is practically complete at 0.52 per cent NaCl. The third curve (Mediterranean anemia) is low, broad and multiphasic with hemolysis beginning at 0.52 per cent NaCl, complete very close to distilled water.

Normal individuals almost invariably yielded monophasic curves. The breadth of the curve was less than 0.20 per cent; that is, most of the red blood cells (90–95 per cent) hemolyzed within solutions differing by only 0.20 per cent sodium chloride. None or very little hemolysis occurred in the 0.60 per cent solution. Less than 5 per cent hemolysis occurred in the 0.56 per cent solution.

The highest hemolytic increment occurred normally in one of the salt solutions between 0.52 and 0.44 per cent and represented 40 to 50 per cent of the red blood cells. The mean corpuscular fragility (the solution in which 50 per cent R.B.C. hemolyzed) was normally at 0.50 or 0.48 per cent. Ninety per cent of the red blood cells hemolyzed above the 0.42–0.40 per cent solutions. As already stated, in many subjects, both normal and pathologic, a further increase in hemolysis occurred at or close to the distilled water level. While no explanation for this can be offered, one can speculate that this is due to the presence of a certain number of "new" red cells, i.e., reticulocytes, which because of their unusual thinness are unusually resistant to hypotonic solutions.

Figures 1, 2 and 3 are examples respectively of a normal curve, a curve from a
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Fig. 4

Fig. 5
Figure 6

Fig. 6, 5, 6. Photoelectric Hypotonic Fragility Curves with the Simplified (7 tube) Method in a Normal Subject, a Subject with Hemolytic Anemia and a Subject with Mediterranean Anemia.

The curves show the range of hypotonic salt solutions in which hemolysis occurs with the typical shift to the "right" (leptocytosis) and to the "left" (spherocytosis) in the abnormal cases. These curves, although sufficient for most clinical diagnostic work, fail to give a detailed picture of the red cell population.

A case of acquired hemolytic anemia, and a curve from a case of severe Mediterranean (Cooley's) anemia. The normal curve shows all of the above mentioned characteristics; i.e., the level at which hemolysis first takes place, the width of the base of the curve, and the height of the highest peak. The conventional type of hemolysis curve is also given for comparison. The curve from the case of hemolytic anemia shows a definite shift to the "left" (i.e., increased hemolysis in relatively concentrated solutions of NaCl) and a tendency to a very high peak as well as to a rather narrow base. The third curve, a severe case of Mediterranean anemia, shows a markedly broadened base, extending practically from the level at which hemolysis first begins in a normal subject, down to the distilled water level. The mean corpuscular fragility and the point at which 90 per cent of the red blood cells hemolyze shows a marked shift to the right. There are no high peaks but many small ones, indicating not only a great diversity in red cell thickness, but (indirectly) a marked fault in the production of red blood cells of uniform degrees of thickness.
III. Simplified Method

After eighty cases had been performed in both normal and abnormal subjects, a simplified test for clinical purposes was devised. In this test the following solutions were used: 0.80, 0.56, 0.48, 0.44, 0.40, 0.32 per cent NaCl solution and distilled water. The 0.80 per cent solution was used as a blank since hemolysis of both normal and abnormal bloods did not usually occur in this solution. For the determination of total hemoglobin, distilled water was used. In the 0.56 per cent solution, normal subjects showed less than 5 per cent red blood cell hemolysis; a much higher percentage of hemolysis in this tube occurred in the various types of hemolytic anemia. The 0.48, 0.44, and 0.40 per cent solutions showed the greatest degree of hemolytic increment in normal subjects; i.e., 45 to 75 per cent, 15 to 35 per cent, and 2.5 to 15 per cent respectively. The 0.32 per cent saline gave hemolysis of close to 100 per cent red blood cells in normal subjects, but of lesser degree in the abnormal states with increased hypotonic resistance.

The results of this simplified method were plotted in curves in the same manner as outlined above for the complete test: Figures 4, 5 and 6 are examples of 3 curves obtained with the simplified "seven tube" method in a normal subject, a case of hemolytic anemia and a case of Mediterranean anemia, respectively. The different characteristics of the curves are obvious at a glance, with the shift to the "left" in the case of hemolytic anemia, and the shift to the "right" in the case of Mediterranean anemia.

Several simplified methods, utilizing only two to four tubes, have been devised in the past for the purpose of obtaining prompt information as to the fragility of the red blood cells (Lepeschkin, Berk, and Smith). The use of seven tubes appears to be simple enough to allow for relatively quick performance. It is obvious, however, that it does not give the regular types of curves seen with the complete test, nor details as to the variations in red cell population. It thus appears to be unsatisfactory for investigative purposes, although as a clinical test it appears to have definite merit.

