STUDIES IN HAEMATOLOGY.

THESIS
PRESENTED FOR
THE
DEGREE OF DOCTOR OF MEDICINE
IN THE
UNIVERSITY OF EDINBURGH
BY
ERIC PONDER,
M.B., Ch.B, D.Sc.

October, 1925.
THE STUDIES WHICH COMPOSE THIS THESIS ARE BASED ON WORK CARRIED OUT DURING THE LAST FIVE YEARS IN THE DEPARTMENT OF PHYSIOLOGY OF THE UNIVERSITY OF EDINBURGH.
PART I. THE KINETICS OF THE SIMPLE HAEMOLYTIC SYSTEMS.

PART II. THE SIZE OF THE ERYTHROCYTE.

PART III. THE ERYTHROCYTE AS A BALLOON-LIKE BODY.

PART IV. THE SHAPE OF THE ERYTHROCYTE AND ITS RESPIRATORY FUNCTION.

PART V. SEDIMENTATION AND ROULEAUX FORMATION.
PART I.

THE KINETICS OF THE SIMPLE HAEMOLYTIC SYSTEMS.

Section 1.
The time-dilution curves.

Section 2.
The percentage haemolysis curves.

Section 3.
On the lysis of cells by bile salts.

Section 4.
Acceleration and inhibition.

Section 5.
The inhibition by serum.

Section 6.
Development of the fundamental equations.

Section 7.
The theory of the zone of action.
For convenience we may divide haemolytic systems into three large classes.

(1) Systems in which all the components are known, and in which every component can be subjected to exact and direct measurement, as, for example, a system containing saline, saponin, and cells, or a system containing saline, saponin, serum, cells, and acetic acid. In such systems there is no doubt about the quantity of any component present.

(2) Systems in which there is one component the quantity of which can be ascertained only by indirect methods; as, for instance, a system containing cells, saline, silicic acid, and complement. The quantity of complement present cannot be measured directly in any simple physical units, and can be found only by taking account of (a) the amount of complement-containing serum added, and (b) the activity of this serum. The conception of 'activity' at once introduces a difficulty.

(3) Systems in which more than one component is incapable of direct measurement, as a system of cells in saline, complement, and amboceptor.

A haemolytic system of type (1) we shall call a simple haemolytic system; the kinetics of such a system we propose to study in some detail.
PART I.

SECTION I.

The time-dilution curves.

Special apparatus.

Preparation of suspensions.

Preparation of lysin.

Experimental procedure.

Failure of the qt-law.

The time-temperature relation, with examples.
Arrhenius, the first investigator to attempt to reduce haemolytic phenomena to simple laws, suggested that a quantity or concentration of lysin q takes a time t to haemolysse red cells, so that

\[ q \cdot t = \text{constant}. \]

This is his celebrated qt-law, which he and his pupils often refer to.

Although we shall see that this law is not correct, it forms a convenient starting-off place for this dissertation on haemolysis, which is suitably opened by an investigation of the relation of quantity of lysin to time which it takes to produce a certain effect.

In order to investigate the matter, we first describe a suitable technique.

Special apparatus required.

1. A set of pipettes to deliver 1, 2, 3, 4, and 5 cc.
2. Pipettes, 1cc. capacity, graduated in 0.01 of a cc.
3. Burettes and pipettes of other convenient capacities, and tared flasks of 10, 50, and 100 cc. capacity.
4. Tubes a white glass, 3" by 1/2".
5. Thermometers in 0.1 degrees.
6. A water bath at constant temperature - usually 25°C.

This bath should be fitted with a thermo-regulator, and should have its back and front walls of glass, so that the tubes therein can be seen without removal from the
water. The back of the bath is lit by artificial light.

Preparation of blood suspension.

The suspension used in most of the experiments to be recorded is of an arbitrary, but convenient, strength. It is prepared as follows.

1 cc of blood is drawn from the finger into a pipette, preferably in four portions of 0.25 cc. at a time. The blood must flow freely. This volume of blood is then placed in centrifuge tubes containing 1.5 per cent citrated saline (1.5 per cent sodium citrate in water), the volume of saline in each tube being about 10 cc. The cells are centrifuged, and the supernatant fluid removed. The tubes are again filled, this time with 0.85 per cent NaCl in water. The cells are twice washed with this saline, and finally suspended in 20 cc. of 0.85 per cent NaCl.

The cells in this suspension will keep fully twelve hours, but should be used as soon as possible after preparation.

It is important that the cells should always be derived from the same source; the blood of the experimenter is convenient in this respect, as the cells from a healthy person show very little variation from time to time.

Technique.

To investigate the activity of a haemolytic substance, we plot the time-dilution curve in the foll-
owing way.

The activity of a haemolytic substance depends on (a) the dilution of the substance, (b) the quantity of blood suspension which is to be lysed, (c) the time it takes to complete the lysis, and (d) the temperature at which the experiment is conducted. Each of these variables must be controlled or measured; when a standard strength of suspension is used, (b) may be neglected.

1. A solution of the lysin in 0.85 per cent NaCl is prepared. In the case of the bile salts, 1 cc. of this solution should contain 12.5 milligrammes of the lysin. The reason for this adjustment is that, in the experiment proper, we shall mix

| lysin solution | 0.8 cc. |
| saline | 0.8 cc. |
| suspension | 0.4 cc. |

thus preparing 2 cc. of the mixture; in order to get a round number for the concentration of lysin in the final mixture, we must make the lysin solution 20/8, or 2.5, times as strong as we wish the final concentration to be. Thus if 1 cc. of the original lysin solution contains 2.5 milligrammes, as soon as this solution is brought into contact with the other components of the system in the above proportions, we shall have the system containing in 2 cc. 2 milligrammes or lysin, or a 1 in 1000 dilution of lysin.

To get a 1 in 10,000 or any other dilution is simple, for we have simply to dilute the stock lysin
to the proper extent.

The importance of basing all results on the concentration or dilution of lysin actually in contact with the cells when the haemolytic system is complete cannot be overestimated.

2. The lysin and the suspension being prepared, the actual experiment is carried in the following way. The water bath is brought to a pre-arranged temperature (say 25°C.), and a series of tubes are prepared by adding 0.8 cc of various dilutions of lysin to 0.8 cc of saline. All the tubes are put in the water bath, together with a tube containing the suspension, to come to the required temperature. With them is put a pipette graduated in 0.01's of a cc.

When the tubes have acquired the temperature of the water bath - this they usually do in five minutes - 0.4 cc of suspension is added to the first of them, the moment of the addition being recorded on a stop watch, and the addition being carried out rapidly with the warmed pipette. The tube is observed until complete haemolysis is brought about, the time noted, and the time taken for that particular dilution of lysin to bring about lysis of the particular quantity of cells used is thus known. The time taken by the other dilutions of lysin are found in a similar manner, and finally we have a series of times required for complete lysis to plot against dilutions of lysin.
The plotting of these figures against one another gives a time-dilution curve for the particular lysin acting at the particular temperature on the particular cells. This curve is reproducible with as much accuracy as may be required.

For lysins such as the following

Saponin.
Sodium taurocholate.
Sodium palmitate.
Sodium oleate.
Sodium stearate.
Potassium palmitate.
Potassium oleate.
Potassium stearate.
Ammonia
Lactic acid
Acetic acid

and others of the same type, the time-dilution curve is very characteristic. It is a curve concave to the time axis, and approaching an asymptote at some particular dilution of lysin. In all experiments with the above lysins, it is perfectly smooth, presenting no irregularities, but in the case of some lysins, such as sodium glycocholate, it takes on a curious form which will be described below.

The curve is plainly not represented by the expression \[ q \cdot t = \text{constant}, \]
or, to use the nomenclature to be employed in these
studies, c.t = constant, for since c, the concentration of lysin, and \( \delta \), the dilution of lysin, are reciprocals, we could write in the place of the last expression
\[
t = k \delta,
\]
the constant being \( k \).

This would give a straight line as a time-dilution curve, and not the curve which we have described and figured. The q.t-law of Arrhenius thus does not meet the case; the proper formula for the time dilution curves we shall give at a later stage of this study.

We may, however, at this stage examine the form of the time-dilution curve at various temperatures, in order to find, for any particular concentration of lysin, the relation between the temperature and the time required to bring about complete haemolysis.

If we call \( t \) the time in minutes taken to produce complete haemolysis of 0.4 cc of the suspension, and \( T \) the temperature in degrees centigrade at which the lysis is allowed to occur, we have the relation
\[
T = \frac{x}{\beta} \left\{ \frac{1}{T} \right\} + \frac{T}{\gamma}
\]
This curve is a hyperbola approaching the line \( T = 0 \) as one asymptote. Of the constants, \( x \) and \( \beta \) are the intercepts made by the other asymptote on the \( t \) and \( T \) axes respectively.

In order to find by experiment the values of \( x, \beta, \), and \( \gamma \), for a given dilution of lysin, the following method is adopted.
1. Find by experiment the time taken by this dilution of lysin to produce lysis at \( \tau = \) any value near 0. Call this time \( t_0 \).

2. In a similar way find \( t_h \), when \( \tau = \) any value near 10.

3. Similarly find \( t_h \), when \( \tau = \) any value between 30 and 45.

4. Treat the part of the hyperbola near \( \tau = 0 \) and \( \tau \\geq 10 \) as a straight line; determine its intercepts on the two axes. The intercept on the \( t \) axis is \( \beta \); that on the \( \tau \) axis is \( \alpha \).

