A METHOD FOR DETERMINING THE FORM OF THE DISTRIBUTION OF RED CELL RESISTANCES TO SIMPLE HEMOLYSINS

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THE DETERMINATION of the way in which the resistances of red cells are distributed with respect to hypotonicity (the erythrocyte fragility test) is a familiar laboratory procedure, and methods have been described for finding the distribution of the resistances of red cells to heat,[1,2] to acid,[3] to shaking,[4,5,6] to saponin hemolysis[7,8] and to hemolysis by lysolecithin.[9] It is now recognized that the form of the frequency distribution of red cell resistances to hypotonicity is determined primarily by the variations in the shape of the cells of the system,[10-14] and it is reasonable to think that the forms of the frequency distributions of resistances to lysins such as saponin or digitonin depend on the chemical nature and spatial arrangement of the components of the red cell architecture, i.e., on the "consist"* of the cell or of its surface ultrastructure. Just as it is necessary to have a method by means of which fragility can be described quantitatively before its relation to shape can be appreciated, so it is necessary to have a method by means of which the distribution of resistances to lysins can be described quantitatively before it is possible to study the relation of the resistance to chemical composition, spatial arrangement, etc.

The purpose of this paper is to describe a method for measuring the resistance of red cells to simple hemolysins, to define the range of normal variation, and to give a few illustrations of abnormal variations in resistance which occur under conditions in which it is reasonable to believe that abnormal variations in the consist of the members of the cell population have occurred. Emphasis will be placed on the observations being made and treated so that the maximum amount of information can be extracted from them, because the full potentialities of methods for determining red cell resistances are not realized when they are carried out in simplified form. Further, it should be remarked at the outset that the method to be described is of general application, although it is illustrated by results obtained with only two simple hemolysins, in addition to the results of fragility determinations.

1. Composition of the Hemolytic Systems

To illustrate the procedure, the two lysins saponin and digitonin have been selected for several reasons. More is known about the kinetics of lysis by saponin than by any other lysin, and the flat form of the frequency distribution of resistances makes it particularly useful for picking up bimodalities, etc. Digitonin is selected because it is one of the few powerful lysins which can be obtained in the pure state (unlike the saponins, the activity of which is variable), and free from inhibitory contaminants (unlike most preparations of the bile salts).

(*a*) *Saponin.* Add 10 mg. of quillia saponin (British Drug Houses) to 100 ml. of 1 per cent NaCl;

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* This is a noun, the general use of which has been urged by Schlegel[17]; it is employed in describing the composition of railway trains. Its definition is: "Consist, *n.* The singular elements of which something is constituted together with all the relevant spatial arrangements of these elements." As Schlegel points out, there is no other word in our language which conveys quite the same idea.

this gives a 1 in 10,000, or a 200γ/2 ml. solution of the lysin. A series of dilutions is made from this, so that 100 ml. volumes of a series of solutions of saponin containing 100, 80, 60, 50, 40, 30, 25, 20, 15, 12, 10, 8, 6, 4, 3, 2, and 1 γ per 2 ml. are obtained. The solutions are stored in stoppered bottles at temperature 4 C., and keep for about a month.

(*b*) *Digitonin.* 100 ml. volumes of solutions of digitonin (Merck) in 1 per cent NaCl, and containing 20, 17.5, 15, 12.5, 11.25, 10, 8.75, 7.5, 6.25, 5.0, 3.75, 2.5, and 1.25 γ per 2 ml. are prepared. Because of the steepness of the digitonin percentage hemolysis curve, intermediate concentrations, e.g., 8.125 γ/2 ml., may be needed; if so, 2 ml. of such a concentration can be made by mixing 1 ml. of the concentrations above it and below it in the series, i.e., 8.75γ/2 ml. and 7.5γ/2 ml. As the final values for hemolysis are reached within fifteen to thirty minutes in systems containing digitonin, the need for making additional observations at such intermediate concentrations can be appreciated soon after the experiment is set up; systems containing the intermediate concentrations can then be added if necessary, and the results can still be read at the end of 5 hours without any error having been introduced. The digitonin solutions keep at 4 C. for about a month.