IV. Discussion

Ponder made use of two types of curves in his extensive studies. He used the term "time dilution" curve to designate the curve obtained by plotting the time required for complete hemolysis against each dilution of the lysin used. The percentage of red cells hemolyzed was plotted against time in the second type of curve which he called "percentage hemolysis." He was more interested in hemolytic agents such as saponin, and sodium taurocholate, than in hypotonic salt solutions. Besides, he always introduced the element of time for the hemolytic reaction to occur, and his investigations were for the most part limited to normal erythrocytes. Unfortunately, his contributions have not as yet found widespread application in the clinical field.

Various investigators have questioned the importance and value of the shape of the curve obtained when quantitative methods for determining hemolysis are used. Thus Vaughan expressed the belief that the curve of hemolysis shown by plotting the concentration of saline against the degree of hemolysis, both being
expressed as a percentage, utilizes a method which is clearly unsatisfactory, 'since markedly different curves may lie in the same range.' Creed in 1938 wrote 'it was hoped that the form of a fragility curve constructed from the data obtained might show distinctive differences in various blood diseases and possibly in other conditions. For the most part these hopes have not been realized....' He also stated that there was a 'slight difference in the shape of the curve in pernicious anemia.'

According to Dacie and Vaughan, 'the shape of the curve or span of resistance ... is without great practical significance,' and '... study of the shape of the curve in pathological bloods ... is not in practice very useful.' In a later publication, however, Dacie paid more attention to the shape of the curves, classifying them as 'tailed,' 'diagonal' and of 'normal type.' Berk agreed with the above statements of Dacie and Vaughan.

Waugh and Asherman, as well as Hunter, plotted their results in curves. However, Waugh and Asherman did not attach much importance to them. Hunter wrote: 'If the technique has been carefully carried out the curve will ordinarily be sigmoid in character. In some instances, particularly in the case of blood showing increased fragility, the curve may show more than one maximal slope. The significance of this occasional variation from the usual curve, however, is by no means clear, and requires further investigation.'

Our results indicate that the shape of the curve obtained, particularly as modified by the use of the 'hemolytic increment' method, is of great importance. With our method of plotting, one can readily distinguish between bloods showing spherocytosis and bloods containing target (i.e., thin) red cells. What is perhaps more important is recognition of the concept that the changes in hemolytic increment from tube to tube indicate the hemolysis of different groups of red cells; i.e., differences in red cell population thickness. This in turn might indicate either different types of red cells from the standpoint of their production by the bone marrow or differences in the ages of groups of red cells, since it is our belief that the cells just delivered from the bone marrow, i.e., the reticulocytes, are thinnest, and the oldest red cells the most spherocytic. The reason for the fragility 'range' even in normal subjects is in all probability due to this age difference in red cells, the oldest ones hemolyzing at 0.56 per cent or thereabouts, the youngest at 0.40 or 0.42 per cent.

In normal subjects, a very sharp and regular type of curve is present indicating a relatively uniform type of red cell and of age thickness population. Little 'bumps' in the curve, particularly in the more hypotonic section of the curve may be due to the presence of a certain number of relatively thin reticulocytes.

In subjects with Mediterranean anemia, the marked irregularity in the curve of hemolytic increments from tube to tube almost certainly indicates a marked variation in the type of red cell produced by the marrow. This brings graphically to light the 'fault' in the red cell (and hemoglobin) production which appears to be at the basis of the disease. Thus, a careful reading of the hemolytic increment curves permits some insight not only as regards the variations in red cell population in given cases but also into the pathologic physiology of red cell formation.
itself. The use of the terms ‘‘isohemolysis, anisohemolysis, shift to the left, shift to the right, broad base, etc.’’ appear to be of some value in describing the type of hemolytic curves obtained by this method, and thus in developing further insight into the physiopathologic mechanisms of blood destruction.

V. SUMMARY

A method is described for the determination and charting of the fragility of the red blood cells to hypotonic sodium chloride solution. For plotting the results obtained, a new method was used, employing the principle of ‘‘hemolytic increments,’’ i.e., plotting the additional amount of hemolysis occurring in each successive tube of solutions of decreasing saline concentration.

By this method, curves are obtained somewhat similar to those of Price-Jones curves of red cell diameters. Such curves, when properly analyzed, afford an interpretation of the varying diameter thickness range of the red cell population and indirectly an insight into the types of red cells present, and of the possible hemolytic mechanisms present. Preliminary investigations indicate that they are particularly instructive in the hemolytic anemias and especially in the Mediterranean anemias.

A simplified shorter method was also devised for routine clinical use. This method, although not as useful for research purposes, has proved to be more accurate and yet less time consuming than most methods currently used.

REFERENCES

30 Gallernani: Quoted by Urcelay.
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