5. Filling in the values for \( \alpha \) and \( \beta \) obtained above, in the expression

\[
\tau = \alpha \beta \left( \frac{\beta - t}{\beta - \tau} \right) + \frac{\alpha \beta}{t}
\]

find the value of \( \gamma \).

Experimental and calculated results may then be compared at values of \( \tau \), as, for example, when \( \tau = 10, 20, 30, 40, \) etc.

Below are given a few examples to show the degree of correspondence between the calculated and experimental results.

Saponin.

The following constants were obtained.

<table>
<thead>
<tr>
<th>( \lambda )</th>
<th>( \alpha )</th>
<th>( \beta )</th>
<th>( \gamma )</th>
</tr>
</thead>
<tbody>
<tr>
<td>5000</td>
<td>9.4</td>
<td>25</td>
<td>4.1</td>
</tr>
<tr>
<td>10000</td>
<td>10.4</td>
<td>57</td>
<td>8.3</td>
</tr>
<tr>
<td>20000</td>
<td>12.4</td>
<td>139</td>
<td>16.6</td>
</tr>
<tr>
<td>30000</td>
<td>14.4</td>
<td>252</td>
<td>25</td>
</tr>
</tbody>
</table>
and the following tables compare results:

\[ \delta = 5000 \]

\[
\begin{array}{|c|c|c|}
\hline
\tau & t, \text{exp.} & t, \text{calc.} \\
\hline
10 & 2.3 & 2.5 \\
30 & 0.17 & 0.2 \\
\hline
\end{array}
\]

\[ \delta = 20000 \]

\[
\begin{array}{|c|c|c|}
\hline
\tau & t, \text{exp.} & t, \text{calc.} \\
\hline
12 & 16 & 15 \\
40 & 0.4 & 0.5 \\
\hline
\end{array}
\]

The correspondence is as good as could be expected.

Sodium taurocholate.

\[
\begin{array}{|c|c|c|c|}
\hline
\delta & \lambda & \beta & \gamma \\
\hline
1000 & 15 & 9 & 20 \\
2000 & 19 & 31 & 40 \\
3000 & 23 & 100 & 60 \\
4000 & 27 & 570 & 80 \\
\hline
\end{array}
\]

These values give the following results,

\[ \delta = 3000 \]

\[
\begin{array}{|c|c|c|}
\hline
\tau & t, \text{exp.} & t, \text{calc.} \\
\hline
10 & 60 & 60.7 \\
30 & 7 & 7.0 \\
40 & 3.5 & 3.4 \\
\hline
\end{array}
\]

\[ \delta = 4000 \]

\[
\begin{array}{|c|c|c|}
\hline
\tau & t, \text{exp.} & t, \text{calc.} \\
\hline
30 & 17.5 & 18 \\
\hline
\end{array}
\]
These few results are quite sufficient to show that the expression relating time and temperature is a satisfactory one; indeed, the fit between calculated and experimental results is better in this type of experiment than in any other. The experiments are, moreover, particularly easy to carry out, the only difficulty lying in the very slow haemolysis at low temperatures.
PART I.

SECTION II.

The Percentage haemolysis curves.

Methods.

Results for NaOH, saponin, and taurocholate.

Discussion.

Review of literature.

The NaOH curves.

The saponin curves.

The taurocholate curves.

The accelerating and inhibiting effect of haemoglobin and serum.

Papers referred to.
Most of the observations on the rate of action of haemolytic substances express the time taken under various conditions for the production of complete haemolysis. While such observations are comparatively easy, it is a much more difficult matter to find the number of cells haemolyzed from time to time from the beginning of the reaction until its completion.

The methods used fall into two classes. In one method the reaction is stopped after it has proceeded for a certain length, the stopping being carried out as a rule by cooling, the intact cells are centrifuged off, and the quantity of haemoglobin in the supernatant fluid estimated. From this the percentage number haemolyzed is calculated. In the second method, less used on account of its greater inaccuracy, the degree of lysis is estimated at various times by comparing the appearance of the tube in which the experiment is proceeding with the appearance of a series of standard tubes, in which the percentage haemolysis is known.

The inaccuracy of this second method is obvious; it is not possible to judge the degree of lysis except in the roughest way. The first mentioned method has also its disadvantages, being unsatisfactory in three respects especially. Cooling the tube in which lysis is proceeding does not stop the reaction, but merely retards it; further, the low temperature must be maintained until the cells are all separated on the centrifuge - a difficult
thing to do unless with a specially constructed instrument. During the time taken to centrifuge, lysis proceeds at a slow rate; the result is, therefore, that the values for percentage haemolysis obtained by this method are too high. In the second place, the method is not satisfactory since the centrifuging causes many of the cells, weakened by the haemolytic agent, to haemolyse. This also makes the results too high. The third disadvantage is most important. It is evident, from the nature of the method, that it is applicable only to reactions which proceed slowly, and is therefore of limited application. The study of percentage haemolysis at high temperatures, or by means of haemolytic agents in high concentrations, is accordingly impossible.

It is desirable to have a method whereby the percentage number of cells haemolysed from minute to minute can be rapidly determined without the necessity of stopping the reaction in progress. Such a method is described below.

Principle of method.

Since a suspension of red cells is opaque, whereas haemolysed cells give a clear solution, the former will cut off more light than the latter. The quantity of light passing through a fluid containing cells will therefore vary with the number of cells haemolysed, increasing as haemolysis increases. The quantity of light passing through the fluid may be measured by observing the speed of rotation of a radiometer,
Radiometer.

L ... lamp house
C ... cell
P ... delivery burette
R ... radiometer house
T₁, T₂, T₃, T₄ ... thermometers

Sectional diagram:
L ... lamp house
C ... cell
D, B ... cell water jackets
R ... radiometer
E ... radiometer water jacket
O ... observing lens
exposed to it, and screened from heat.

Description of apparatus.

The apparatus must meet certain requirements.

1. The source of light must be constant.

2. The cell containing the red cell suspension must be water-jacketed, so that the experiments may be carried out at any desired temperature.

3. The radiometer must be screened from heat.

The source of light used is a gas filled lamp giving 1000 candle power. It is enclosed in a box fitted with a ventilator, and mounted on a rod so as to be movable.

The light from the tank passes through a large tank, (A in figure), 9" broad, 8" high, and 2" thick; the front and back are made of glass. The tank has an outlet and an inlet, these are connected to the cold water supply. After passing through the tank the light falls on an aperture 2½ square, in the front wall of the box in which the light is situated. It then traverses the water jackets of the cell and the cell itself. The first and second water jackets have each thermometers, and are connected to circulating cold water or to circulating water at any desired temperature, the water being warmed by a thermostat. The haemolysis cell lies between the two water jackets - its capacity is almost exactly 100 cc. The top of this chamber is open, the bottom possesses an outlet. All the walls in the path of the light are of plate glass; the light passing through them finally
falls on an aperture in the case of the radiometer, and on the vanes of the radiometer itself. The movement of these vanes can be observed through a low power lens in the line of the light.

The radiometer, to screen it from heat, is surrounded by a water jacket connected to the cold water supply.

Above the open top of the haemolysis cell is a water jacketed burette, at the same temperature as the jackets of the haemolysis cell; from this the blood suspension is added when required to the lysin in the cell.

Lastly, into the haemolysis cell passes a fine jet of compressed air to mix the cells.

Technique.

The preparation of the apparatus is carried out by flooding all the water jackets with water, circulating at the desired temperature, the tank marked A and the radiometer jacket always carrying circulating water at room temperature. When the thermometers show the temperatures to be constant, the apparatus is calibrated.

Calibration.

A series of standards is made up as follows. In each of 10 bottles is placed 50 cc of distilled water. To the first bottle is added 0.025 cc of blood from the finger, to the second 0.05 cc, and so on, increasing by 0.025 cc till the tenth bottle has added 0.25 cc. These red cells are, of course, lysed. To each bottle is now added 50 cc of 1.7 per cent NaCl.
so as to make the contents of each bottle isotonic. Blood is again added to each from the finger, so that the total addition to each bottle is 0.25 cc. The ten bottles now contain suspensions and lysed cells equivalent to 0, 10, 20, ..., 90, and 100 per cent lysis in a suspension such as would occur if 20 cc. of a suspension 1/4 the strength of the usual standard were added to 80 cc. of saline or lysin in saline.

Each fluid is in turn placed in the haemolysis cell and the speed of rotation of the radiometer which corresponds to the particular degree of lysis determined.

Experiment proper. The chambers, etc, being at the required temperature, 80 cc of the lysin solution is placed in the haemolysis cell. In the burette above it is placed 20 cc of the 1/4 strength suspension, and the two fluids allowed to take on the temperature of the water jackets.

The suspension is then run into the cell, and lysis begins. From minute to minute the speed of the radiometer is measured as before.

The speed of radiometer rotation from minute to minute is then converted into percentage lysis from minute to minute by means of the calibration curve, and the percentage haemolysis curve thus obtained, with percentage haemolysis on the ordinate, and time on the abscissa.

Results.

We propose to give three curves only, as we shall discuss only their general properties.
Curve 1. NaOH and human cells. Lysin, N/200.
Points are,
(0,0) (5,0) (10,3) (15, 0) (18,75) (20,88)
(22,94) (27,100)

Curve 2. Saponin and human cells. Lysin 1 in 50,000
Points are,
(0,0) (2,0) (3,1) (6,5) (9,25) (12,52) (15,75)
(18,88) (24,98) (27,100).