(*c*) *Hypotonic NaCl.* A series of solutions of hypotonic NaCl is prepared,* starting with a 0.5 per cent NaCl and descending by a common difference of 0.02 units, i.e., 0.50, 0.48, 0.46, ··· per cent NaCl, in the scale of tonicity (the tonicity of a 1 per cent NaCl corresponds to 1 tonicity unit). The lowest member of the series should be 0.14 per cent NaCl. These solutions are stored at 4 C. When 0.5 ml. of a red cell suspension, with a tonicity of 1.0, is added to 2 ml. of each member of the series, we get a new series for the final tonicity T_1 of the mixture; this is

$$T_1 = \frac{2T_0 + 0.5}{2.5}$$

where T_0 is the tonicity of the member of the first series. The common difference on the new series for the final tonicity is 0.016, so that the new series runs 0.60, 0.584, 0.568, ··· with a lowest member of 0.312 per cent NaCl or tonicity units. For convenience in differentiating the experimental curves, the members of the new series can be described by integers starting with 0.60 = 0, so that 0.584 = 1, 0.568 = 2, ··· 0.312 = 18, as in the subsidiary scale on the abscissa of the curve marked T in figure 2.

(*d*) *Cell suspensions.* The volume concentration ρ of the red cells in freshly drawn heparinized blood is found with a high speed hematocrit, and the cells of 2.5 (0.4/ρ) ml. of blood, after being washed three times with 1 per cent NaCl, are finally suspended in 25 ml. of NaCl-buffer at pH 7.0.†

(*e*) *The hemolytic systems.* The hemolytic systems, contained in three series of 100 x 13 mm. tubes, are prepared by adding 0.5 ml. of the red cell suspension to 2 ml. volumes of the various concentrations of saponin, digitonin, and hypotonic NaCl. The lysins are put into the tubes first, and allowed to reach the temperature at which the determinations are to be made; 0.5 ml. of suspension is then added to each tube, with immediate shaking to produce mixing. Any desired temperature can be obtained by using water

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* The NaCl used must be silver-free,[18] as most C.P. preparations now are. It must also be dry. Commercial preparations may contain as much as 10 per cent of water after standing around the laboratory; they should be dried for twenty-four hours at 120 C. before use.

† The NaCl-buffer is made by mixing 75 ml. of 1.2 Gm./100 ml. NaCl with 25 ml. of a mixed buffer composed of 72 ml. of M/15 Na_2HPO_4 and 28 ml. of M/15 NaH_2PO_4. The depression of freezing point of this NaCl-buffer is the same as that of 1 Gm./100 ml. NaCl (i.e., its tonicity is 1.0), and its pH is 7.0 at 25 C.

The pH of the systems requires to be controlled at a known value because the activity of many lysins, e.g., saponin and the bile salts, has a marked pH dependence. It is usual to make measurements of fragility in systems containing unbuffered hypotonic NaCl on the grounds that the situation may be complicated by the addition of the ions in the buffer mixtures. This is true, but the situation is also liable to be complicated by uncontrolled variations in pH, especially if the red cell suspension is dilute.[19] If the suspension is more concentrated, the effects may be very much less,[20] but, since a complete investigation of the distributions of resistances to simple lysins and to hypotonicity would include measurements of resistances at a variety of pH's, one may as well buffer the systems from the start.

baths, but 25 C. is a convenient temperature which can easily be maintained to within ±1 C. for five hours at a time in the average laboratory room.

The hemolytic systems are mixed, either by rotary shaking or by inversion, every fifteen minutes, and the amount of hemolysis in each is determined at the end of five hours.

2. Measurement of Percentage Hemolysis

To find the percentage of complete hemolysis present in any one of the hemolytic systems at the end of five hours at 25 C., the cells are thrown down by spinning for a few minutes at 2000 r.p.m. The supernatant fluid can then be poured off into a dry vial without disturbing the cells. Two ml. of this supernatant fluid is added to 10 ml. of distilled water, and the optical density is measured at a wave length of about 4500 Å (blue filter) with a Lumetron photometer or with any other photometer of a similar type. This measurement is converted into a value for percentage