Curve 3. Taurocholate and human cells. Lysin, 1 in 7,000.
Points are,
(0,0) (4,0) (5,1) (7,5) (13,24) (15,35) (16,52)
(17,65) (18,78) (19,86) (20,92) (21,98) (22,100)

All these curves are in general S-shaped, and are shown in
the figures in the following pages.
Discussion.

Before discussing the results obtained it will be convenient to review the results of other workers. Many observers have stated that the formula describing monomolecular reactions

\[ n_t = n_0 e^{-\beta t} \]

applies to the lysis of red cells and of bacteria, \( n_0 \) being the number of cells at the beginning of the experiment, \( n_t \) the number at time \( t \), and \( \beta \) a constant. Arrhenius gives this expression as applicable equally to red cells and to bacteria; he explains, too, that it is to be expected that bacteria or cells shall have different degrees of resistance to lysis, some being very resistant, others less resistant, and others again exceedingly weak. He points out that if this were the case, instead of the straight line which results if the logarithm of the number of surviving bacteria or cells be plotted against the time in cases where the monomolecular formula applies, a curved line with a double inflexion would result. This latter curve Arrhenius did not find in the case of bacteriolysis, and it is therefore to be concluded that the natural resistance of the cells does not affect the reaction in any way. Arrhenius shows that the monomolecular formula applies to haemolysis but he also shows that red cells possess different natural resistances, the resistances being distributed according to a probability curve. How these two findings are to be reconciled with the fact that the logarithm of the surviving cells...
against the time results in a straight line is not explained. In fact, a contradiction is involved. The experiments by which it was shown that the monomolecular formula is applicable are very unsatisfactory, since the reaction was frequently not observed throughout its whole course, but only for a limited time. This is a frequent fallacy in Arrhenius' experiments on lysis. Henri also finds the monomolecular formula applicable; he neglects the influence of individual variations of resistance on the reaction, for which he is severely criticised by Mioni, whose experimental evidence, however, is equally inconclusive. Dryer and his co-workers observe that, after a period which they call the induction period, the monomolecular law is followed. This "period of induction" is to be carefully distinguished from the "period of incubation" referred to by Arrhenius; during the former, lysis proceeds, but not according to the monomolecular law—during the latter, there is no lysis at all. The "period of induction" cannot be regarded as anything but a part of the curve describing the reaction, to which it is not possible to fit a convenient formula. Several explanations have been advanced to explain the fact that the lysis of cells which are of varying resistances can be described by a typical monomolecular formula—to explain, in fact, an error of observation.

Allied to the problem of percentage haemolysis is that of percentage bacteriolysis. Madsen and Nyman, Chick, and Phelps, state that this process can be des-
cribed by the formula for monomolecular reactions. Most of these observers recognise that the typical monomolecular formula ought not to apply, since the bacteria are of different resistances to the destructive agents. Madsen and Nyman admit the fact and then ignore it. Chick imagines the cells to undergo rapid cyclic changes in the resistances, and explanation which Eijkman and Phelps adopt. The explanation is so unsatisfactory that Chick herself modifies it in a later paper - it has, indeed, no evidence to support it. The modification is not an improvement.

Brooks investigated the action of ultra-violet light on the red cells, and also the action of haemolytic sera. He found that the curves describing the reaction were sigmoid, and concluded that the course of the process was largely dependent on variations of resistance of different cells to the haemolytic agent. He also concluded that the evidence for the monomolecular reaction is very weak, and that there is little reason to think that such a reaction enters into the lytic process at all. Brock's paper contains a clear discussion of the theory and an excellent set of references.

Brinkmann finds that the type of curve obtained in experiments dealing with the resistance of red cells to osmotic influences shows that some of the cells possess a different degree of resistance from others, the majority lying between the two extremes.

The recent work of Peters must also be referred to. Peters lays stress on the fact that organisms have
resistance probably distributed according to a skew frequency curve. His results were obtained by counting the organisms, so that the figures are based on small numbers. There is, however, no doubt about the type of curve or about the fact that the explanation lies in the difference of resistance of the groups of organisms. Peters noted that a considerable part of the curve is described by the monomolecular formula. This part corresponds to that outside Dryere's induction period; a purely artificial division of the curve in this way cannot have any advantage.

It is remarkable that only one of these observers comment on the possibility that the haemolysis may result in the production or liberation of some substance which may influence the velocity of the reaction. Brooks observes that the symmetry of the sigmoid curves may be affected by an inactivation of the lysin, causing a slowing of the reaction.

Summarising these results, it appears that one group of observers consider that haemolysis and bacteriolysis may be described completely by the monomolecular formula, and that the curve obtained when percentage haemolysis is considered are such as would be expected if a reaction of this sort were in progress. A second group considers that the sigmoid curves are typical, and explain these as the result of a distribution of the resistances of the cells according to one or another form of frequency curve. The only satisfactory attempt to
Sodium hydrate.

Fig. 3.

Fig. 4.
discover the type of reaction which, taken together with the distribution of the resistances, would result in these sigmoid curves, is that of Brooks, who, however, rightly limits his discussion to theory since, as he states, inhibitory of other influences may alter the form of the experimental curves.

Considering now the curves given above in the results, it will be convenient first to deal with the observations on the percentage haemolysis curve obtained when NaCH is used as a lysin. The curve is shown in the figure.

There is a distinct latent period, during which no haemolysis occurs. This is commonly found in experiments with lysins which act slowly. After this period the reaction resulting in haemolysis commences, and is described by a sigmoid curve, almost symmetrical. The deviation from symmetry is so small as to fall within the range of experimental error. The median nearly coincides with the ordinate erected half way between the point of the commencement of lysis and its completion. If the position of the quartiles be examined they will be found to be very nearly equidistant from the median. From this curve, the first derived function can be obtained by graphical differentiation. It is shown in the subjoined figure, and is obviously very like a curve of a symmetrical frequency distribution; the resistances of the cells, measured in the terms of time taken for their lysis, being ideally distributed. As follows from the remarks made about the integral curve, the median
Fig. 5.

Saponin.

Fig. 6.
lies very close to the ordinate erected at the point half way between the time for the commencement of lysis and its completion.

The chief difficulty underlying the attempt to ascertain the nature of the reaction lies in the fact that in the case of most haemolytic reactions the percentage haemolysis curve is not symmetrical. This fact may be explained according to one of two hypotheses - that the reaction proceeds with a constant velocity, while the distribution is asymmetrical, or that the reaction is monomolecular, and the distribution a symmetrical one. In the case under consideration there is no asymmetry; we can therefore say that, given a symmetrical distribution, the reaction has a constant velocity.

Unfortunately, a monomolecular reaction can be of so very nearly constant velocity that it is indistinguishable from a reaction of uniform rate; this may be a case in point.

Proceeding now to the curve relating percentage haemolysis to time when saponin is the lysis, and as shown in the figure, it will be observed that the curve is of the type usually associated with haemolytic substances such as sera and ultra-violet rays. There is a considerable amount of asymmetry, the number of red cells destroyed in the first half of the reaction being greater than that destroyed in the second half. The form of this curve might be explained by assuming a reaction of constant velocity together with an asym-
Sodium taurocholate.
metric distribution, or by assuming an ideal distribution together with a monomolecular reaction. We shall see later that this latter assumption is a completely satisfactory one - indeed, the former explanation may be at once set aside by stating that there is no evidence whatever for the asymmetry of the distribution, since this appears to vary in the experimental curves according to the concentration of lysin used. If the lysin is sufficiently concentrated (saponin 1 in 20,000) the percentage haemolysis curve is almost symmetrical; only when the lysin is dilute does the asymmetry appear.

The curve under discussion may be differentiated to give a curve for a resistance distribution such as is seen in the adjoining figure. The skewness is slight, and all the appearance of the curve suggests that this skewness is due to a retardation of the reaction as it proceeds. It will be noticed that there is a distinct latent period.

The results for the lysin sodium taurocholate may now be considered. The curve, which is different from that obtained with NaCH or with saponin, is shown in the affixed figure. Its asymmetry is marked, but not in the same direction as that of the saponin curve; this is well seen in the figure showing the first derived function. The number of cells haemolysed during the first parts of the reaction is far less than the number haemolysed during the later stages. It is scarcely possible to regard this result as the result of a monomolecular reaction moving through a symmetrical population.
The following explanation is, however, possible. We may imagine the distribution to be ideal, and the reaction to proceed either at a constant velocity or according to the formula for a monomolecular reaction, and add that as the reaction proceeds some liberation of substance - as for example, haemoglobin - causes an acceleration of the reaction or an alteration in the frequency distribution. By an assumption of this sort strained hypotheses are avoided, for the assumption may readily be put to the test of experiment.

It is, however, known that haemoglobin has an inhibitory effect on haemolysis, and does not act, under the usual circumstances, as an accelerator. Indeed the inhibitory effect of haemoglobin on taurocholate haemolysis is even greater than it is on saponin haemolysis. It might be accordingly considered absurd to explain an acceleration, such as appears in the percentage haemolysis curves for taurocholate, by suggesting that it is that very substance which produces inhibition which gives the acceleration. Attention must, however, be drawn to a very important point. The demonstration that haemoglobin is inhibitory has always been made in a particular way - by adding the lysin to the haemoglobin, and thereafter determining the delay produced in the lysis of added red cells. If we are to consider the occurrences which take place when cells are haemolysed in a haemolytic system, it will be seen that there is an important difference between this lysis and the lysis in the system determining the inhibition.
In the case of the experiment showing the inhibition, the lysin has added to it haemoglobin or serum, and cells are thereafter added. In the case of the cells undergoing lysis, the lysin is first in contact with the cells, the haemoglobin being added as lysis proceeds, by the destruction of the cells. Such a difference as this cannot be ignored, for the order in which substances are introduced into a system is exceedingly important in determining the final result. A substance such as histamine, for example, behaves as an inhibitor if brought into contact with sodium taurocholate before the addition of the cells, but as an accelerator if added to a system containing cells and taurocholate.

It is possible that haemoglobin behaves similarly, acting as an inhibitor when mixed with the lysin, and as an accelerator when added to the cell-lysin system. To show this the following experiment was devised.

A red cell suspension, one quarter the strength used in the radiometer experiments, was prepared, for use in haemolytic systems containing taurocholate. In those cases where serum or haemoglobin had to be added, the addition was made after 30 seconds. The haemoglobin solution was prepared from washed red cells, so that serum proteins should be removed; to remove any envelopes of red cells it was passed through a Berkefeld filter, and to remove fats, was extracted with ether.
The results are expressed in the following table.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 in 3000 taurocholate</td>
<td>3.5 mins</td>
</tr>
<tr>
<td>1 in 3000 taurocholate plus 0.025 cc. serum added before addition of cells</td>
<td>32.0 mins</td>
</tr>
<tr>
<td>1 in 3000 taurocholate plus 0.025 cc. Hb. solution, added before addition of cells</td>
<td>12.0 mins</td>
</tr>
<tr>
<td>1 in 3000 taurocholate plus 0.025 cc. serum added after addition of cells</td>
<td>0.6 mins</td>
</tr>
<tr>
<td>1 in 3000 taurocholate plus 0.025 cc. Hb. solution added after addition of cells</td>
<td>1.2 mins</td>
</tr>
</tbody>
</table>

The temperature at which this experiment was carried out was 25°C. The quantity of haemoglobin added was just that amount which would be liberated by the lysis of 0.5 cc. of the standard suspension if haemolysed. The results show plainly that (a) the effect of the addition of serum or haemoglobin to taurocholate before the addition of the cells is to inhibit the lysis, and (b) that the effect of the addition after the addition of the cells is to accelerate the reaction.

It now remains to be shown that this peculiar phenomenon is not observed with saponin as a lysis, and that serum and haemoglobin act as inhibitory agents whether added before or after the addition of the cells to the system. This is shown in the following experiment, conducted in a manner similar to the experiment just described.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 in 3000 saponin</td>
<td>3.25 mins</td>
</tr>
<tr>
<td>1 in 3000 saponin plus 0.025 cc serum, added before addition of cells.</td>
<td>37.0 mins</td>
</tr>
<tr>
<td>1 in 3000 saponin plus 0.025 cc Hb. solution added before addition of cells</td>
<td>3.7 mins</td>
</tr>
</tbody>
</table>
1 in 30000 saponin plus 0.025 cc. serum added after addition of cells | 37.0 mins.
1 in 30000 saponin plus 0.025 cc. Hb., added after addition of cells | 3.7 mins.

These results show that (a) serum and Haemoglobin act as inhibitory agents to saponin haemolysis whether added before or after the saponin has come into contact with the red cells; (b) that the inhibitory effect of haemoglobin is very slight.

These two experiments cannot be considered as anything but illustrative.; a complete study of the subject requires to be made, although the fundamental point is readily shown.

It appears, then, that the acceleration of the reaction which occurs with sodium taurocholate as a lysin may be explained by considering the effect of the liberated haemoglobin. Thus we arrive at the following conclusions -

The reaction between the lysin and the cells may be explained by assuming that the fundamental reaction proceeds with a constant velocity of according to the formula for a monomolecular reaction, among a population described by a frequency curve as regards their resistance to the lysin.

Deviations from symmetry are to be best accounted for by the effect of the haemoglobin liberated from the cells themselves.
Papers referred to.

Arrhenius "Quantitative laws in biological chemistry". N.Y. p.78, 1915.


Dreyer and Hansen C.R. de l'Acad. cvi, 999, 1907.

Madsen and Nyman Zeitschr. Hyg. lvii, 388, 1907.

Chick Zeitschr. Hyg. viii, 27, 1911; and x, 237. 1910.


Bijkman Folia Microbiol. i, 359. 1912.


Brinkmann Dissert. Gronigen. 1922.

Peters Journal of Physiol. liv, 260, 1921.
PART I.

SECTION III.

On the Lysis of cells by bile salts.

The physical condition of the bile salts.
The haemolytic action of sodium glycocholate.
The effect of blood serum.
The effect of serum on glycocholate lysis.

Methods of mixing.
Variation of lysin.
Sensitivity.
Variation of serum.

The protective action of the serum proteins.

Summary.
It so happens that the bile salts, sodium taurocholate and sodium glycocholate, present some very unusual features when used as haemolytic, being, in fact, excellent examples of a class of lysozyme which is both a semi-colloid and unstable in physical state. Of the two the most curious is the glycocholate, and this section is concerned with some of these peculiarities.

Physical properties of the bile salts.

Although there is no very definite statement on the matter, the general opinion appears to be that the bile salts pass into true solution in water. If sodium taurocholate be dissolved in water, a clear yellow solution results; this clearness soon disappears, and the solution becomes quite opalescent, indeed, after 12 hours or so, opaque. The opalescence is caused by the salt passing into some state other than that in which it was initially. The opalescent solution has all the properties of a taurocholate solution, its haemolytic activity being comparable with that of a clear solution of the same strength. A difference appears on filtering the solution - the filtrate of the opaque solution has a much reduced haemolytic power, and that of the clear solution a somewhat less reduced activity. In fact, the opalescence suggests that the salt has passed into a less disperse form.

The more dilute the taurocholate solution is made, the more rapidly does the opalescence appear; in
solutions in 1 per cent saline a similar occurrence takes place, but less rapidly than in aqueous solution.

Sodium glycocholate behaves in a similar way, but the appearance of the opalescence is not so rapid. In a 1 per cent solution in saline it takes days to appear.

Both salts possess a property peculiar to colloids— they protect a gold sol against precipitation. The protective power is shown in the following way. Using a sol prepared by the formaldehyde method, 1 cc. is precipitated by 0.1 cc. of 10 per cent NaCl within 10 seconds. If small quantities of bile salts are added, we find that precipitation is prevented. This protective power is apparent before the solution becomes opaque, and becomes less as opalescence proceeds, attaining a minimum after about 36 hours. The quantities of the two bile salts which are required to protect 1 cc. of gold sol against 0.1 cc. of 10 per cent NaCl are shown below.

<table>
<thead>
<tr>
<th>Hours after preparation</th>
<th>Taurocholate 1 in 1000</th>
<th>Glycocholate 1 in 1000</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.2 cc.</td>
<td>0.35 cc.</td>
</tr>
<tr>
<td>12</td>
<td>0.3</td>
<td>0.4</td>
</tr>
<tr>
<td>24</td>
<td>0.35</td>
<td>0.55</td>
</tr>
<tr>
<td>36</td>
<td>0.4</td>
<td>0.6</td>
</tr>
<tr>
<td>46</td>
<td>0.4</td>
<td>0.6</td>
</tr>
</tbody>
</table>

It will be observed that (a) taurocholate is twice as protective as glycocholate, speaking approximately, and (b) as opalescence becomes established
there is a loss of protective power, the protection falling to about half its original value.

The haemolytic action of sodium taurocholate.

This lysin is peculiar in that it produces lysis in dilutions of 1 in 1000 and more, exactly as does sodium taurocholate or saponin; the time-dilution curve is smooth and uniformly concave to the time axis. In dilutions less than 1 in 1000, however, peculiarities appear; these are principally the following.

1. There is great difficulty in obtaining consistent results; the time for complete lysis appears to vary rather more than usually, even if the temperature, etc., is carefully controlled.

2. The salt haemolyses more rapidly in dilutions of 1 in 1000 than in dilutions of 1 in 100.

3. A freshly prepared suspension of red cells seems to be less rapidly haemolysed by the salt in all dilutions up to 1 in 1000, and especially in the dilutions 1 in 100 and 1 in 50, than a suspension which is 12 hours old. This is not unexpected; the envelopes of the old cells may be weak.

The following table gives the times taken for complete haemolysis of 0.2 cc. of blood suspension, by various dilutions of the salt.

<table>
<thead>
<tr>
<th>Dilution 1 in</th>
<th>Time mins.</th>
<th>Dilution 1 in</th>
<th>Time mins.</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>25</td>
<td>400</td>
<td>40</td>
</tr>
<tr>
<td>100</td>
<td>90</td>
<td>600</td>
<td>30</td>
</tr>
<tr>
<td>Dilution 1 in</td>
<td>Time mins.</td>
<td>Dilution 1 in</td>
<td>Time mins.</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>200</td>
<td>65</td>
<td>1000</td>
<td>10</td>
</tr>
</tbody>
</table>

These results may be taken as representative of the general behaviour of the salt; the times are not, of course, exactly reproduced with different suspensions.