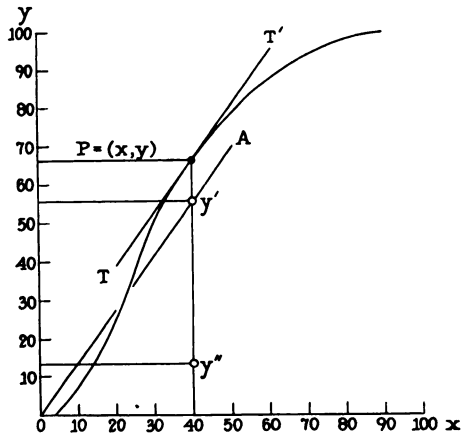

Fig. 1. Diagram to Illustrate the Method of Graphic Differentiation
For description, see text

hemolysis by referring to a calibration curve, prepared by measuring the optical densities corresponding to 100, 50, 25, and 12.5 per cent of complete hemolysis of the cells contained in the hemolytic systems. When measured at 4500 Å, the optical density is so related to the concentration of Hb present that percentages of complete hemolysis are found with about the same degree of accuracy over the whole percentage hemolysis range.

A separate calibration curve must be prepared for each suspension used; in practice, one uses the pooled contents of several completely hemolyzed systems for obtaining the 100 per cent point, and finds the other points by successive dilutions by powers of 2.

3. Plotting and Differentiation of the Curves

The values of percentage hemolysis P are plotted against the quantity of lysin in γ contained in the system, using the same scales for ordinates and abscissae as those used in figures 2, 3, and 4 The choice of the scales is important, as it de-

termines the ease and accuracy with which differentiation can be carried out. Smooth sigmoid curves are drawn through the experimental points, a possible error of about ±2 per cent being allowed in the case of each. If inspection shows that the curve is bimodal or polymodal, the procedure is still to draw the smoothest curve through the experimental points. It will be obvious that the more points

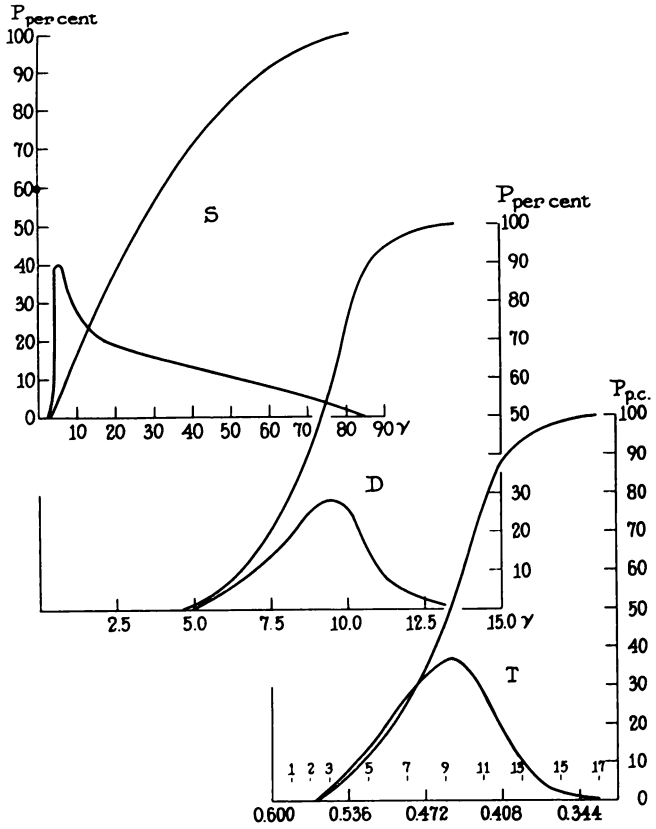

FIG. 2. NORMAL FREQUENCY DISTRIBUTIONS OF RESISTANCES, TOGETHER WITH THEIR INTEGRALS (THE EXPERIMENTAL CURVES)

Ordinates, percentage of complete hemolysis; abscissae, quantity of lysin in system in γ except in curve *T*, where the abscissa shows the tonicity in tonicity units. Subsidiary scale on abscissa of curve *T* is that used in differentiating. Curve *S*, results for saponin, curve *D*, results for digitonin, curve *T*, results for hypotonic NaCl. Reduction, 2×.

there are, the more certainly can the course of the curve be determined, and in some cases of suspected polymodality it may be necessary to repeat the entire set of determinations with the addition of new and strategically placed concentrations of the lysin.

The sigmoid curve is now differentiated graphically by using the following principle. If TT′ is the tangent at a point P with coordinates x and y (fig. 1), and if OA, the parallel through the origin, cuts the ordinate of P at y′, the value of

dy/dx at the point P is y″ = y′/x. Proceeding along the sigmoid curve systematically, tangents and their parallels through the origin are drawn with the aid of rulers fixed so as to move in parallel; these can be purchased from any dealer in draughtsman's materials. The value of y′ (read off on the ordinate) at which the parallel through the origin cuts the ordinate of the point under consideration is noted and is divided by the value for the x-coordinate of the point (read off on the

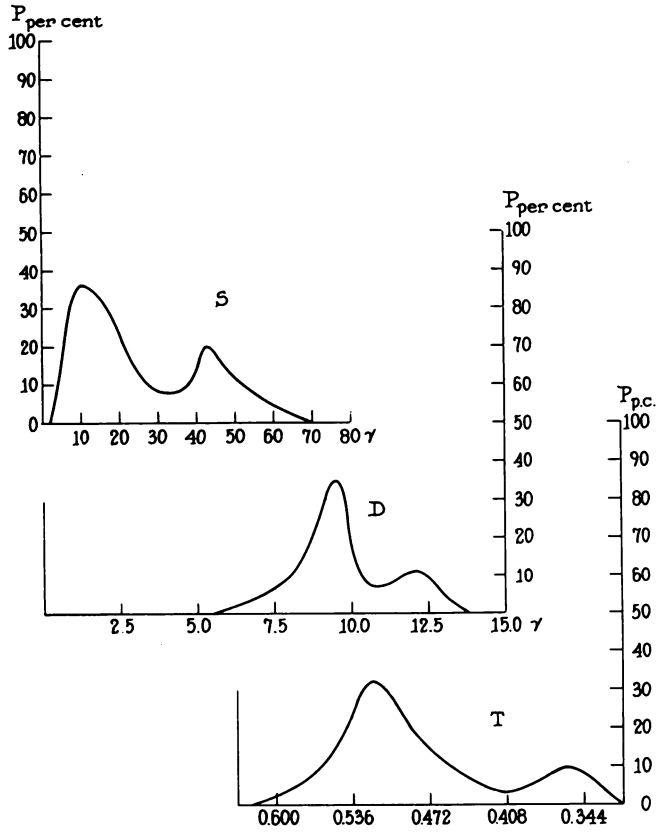

FIG. 3. FREQUENCY DISTRIBUTIONS AND THEIR INTEGRALS FOLLOWING MASSIVE HEMORRHAGE

Ordinates, abscissae, etc., as in fig. 1

abscissa); the result of the division is a value of y″, the ordinate of the corresponding point on the differential curve. To change the scale on which the differential curve is plotted, the value of y′ can be divided, not by x, but by x/s , where s is a convenient scaling constant; the values s = 10 for saponin, s = 1 for digitonin, and s = 2 for hypotonic NaCl are convenient because the frequency distributions are then plotted on such a scale that their shape and variations in it can be easily appreciated by eye. These values have been used in constructing the differential curves in figures 2, 3, and 4. If s = 1 were used for saponin, for example, the differential curve would be so flat that variations in its shape would not be apparent on simple inspection.

This process of differentiation can be carried out quickly and easily, and is all done on the same piece of graph paper. The number of tangents which require to be drawn depends to some extent on the shape of the sigmoid curve, but one should be drawn at each experimental point for a start. The process is completed by drawing a smooth curve through the points y'' obtained by differentiation. In each case, the result is a frequency curve showing the distribution of red cell resistances to the