This finding must be regarded as rather a remarkable one, haemolysis occurring more rapidly in a dilution of 1 in 1000 than in a dilution of 1 in 100. It is obvious that it depends on some other condition than the mere solution of lecithin and cholesterol from the envelope. With this lysis in comparatively concentrated solutions this section is principally concerned.

The effect of blood serum.

Blood serum exerts a powerful inhibitory effect on the action of sodium taurocholate and of sodium glycocholate. This is illustrated by the following figures, obtained by experiment at 18 °C.

<table>
<thead>
<tr>
<th>Sodium taurocholate, 1 in 1000</th>
<th>3 minutes.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium taurocholate, plus 0.1 cc serum</td>
<td>16 minutes.</td>
</tr>
<tr>
<td>Sodium glycocholate 1 in 1000</td>
<td>10 minutes.</td>
</tr>
<tr>
<td>Sodium glycocholate plus 0.1 cc serum</td>
<td>37 minutes.</td>
</tr>
</tbody>
</table>

It is of interest to note in passing that the bile salts cannot exert a haemolytic effect in the blood stream, for the cells are completely protected against their action by the plasma, which inhibits exactly as does serum.
The effect of serum on lysis by glycocholate.

The following solutions are used.

1. A series of solutions of sodium glycocholate in saline (0.85 NaCl.) The strength of the solutions is shown in the following table.

| Glycocholate per cent. | Final concentration in | Value of l |  |
|------------------------|------------------------|-------------|
| 2.5                    | 100                    | 100         |
| 1.25                   | 200                    | 200         |
| 0.833                  | 300                    | 300         |
| 0.625                  | 400                    | 400         |
| 0.5                    | 500                    | 500         |
| 0.417                  | 600                    | 600         |
| 0.25                   | 1000                   | 1000        |

The second column gives the concentration of the lysin in a tube if, of any of these dilutions, 0.8 cc. is taken and added to 0.8 cc. of saline, 0.4 cc. of red cell suspension being added thereafter. We are thus working with solutions which give the concentration in actual contact with the cells themselves. Each solution is, in fact 2.5 times as strong as this final concentration in contact with the cells.

2. A solution of serum albumin. This was prepared from dried blood; the strength of the solution (in saline) was 1 per cent.

3. A red cell suspension, prepared as in Section 1.

It is advisable that all these solutions should be freshly prepared, and kept on ice for the few
hours which may elapse between their preparation and their use.

If to 0.8 cc. of 2.5 per cent glycholate be added 0.4 cc. of suspension, and, after an interval of 5 seconds, 0.4 cc. of serum albumin be added to the mixture, lysis occurs very rapidly in about 30 seconds. It has been noted above that a 1 in 100 dilution of glycholate takes about 60 minutes to bring about lysis by itself; there is therefore an enormous acceleration produced by the added protein. It should be observed that the serum albumin itself in non-haemolytic, and that the rapid lysis cannot be explained by the fact that the cells remain in contact with the glycholate for the short period of 5 seconds, for no lysis occurs in that time.

The occurrence of this rapid lysis depends on several factors; since it occurs in the mixture of three components, we may see the effect of mixing them in different orders.

Method 1. Put 0.8 cc. of lysin, 2.5 per cent, in a tube, add 0.4 cc. of red cell suspension, and then, after an interval add 0.8 cc. of serum albumin solution.

Method 2. Put 0.8 cc. of serum albumin solution in the tube, add 0.4 cc. of the suspension, and, after an interval, 0.8 cc. of the lysin.

Method 3. Put the lysin and the serum albumin in the tube, 0.8 cc. each; mix, and add 0.4 cc. of the suspension.

In all the cases mentioned below, the interval was 5 seconds.
Yet very different results appear.

By method 1, lysis is complete in 30 seconds.

By method 2, lysis is complete in 25 minutes.

By method 3, lysis is complete in a time intermediate between the time by method 1 and that by method 2.

It is thus obvious that two different phenomena are being observed according to the order in which the components of the systems are mixed. It further becomes obvious that method 3 is not a satisfactory one; it gives irregular results, and is really a compromise between methods 1 and 2.

We shall accordingly vary the amount of lysozyme, mixing the system as in method 1, and observe the effect. In each of a series of tubes is placed 0.8 cc. of 1 in 100 sodium glycocholate; to each tube is added 0.4 cc. of suspension, and after 5 seconds, 0.4 cc of the 1 per cent solution of serum albumin.

The results are as under; the temperature was 18° C.

<table>
<thead>
<tr>
<th>Dilution 1 in</th>
<th>Time mins.</th>
<th>Dilution 1 in</th>
<th>Time mins.</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>1</td>
<td>400</td>
<td>0.95</td>
</tr>
<tr>
<td>200</td>
<td>2.5</td>
<td>600</td>
<td>0.85</td>
</tr>
<tr>
<td>300</td>
<td>1.5</td>
<td>1000</td>
<td>6.0</td>
</tr>
</tbody>
</table>

It will be found that very rapid lysis occurs in all the dilutions of glycocholate used.

If the same suspension be tested in a similar way after it has stood for some hours, the cells will be found to have undergone a change on standing.
This surprising result occurs with great regularity, and with care the stages through which the change passes can be made out. Below is an example in tabular form; the temperature at which the experiment was conducted was 18 °C, and the mixing performed by method 1.

<table>
<thead>
<tr>
<th>Dilution 1 in</th>
<th>Time after preparation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5 mins.</td>
</tr>
<tr>
<td>100</td>
<td>1.5</td>
</tr>
<tr>
<td>200</td>
<td>2.0</td>
</tr>
<tr>
<td>300</td>
<td>1.0</td>
</tr>
<tr>
<td>400</td>
<td>0.9</td>
</tr>
<tr>
<td>600</td>
<td>0.7</td>
</tr>
<tr>
<td>1000</td>
<td>2.0</td>
</tr>
</tbody>
</table>

All the times for lysis are in minutes.

To summarise this table in words, it appears that there may be two types of suspension; one which we may call sensitive, and which is haemolysed by the serum-albumin glycocholate mixture with great rapidity for all dilutions of lysin, and another which we may call insensitive which is not rapidly haemolysed by the mixture except in the region of 1 in 500 dilution. A sensitive suspension becomes insensitive on standing.

At this point it is convenient to mention one essential difference between a sensitive and an insensitive suspension. When Blood is drawn into citrated saline for the preparation of the suspension of cells, the resulting suspension is always sensitive. If the blood be drawn into saline (NaCl) instead of into cit-
rate, an insensitive suspension results. It may be suggested that the act of clotting has an influence on the suspension in this respect, for if the blood is drawn slowly from the finger, so that some clotting begins to occur, insensitive suspensions invariable result.

It is a curious thing that there is no difference between a sensitive and an insensitive suspension as regards the action of sodium glycocholate or of sodium taurocholate upon them; the difference exists only towards the mixture of serum-albumin and glycocholate. The above phenomenon occurs with the blood of man, of the dog, of the cat, the guinea pig, and the rabbit. The length of time during which a suspension remains sensitive varies; some remain sensitive for hours, others for less than 30 minutes.

We may now vary the concentration of serum-albumin in the mixture. As before the experiment was carried out at 18°C; the mixing by method 1.

<table>
<thead>
<tr>
<th>Albumin per cent.</th>
<th>Glycocholate, 1 in 100</th>
<th>400</th>
<th>600</th>
<th>1000</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>0.5</td>
<td>6.3</td>
<td>0.6</td>
<td>5.0</td>
</tr>
<tr>
<td>1</td>
<td>23.0</td>
<td>0.5</td>
<td>1.4</td>
<td>15.0</td>
</tr>
<tr>
<td>0.5</td>
<td>43</td>
<td>4.0</td>
<td>0.3</td>
<td>35.0</td>
</tr>
<tr>
<td>0.2</td>
<td>56</td>
<td>12</td>
<td>1.5</td>
<td>17.0</td>
</tr>
</tbody>
</table>

It will be plain that the phenomena are very complex, and incapable of analysis at this stage.
The protective action of the serum proteins.

Without going into detail, the following points may be noted.

1. Serum albumin, if added to a sensitive suspension, renders it insensitive; the albumin thus protects against the albumin-lysin mixture.

2. The same occurs with fresh serum.

3. The degree of insensitiveness conferred depends on several factors, among which is the quantity of serum or albumin added.

4. If the added albumin or serum be washed off in the centrifuge, the original state of the suspension is restored. The effect on the cells - if it is indeed an effect on the cells and not a more general effect in the haemolytic system - is accordingly not permanent.

5. The blood drawn from a rabbit's vein is normally productive of a sensitive suspension. If 5 cc. of a 2 per cent solution of serum albumin is injected into the vein of a distant part, the suspension prepared will be insensitive. The serum-albumin therefore confers a protection in vivo. It is as well to wash the cells only once in carrying out this experiment; if they are washed several times the protection appears to be lost, as we should expect from (4) above.

This very incomplete investigation outlines a large subject, and is introduced here because the point mentioned above recur again and again in the consideration of the action of the simple lysins. The most essential
observation is that the same substances - glycocholate and serum, for example - can be combined to produce an inactive haemolytic system, or a very active one, according to the way in which they are brought into contact with the cells.

When the serum is first mixed with the cells and the lysin added afterwards, the explanation is a simple one - lysin and serum combine to form an inactive compound. This will be proved beyond doubt in a following section.