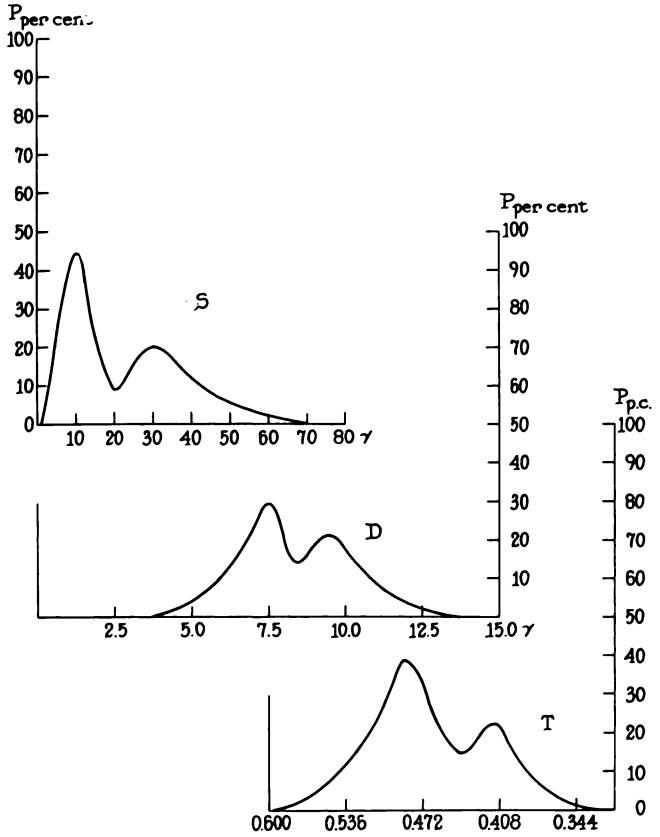

FIG. 4. FREQUENCY DISTRIBUTIONS AND THEIR INTEGRALS FOLLOWING A HEMOLYTIC EPISODE

Ordinates, abscissae, etc., as in fig. 1

lysin, on an abscissa showing the amount of lysin in the system in γ (or the tonicity of the system in tonicity units in the case of hypotonic NaCl).

4. NORMAL VALUES

To explore the range of normal variation, the frequency distributions of resistances to saponin, digitonin, and hypotonic NaCl have been obtained at 25 C. for the red cells of 12 normal blood donors. Table 1 shows the average, lowest, and highest values found for various statistics of the curves. The interdecile difference between the lowest and highest deciles, D_1 and D_2, has been used as a measure of

scatter, and the skewness has been calculated from the deciles D_1 and D_2 and the median M, as

$$\frac{(D_2 - M) - (M - D_1)}{(D_2 - M) + (M - D_1)}$$

by analogy with Bowley's measure of skewness. The three distribution integrals with average values are plotted, together with the distributions obtained from them by differentiation, in figure 2.

The distribution of resistances to saponin is normally negatively skew, with a long tail spreading out towards its upper extreme. The resistance distribution to digitonin is usually positively skew, and its scatter is relatively small. The normal

TABLE 1

	Average	Lowest	Highest
Saponin			
Median	26.5	22.0	28.5
Lower extreme	3.5	3.0	4.5
Upper extreme	85.0	73.0	96.0
Inter-decile diff.	52.5	44.0	57.5
Skewness	−0.30	−0.15	−0.41
Digitonin			
Median	9.20	9.05	9.45
Lower extreme	4.70	3.80	5.35
Upper extreme	13.5	12.5	14.9
Inter-decile diff.	4.25	4.05	4.50
Skewness	0.29	0.05	0.36
Hypotonicity			
Median	0.448	0.424	0.472
Lower extreme	0.566	0.584	0.552
Upper extreme	0.328	0.360	0.312
Inter-decile diff.	0.131	0.103	0.139
Skewness	0.14	−0.06	0.22

distribution of resistances to hypotonic NaCl at pH 7.0 is usually symmetrical, although it may have a small skewness in either direction. None of the observed distributions shows any great individual variation from the average distribution obtained for normal red cells.

5. Discussion, with Examples

The frequency distributions obtained experimentally with each lysin represent the distribution of the resistances of the N cells in the general circulation at the time the blood is drawn. This number is maintained at a constant level in the normal individual by the addition of new cells at a rate P and by the removal of old cells at a rate Q, so that $dN/dt = P - Q$. Changes in the rates of production and destruction result in changes in the value of N accompanied by *transient* changes in the form of

the resistance distribution; variations in the distribution of the resistances of the cell in the population N can, however, be brought about in another way. The P cells which are added to the population N in each unit of time themselves constitute a population with resistances distributed according to some form of frequency distribution; the same applies to the Q cells which are removed from the population N in unit time, and it can be shown that in the steady state the distribution of resistances in the population P is the same as that in the population Q, that the form of these distributions determines the form of the distribution in the population N, and that, if the life of the red cell in the circulation depends on its resistance to the lysin in question, the P and Q distributions must be more negatively skew than the N distribution.[15] It follows that *persistent* changes in the form of the distribution of resistances in the population N are reflections of persistent changes in the form of the distribution of resistances in the populations P and Q, and that *transient* changes in the distribution of resistances in the population N are due to sudden changes in the rate at which new cells are added to it, or in the form of the resistance distribution in the population P. Sudden changes in the rate of removal of old cells or in the distribution of resistances in the population Q can conceivably produce similar transient changes in the population N.