When the lysin is added first, and the serum afterwards, the acceleration which occurs in much more difficult to explain. One thing is certain - the lysin has begun to attack the cells within the first short space of time; the addition of the serum apparently either increases the activity of the lysin, or weakens the cells. We shall see later that the latter explanation is probably the correct one, and that the added serum alters the resistance distribution of the cell population.

A very essential point is to remember that haemoglobin has an action similar to serum; this has been mentioned in Section 2. As haemoglobin is continually being liberated from the cells during haemolysis, it is obvious that we have in its action a complication of the reaction which takes place in the system.

Although we have dealt principally with the phenomena observed with glycocholate as a lysin, it is to be remembered that not dissimilar occurrences are
met with when the taurocholate is used. In many ways it is better to work with the glycocholate, owing to its greater stability, for with this lysin the phenomena are shown in a very striking way. For a complete investigation both lysins would require to be used; this we have not yet attempted, and, indeed, it is very doubtful whether the results would be worth the immense labour.
PART I.

SECTION IV.

Acceleration and inhibition.

General division of accelerators and inhibitors.

Group 1. Inhibitors.
   Sucrose.
   The temperature effect.
   Sodium hydrate.
   Arginine.

Group 2. Accelerators.
   Acetic acid.
   Glutaminic acid.
   Histidine monohydrochloride.
   Aspartic acid.
   Potassium chloride.

The mechanism of the inhibition.

Methods.
   Arginine and saponin
   Sucrose and saponin.
   Aspartic acid and saponin.
   Glutaminic acid and saponin.
In general, we can divide accelerators and inhibitors of haemolysis into four classes.

1. Inhibitors such as the sugars and certain bases; these act principally on the cells.

2. Accelerators such as the acids, acting principally on the cells.

3. Inhibitors acting on the lysin, such as serum, haemoglobin, etc; these are mainly colloids.

4. Accelerators acting probably on the cells, but in a curious way - as an instance we may take the action of serum on a haemolytic system in which glycочекolate is the lysin, when the serum is added after the addition of the lysin. Some mention of this phenomenon has been made in Section 3.

In this Section we propose to deal with classes 1 and 2 of the above - the simplest cases of inhibition and acceleration.

Group 1. Inhibitors.

Of the many substances which fall into this group we shall consider only one or two, leaving other cases to be considered afterwards.

1. Sucrose.

This substance in suitable concentration inhibits the action of saponin. In order to obtain quantitative results, we use the following technique.

The curve showing the relation of the time taken for complete haemolysis to the dilution of the
lysin acting is first obtained, the experiment being conducted, as are all mentioned in this section, at 25 C. A series of dilutions of saponin in saline (0.85 per cent NaCl) is prepared, each solution being 2.5 times as concentrated as is finally desired. Of each dilution 0.8 cc. is mixed with 0.8 cc of saline, the mixture warmed to 25° C., and 0.4 cc of cell suspension added. This cell suspension consists of the thrice washed cells from 1 cc of human blood, received into citrate and finally suspended in 20 cc of saline. The curve for saponin alone passes through the following points; (20,000, 0.75), (30,000, 1.75), (40,000, 3.4), (50,000, 5.6), (60,000, 10).

The curve for the lysin acting on the cells in the presence of 2 per cent sucrose is next obtained by exactly the same procedure, except that 0.8 cc of 5 per cent sucrose is substituted for the 0.8 cc of saline in the above. This concentration of sucrose is necessary in order to maintain the correct tonicity in the system. The curve passes through the following points; (20,000, 1.35), (30,000, 3.1), (40,000, 5.5), (50,000, 11).

We now draw up a table. Call $S$ the dilution of lysin added and $c$, its corresponding concentration - that is, the number of milligrammes actually in the tube of 2 cc content. The dilution of lysin, which, acting alone, would take the same time for the completion of lysis as is observed with the lysin plus the inhibitory agent, is $S_0$, and its corresponding concentration $c_0$. 


Call \((c_1 - c_2)\), \(x\).

Arranging the table, we get,

\[
\begin{array}{|c|c|c|c|c|}
\hline
\delta_1 & c_1 & \delta_2 & c_2 & x \\
\hline
20,000 & 0.100 & 27,000 & 0.074 & 0.026 \\
30,000 & 0.066 & 38,000 & 0.052 & 0.014 \\
40,000 & 0.050 & 50,000 & 0.040 & 0.010 \\
50,000 & 0.040 & 61,000 & 0.033 & 0.007 \\
\hline
\end{array}
\]

All these figures (except those for the dilutions) are in milligrammes.

Plotting \(x\) against \(c_2\), we have a series of points lying on a straight line, and since \(x = (c_1 - c_2)\) we may plot \(c_1\) against \(c_2\), when we shall get another straight line. It makes a small intercept on the \(c_2\) axis, and its equation is

\[c_1 = R(c_2 - k)\]

in which \(R\) is 1.46 and \(k\) is 0.005. This is the general expression which it will be shown is applicable to all haemolytic systems involving accelerators or inhibitors of classes 1 and 2.

Supposing that \(k\) were zero, the meaning of this expression would be that in a haemolytic system containing sucrose a concentration of lysin \(c_1\), produces haemolysis as if it were of concentration \(c_1/R\) and acting of the cells in the absence of sucrose — or, what amounts to the same thing, that the addition of sucrose increased the resistance of the cells to the lysin \(R\) times. It is not possible at this stage of the enquiry to say that the
The action of the inhibitor is exclusively on the cells; it is equally possible that the substance affects the activity of the lysin, or affects the manner in which the lysin is taken up by the cells. The best way to regard R is as measuring the resistance of the cells in the cell-saponin-sucrose system as compared with that of the cells in the cell-saponin system, without specifying which component of the system is affected by the addition of the sucrose.

The meaning of the constant k is less clear; it is sufficient to regard it as a small empirical constant.

The next point of interest is to enquire whether the values of R and k are the same at a different temperature. The curve for saponin acting alone at 35°C. is as follows; (30,000, 0.6), (40,000, 1.25), (50,000, 2.6), (60,000, 4.5), (70,000, 10), (80,000, 25). The curve for saponin plus 2 per cent sucrose is; (30,000, 1.2), (40,000, 2.6), (50,000, 5.5), (60,000, 13). From these curves the following data are compiled.

<table>
<thead>
<tr>
<th>$\delta_1$</th>
<th>$c_1$</th>
<th>$\delta_2$</th>
<th>$c_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>30,000</td>
<td>0.066</td>
<td>39,000</td>
<td>0.051</td>
</tr>
<tr>
<td>40,000</td>
<td>0.050</td>
<td>50,000</td>
<td>0.040</td>
</tr>
<tr>
<td>50,000</td>
<td>0.040</td>
<td>62,000</td>
<td>0.0322</td>
</tr>
<tr>
<td>60,000</td>
<td>0.033</td>
<td>73,000</td>
<td>0.0274</td>
</tr>
</tbody>
</table>

Plotting $c_1$ against $c_2$, we get a straight line, with the values of R as 1.46 and k as 0.005. The values of the two constants are therefore the same at the two
temperatures.

Proceeding in a similar way to the above we have investigated the inhibitory effect of concentrations of sucrose other than 2 per cent. In these cases it is most important to dissolve the sucrose in such a concentration of NaCl as will give a correct tonicity for the mixture, for the sucrose exerts an osmotic pressure of its own. This being done, the following values of R and of k were obtained for different concentrations of the inhibitory agent.

<table>
<thead>
<tr>
<th>Sucrose concentration per cent.</th>
<th>R</th>
<th>k</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>1.25</td>
<td>0.005</td>
</tr>
<tr>
<td>2.0</td>
<td>1.46</td>
<td>0.005</td>
</tr>
<tr>
<td>3.0</td>
<td>1.51</td>
<td>0.005</td>
</tr>
<tr>
<td>4.0</td>
<td>1.57</td>
<td>0.005</td>
</tr>
</tbody>
</table>

In all these cases it is to be noted that the concentrations are those in the 2 cc. of haemolytic system after the mixture of the components in complete. It will be seen that as the concentration increases R increases, but not in simple proportion; so far as the findings of the method show, k is constant. The method is, however, not such as would detect small variations in this latter constant.

In treating the curve obtained by plotting c₂ against c₁ we have considered the possibility of its being in reality a very flat curve. The most careful experiments have not, however, lent any
support to this possibility. It is to be remembered, as a most important point, that the line does not pass beyond a point whose coordinates are given by the positions of the asymptotes of the two experimental curves; it has no real existence near the origin at all.

2. Sodium hydroxide.

The addition of small amount of NaOH is enough to inhibit lysis by saponin or by the bile salts. In the case of sodium taurocholate as little as N/5,000 NaOH produces the effect, which is unmeasurable by these methods.

In the case of saponin, N/1250 NaOH gives results much like those given by sucrose. The values of the constants are \( R = 1.5 \) and \( k = 0.008 \). The pH of the solution was found to be 9.90.

3. Arginine.

In the course of the examination of a number of amino-acids for inhibition and acceleration effects, arginine was found to inhibit the lytic action of saponin and of sodium taurocholate. A solution of this amino-acid is alkaline, and the inhibition may therefore be compared with that of NaOH.

Saponin acting with N/1250 and saponin acting with 1 in 1250 arginine were found to give exactly the same curve, the plotting of \( c_1 \) against \( c_2 \) giving the same straight line with a value of \( R \) of 1.5 and a value of \( k \) of 0.008. As regards their inhibitory effects these two substances are therefore identical, in these concentrations. As regards their pH, how-
ever, they are by no means the same, for while the pH of the NaOH solution was found to be 9.90, that of the arginine solution of the same inhibitory power was 8.94. It is therefore to be concluded that the pH is not the only factor in the inhibition.

In view of findings to be recorded below, an important point should be noted. If the arginine be allowed to remain in contact with the cells for some time before the lysin is added, the resulting inhibition is very much greater than if the lysin and the arginine be mixed and the cells added. That this should be so is not in any way surprising, but it is rather remarkable that under these circumstances the amount of inhibition tends to be independent of the amount of inhibitor used. Arginine 1 in 600, 1 in 2500, and 1 in 4000 all give the same amount of inhibition if the cells are left in contact with these concentrations for about 5 minutes at 25°C before the addition of the saponin. When the experiment is performed with the additions in this order, it is not possible to produce a greater than a greater inhibition than is exerted by one of these concentrations; the full effect seems to be attained by such a low concentration as 1 in 4000, below which the inhibition fails. We are unable to explain this result, and merely note it in passing. A similar result has been obtained for several other cases of inhibition, the effect appearing to be independent of the concentration of inhibitory substance over
quite a considerable range.

These are only three of the many substances which produce inhibition and whose action can be described by a linear expression. We have found this expression applicable to the inhibitory effect of most of the sugars and also to the action of many salts. It is unnecessary to deal with these here—they are all examples of the same type of inhibition.

It may be mentioned that the following amino-acids are inert:

<table>
<thead>
<tr>
<th>Glycine</th>
<th>Alanine</th>
<th>Phenylalanine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Valine</td>
<td>Cystine</td>
<td>Sarcosine</td>
</tr>
<tr>
<td>Cystine</td>
<td>Taurine</td>
<td>Leucine</td>
</tr>
<tr>
<td>Guanidine</td>
<td>Carnosine</td>
<td></td>
</tr>
</tbody>
</table>

and that the following sugars produce inhibition,

<table>
<thead>
<tr>
<th>Sucrose</th>
<th>Glucose</th>
<th>Mannose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trehalose</td>
<td>Lactose</td>
<td>Maltose</td>
</tr>
<tr>
<td>Galactose</td>
<td>Fructose</td>
<td></td>
</tr>
</tbody>
</table>

while xylose is inert, and also arabinose.

Group 2. Accelerators.

Acceleration of lysis may be produced by most acid substances, some of which we shall consider here.

1. Acetic acid.

The investigation of the acceleration brought about by this, or any other, accelerating agent is similar to that of an inhibition. The curve for the lyasin acting alone is first obtained, and thereafter the curve for the lyasin together with the accelerator.
To give an example, take the case of the accelerator N/500 acetic acid, acting with saponin.

The saponin-cell system gives the curve; (20,000, 0.75), (30,000, 1.2), (40,000, 2.3), (50,000, 3.5), (60,000, 11), (70,000, 20).

The saponin-acetic-acid-cell system has the curve; (40,000, 0.5), (50,000, 0.75), (60,000, 1.1), (70,000, 1.75), (80,000, 2.3).

From the two curves we get the following data. Here \( x = (c_2 - c_1) \).

<table>
<thead>
<tr>
<th>( c_1 )</th>
<th>( \delta_1 )</th>
<th>( c_2 )</th>
<th>( \delta_2 )</th>
<th>( x )</th>
</tr>
</thead>
<tbody>
<tr>
<td>40,000</td>
<td>0.050</td>
<td>15,000</td>
<td>0.133</td>
<td>0.073</td>
</tr>
<tr>
<td>50,000</td>
<td>0.040</td>
<td>20,000</td>
<td>0.100</td>
<td>0.060</td>
</tr>
<tr>
<td>60,000</td>
<td>0.033</td>
<td>25,000</td>
<td>0.071</td>
<td>0.038</td>
</tr>
<tr>
<td>70,000</td>
<td>0.028</td>
<td>30,000</td>
<td>0.058</td>
<td>0.030</td>
</tr>
<tr>
<td>80,000</td>
<td>0.025</td>
<td>40,000</td>
<td>0.050</td>
<td>0.025</td>
</tr>
</tbody>
</table>

Plotting \( c_1 \) against \( c_2 \) there results, as in the case of an inhibition, a straight line. The equation is

\[ c_1 = R(c_2 - k), \]

where \( R = 0.29 \) and \( k = -0.039 \).

This is the same type of expression as is applicable to an inhibition; where inhibition occurs, \( R \) is greater than 1, and \( k \) is positive; where acceleration occurs, \( R \) is less than 1, and \( k \) is negative. The meaning to be attached to \( R \) is similar to that meaning which it has in the case of an inhibition; \( k \) we regard meantime as a small empirical constant.

The pH of this acetic acid in saline was found to be 3.73.
I. Sucrose, 2 per cent.—Inhibition.
II. Acetic Acid, N/500—Acceleration.
III. Serum, 0.02 c.c.—Inhibition.
IV. Serum, 0.0025 c.c.—Inhibition.

Graph showing relation of c₁ and c₂ in the case of several inhibiting and accelerating.
Note the curve for serum, as compared with the straight lines for the other substances.
2. Glutaminic acid. In concentrations of 1 in 10,000 this amino acid, acting with saponin, gives a remarkable acceleration. The relation between \( c_1 \) and \( c_2 \) is, as before, a linear one, with the values of \( R \) of 0.373 and \( k \) of 0.027. Experiments with this accelerator are very satisfactory, and to show how excellent is the fit of the formula, we give the following results.

<table>
<thead>
<tr>
<th>( \text{c, exper.} )</th>
<th>( \text{c, calc.} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.066</td>
<td>0.064</td>
</tr>
<tr>
<td>0.056</td>
<td>0.047</td>
</tr>
<tr>
<td>0.040</td>
<td>0.040</td>
</tr>
<tr>
<td>0.033</td>
<td>0.035</td>
</tr>
<tr>
<td>0.030</td>
<td>0.029</td>
</tr>
<tr>
<td>0.025</td>
<td>0.025</td>
</tr>
<tr>
<td>0.020</td>
<td>0.021</td>
</tr>
</tbody>
</table>

The errors are small, considering the amount of calculation necessary in deriving the values of the constants.

3. Histidine monochlorohydrochloride. This acid substance accelerates haemolysis by saponin, and the relation between \( c_1 \) and \( c_2 \) is of the type described above. The value of \( R \) is 0.52, and the value of \( k \) 0.017, when 1 in 2500 histidine is used.

4. Aspartic acid. This amino acid behaves much like glutaminic acid, accelerating the lysis of the bile salts, of saponin, or of the soaps. Quantitative work
must be confined to saponin, for the acid precipitates both the bile salts and the soaps. With saponin 1 in 2500 aspartic acid produces a typical acceleration, giving values of $R$ of 0.27 and of $k$ of 0.015. Half the quantity of accelerator gives the values $R = 0.35$, $k = -0.014$. Even such a low concentration as 1 in 20,000 of aspartic acid gives appreciable acceleration; greater concentrations than 1 in 2500 produce not only acceleration, but lysis of themselves.

5. Potassium Chloride. In the case of human cells the replacement of Na by K in the saline results in an acceleration.

This may be investigated by plotting the curves for the two systems (1) saponin, 0.8 cc, 0.85 per cent NaCl, 0.8 cc, suspension, 0.4 cc; (2) saponin, 0.8 cc, M/6.8 KCl, 0.8 cc, suspension 0.4 cc. The tonicities of the two systems is the same - but in the second 2/5ths. of the Na content is replaced by K.

In a large number of experiments the following values were obtained for $R$, the resistance constant - 0.8, 0.75, 0.77, 0.81, 0.79, 0.83. Values of $k$, lying between 0.003 and 0.006 were obtained in conjunction with these values of $R$.

In order to show the type of graphical result obtained, the lines for sucrose and for acetic acid are shown in the graph attached. With them are shown two curves for serum as an inhibitory agent - a matter to be discussed.
in the next section.

The mechanism of the inhibition.

In a haemolytic system such as these discussed there are only three components - the cells, the lysin, and the added substance, accelerator of inhibitor. The observations on the last component may now be amplified by a method which indicates its mode of action. The essential point is whether the added substance acts on the lysin or on the cells.

The principle of the method used is an obvious one. The cells are allowed to remain in contact with the accelerator or the inhibitor for a certain period under certain conditions, and are then brought into a haemolytic system in which none of the accelerator or inhibitor are present. If in this system there is acceleration or inhibition, this is taken as evidence that the effect of the added substance is on the cells themselves; if the haemolytic system is the same as one containing untreated cells, it is taken that the cells have not been affected.

Below the method is shown worked out in detail.

1. Arginine and saponin.

To 10 cc. of 1 in 1000 arginine in saline is added 5 cc. of the standard cell suspension. The mixture is allowed to remain at 25°C. for 30 minutes, occasional stirring being advisable; at the end of this
time 10 cc. of the mixture is transferred to a tared flask, great care being taken that the cells are uniformly distributed when the transference is made. The fluid from the flask is divided into two centrifuge tubes, each of 10 cc. capacity, and 5 cc. of saline added to each. The contents of the tubes are centrifuged, the supernatant fluid pipetted off, and the cells together with the small amount of fluid which remains replaced in the flask. The tubes are washed out with saline to remove all cells, and the washings added to the flask. The 10 cc volume is then made up with saline, the cell thoroughly distributed throughout the fluid, and the suspension used to plot the time-dilution curves.