Hemorrhagic or hemolytic episodes supply the clearest instances of transient changes in the form of the distribution of resistances to saponin, digitonin, and hypotonic NaCl, as is illustrated by the following two examples.

Example 1. Hemorrhage from duodenal ulcer seven days before. Red cells 2.2 millions; Hb 5.7 Gm.; reticulocytes 6 per cent. All three resistance distributions bimodal. Saponin distribution has modes at 10γ and 43γ; digitonin distribution has modes at 9.5γ and 12γ; hypotonic NaCl distribution has modes at 0.520 and 0.360 tonicity units (fig. 3).

Example 2. Hemolytic anemia accompanying metastatic carcimoma with pylorus as the primary site. Red cells, 1.8 millions; Hb 4.6 Gm.; reticulocytes 13 per cent. All distributions bimodal. Saponin modes, 10γ and 30γ; digitonin modes, 7.5γ and 9.4γ; hypotonic NaCl modes, 0.488 and 0.416 tonicity units (fig. 4).

In these cases, the bimodalities are presumably due to a new population with its own frequency distribution of resistances having been added to the existing one at the time of the hemorrhagic or major hemolytic episode. The existing data do not allow the situation to be analyzed further. It would be almost certainly wrong, for example, to identify one of the modes as being produced by the reticulocytes in the population. All that can be said is that *at least* two distinguishable frequency distributions are superimposed.

Illustrations of persistent abnormalities in the form of one or more of the frequency distributions are less striking and not so easy to supply. Apart from the marked change in the distribution to saponin found in pernicious anemia in relapse[16] and to lysolecithin found in congenital hemolytic icterus,[9] the abnormalities are usually encountered during routine determinations of the resistance distributions in hypoplastic and toxic anemias, and in the anemias associated with leukemia, the various forms of lymphoma, etc. The italicized values found in the following three cases are outside the normal range of variation as given in Table 1.

Example 3. Hodgkin's disease. Red cells, 3.9 millions; Hb 10.4 Gm. Saponin distribution: median *18γ*, lower extreme, *2γ*, upper extreme, *100γ*, skewness, *−0.60*. Digitonin distribution: median, *8.0γ*, lower extreme, *3.0γ*, upper extreme, *12.5γ*. Hypotonic NaCl distribution: median 0.456, lower extreme, *0.636*, upper extreme, *0.294*, interdecile difference, *0.233*.

Example 4. Aplastic anemia. Red cells, 1.9 million; Hb, 5.2 Gm. Saponin distribution: median, *20γ*, lower extreme 4γ, upper extreme, *56γ*, interdecile difference, *40γ*, skewness, −0.35. Digitonin distribution: median, 9.1γ, skewness *0.45*. Hypotonic NaCl distribution, no abnormalities.

Example 5. Normocytic anemia due to chronic infection. Red cells, 3.8 millions; Hb, 9.2 Gm. Saponin distribution: median, *31γ*, lower extreme, *2γ*, upper extreme, *63γ*, skewness, *−0.03*. Distributions to digitonin and to hypotonic NaCl substantially normal.

Much more data will have to be accumulated before it is possible to classify these persistent abnormalities in the form of the frequency distributions to lysins. It is significant that all those which I have observed have been associated with anemia. In the meantime the persistent abnormalities can be looked upon as having much the same significance that abnormal Price-Jones curves would have, i.e., as showing that the normal red cell population has been partially or wholly replaced by populations having different resistance characteristics.

Summary

A method is described for determining quantitatively the form of the resistance distributions of red cell resistances to simple hemolysins. The normal range of variation is given for the resistance distributions to saponin, digitonin, and hypotonic NaCl, all at pH 7.0, and examples of departures from the normal, which may take the form of bimodalities after hemorrhagic or hemolytic episodes or of more persistent changes in the characteristics of the distributions, are provided.

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