Three curves are now plotted. The first is that of saponin acting alone on the untreated cell suspension. The second is obtained by adding to 0.8 cc. of a series of saponin dilutions 1.2 cc. of the mixture of the cells and arginine which is left over from the 15 cc. of the mixture after withdrawal of the 10 cc. required to fill the tared flask, and the third by mixing with a series of saponin dilutions the suspension obtained after the washing of the cells. In each of these systems the quantity of cells is the same - in the first they are untreated, in the second they are in the presence of arginine, and in the third they have been treated with arginine, but the inhibitory agent washed away.

From the plotted curves is then determined the value of R for the haemolytic system containing
arginine, and the value of R for the system containing the cells which have been treated with arginine and subsequently washed. As an illustration, the figures for one experiment may be given.

<table>
<thead>
<tr>
<th>Arginine-saponin-cell system.</th>
<th>Saponin-washed-cell system.</th>
</tr>
</thead>
<tbody>
<tr>
<td>( c_1 )</td>
<td>( c_2 )</td>
</tr>
<tr>
<td>0.066</td>
<td>0.049</td>
</tr>
<tr>
<td>0.050</td>
<td>0.038</td>
</tr>
<tr>
<td>0.040</td>
<td>0.031</td>
</tr>
</tbody>
</table>

Gives \( R_1 = 1.55 \) \( k_1 = 0.005 \) \( R_2 = 1.4 \) \( k_2 = 0.005 \)

In this experiment the concentration of arginine added to the cells was 1 in 1000; the concentration acting was therefore 1 in 1500.

This result shows that the effect of the arginine is principally on the cells themselves for it persists after by far the greater part of the inhibitor has been washed from the treated cells. The whole of the arginine is not however removed, and it is necessary for us to know how much of it remains with the cells after the washing.

If a represents the quantity of fluid in which the cells are suspended before the washing, and \( x_0 \) the concentration of inhibitor therein, the absolute amount is \( ax_0 \). A quantity of washing fluid \( m \) is added, centrifuging carried out, and a quantity of fluid \( b \) kept in contact with the cells.
the amount of inhibitor in this fluid will be

\[ x = \frac{b}{a} \left( \frac{a}{m + a} \right) x_0 \]

This will also be the amount of inhibitor in the suspension of washed cells.

In this case we have \( b \) equal to 10 cc, and \( x \) equal to 1 milligramme per cc. The value of \( m \) is 10 cc., and \( b \) may be estimated as no greater than 0.5 cc. Hence \( x = 0.25 \) milligrammes - the amount of arginine in the fluid in which the cells are resuspended, i.e. 10 cc. This gives a dilution of arginine of 1 in 40,000 in the washed cell suspension - an amount which is negligible. So far as the inhibitory effect is concerned, it may be taken that the arginine is all removed in one washing. Any increased resistance in the cells is accordingly due to some change produced in them during their 30 minute contact with the arginine.

The removal of the inhibitor from the haemolytic system and the washing of the cells result in a fall in the inhibition from a value of \( R_l \) = 1.55 to \( R_l \) = 1.4. To compare the inhibitory effects described by the two constants, we take the ratio

\[ \frac{(1 - 1/R_l)}{(1 - 1/R_l)} \]

a formula which is correct only is \( k_1 = k_2 \). In this case it is 0.8; we may therefore conclude that of the total inhibition produced by the arginine, 0.8 can be explained by the effect on the cells themselves, while the remainder, 0.2 of the total, is unaccounted for.

2. Sucrose and saponin.

Proceeding in the same way, the
effect of a 10 per cent solution of sucrose was determined. The cells were allowed to remain in contact with the sucrose for 30 minutes at 25°C, and the effect determined. Comparison of the curves gave $R_1 = 1.35$, $R_2 = 1.3$, with $k_1 = k_2 = 0.004$. Of the total inhibition brought about 0.9 can be accounted for by a direct action on the cells, while 0.1 disappears on washing the cells.

4. Aspartic acid and saponin.

The method outlined above can be applied equally well in the case of an accelerator. For 1 in 3000 aspartic acid we obtained $R_1 = 0.31$, $R_2 = 0.5$, and $k_1 = k_2 = 0.004$. Thus only 0.46 of the total effect remains on washing – the effect of the acid appears to be on the cells themselves to some extent.

5. Glutaminic acid and saponin.

Here we found 0.58 of the total acceleration remaining after washing of the cells. This is a small figure compared with the large effect on the cells in the cases of the inhibitors.

These methods are, of course, applicable to a great number of substances – indeed, to any two haemolytic systems, in which the lysin is the same, and which differ from one another in activity. In the following sections many examples will be found of the solution of problems in terms of the resistance constant, $R$. 
Time Solution curves for the systems:

Saponin - cell.
Saponin - cell - sucrose
Saponin - cell - sucrose.

at 25°C.
Relation of $c_1$ and $c_2$ in saponin sucrose system.
The slope of the line is $R$, and equals to 1.46.
Relation of $c_1$ and $c_2$ for saponin - cell-
acetic acid system.
Relations for 3 accelerators.
Relations for a number of chlorides.
PART I.

SECTION V.

The inhibitory effect of blood serum.

The nature of the inhibitory substance.

Methods.

The inhibitory power of normal human serum.

The inhibitory power of the serum of animals.

The effect of drying the serum.

Diurnal variation.

Changes on exposure to air.

Inhibition to saponin and to taurocholate.

Quantitative study.

The expression for the relation of the lysin inactivated.

Values of constants for various dilutions.
It has been long known that blood serum has an inhibitory effect on the haemolysis produced by many substances, notably saponin and the bile salts. Ramson in 1901 observed that cholesterol inhibits the action of saponin, and attributed the inhibitory effect of serum to the contained cholesterol. The quantities of cholesterol contained in the solutions used in his experiments are far greater than occur in serum, and the experiments are inconclusive for that reason. Bayer, in 1907 investigated the inhibitory effect produced by serum on the lysis of the bile salts. He found that lecithin, but not cholesterol, inhibits this action, but not in the quantities which occur in the blood, and that the proteins of serum are responsible for the effect. He calls attention to the results of v. Eksler who found that serum globulin inhibits the action of tetanoylsin, and also those of v. Liebermann, who finds that the lysis by soaps is prevented by serum albumin. Bayer's results are in the main confirmed by Sellards.

In all these cases the results are not reliable, for adequate quantitative methods were not used.

The nature of the inhibitory substance.

Before proceeding to the quantitative estimations, it is necessary to know which constituents of serum are responsible for the inhibition of the action of saponin and of the bile salts respectively. Bayer's
results might be taken as conclusive were it not for two considerations; (1) Bayer filtered most of the solutions of bile salts and the lecithin-bile salt mixtures, whose haemolytic activity he wished to determine, through a Berkefeld filter. He states that this procedure has no effect on the time taken by these solutions to bring about lysis. This is a fallacy, for a solution of sodium taurocholate will not pass even through a filter paper without losing some of its haemolytic activity, while passage through a Berkefeld causes a great change. (2) Bayer's quantitative methods were very rough; he refers to 'slight haemolysis', 'considerable haemolysis', etc., and accordingly would be able to detect only very marked degrees of inhibition. The same remark applies to the experiments of Seilards.

The experiments of Bayer have accordingly been repeated, using the more exact methods outlined below. The results may be expressed as a series of conclusions.

1. Deproteinised serum exercises very much less inhibition than does serum before deproteinisation, and this is the case whether the lysin is saponin or taurocholate. Serum diluted 1 in 200 to 1 in 300 will give quite a measurable degree of inhibition; after deproteinisation, it will not inhibit in a dilution greater than 1 in 10. Bayer's statement that the proteins are the principal inhibitory substances is thus confirmed.

2. As Bayer observes, euglobulin, pseudo-globulin, and
serum albumin are all inhibitory.

3. The digestion of serum protein by trypsin or pepsin, resulting as it does in the disappearance of the inhibitory power, must not be taken to be a direct proof that the proteins are the inhibitory substances. The inhibitory power disappears even when there is still a good deal of protein undigested. The explanation is that the digestion results in the production of substances which accelerate haemolysis by saponin and the bile salts, this acceleration balancing the inhibition caused by the remaining proteins.

4. If the deproteinised serum be extracted with ether, the etherial extract, when evaporated to dryness, yields a residue which when taken up in saline proves to be inhibitory to both saponin and to taurocholate. The non-protein inhibitory substances are therefore ether soluble.

5. If the etherial extract be evaporated to dryness and extracted with acetone, the acetone will extract a substance which is inhibitory to saponin lysis, but which has no effect of bile salt lysis. This substance is probably cholesterol.

6. The substance not extracted by acetone has an inhibitory effect on taurocholate lysis; it is probably lecithin. It has no effect on saponin haemolysis.

It thus appears that there are three inhibitory substances in serum at least - proteins, lecithin, and cholesterol. The proteins inhibit both lysis by
Saponin and lysis by taurocholate; the lecithin inhibits the bile salts and not saponin, while the cholesterol inhibits saponin and not the bile salts. The inhibition produced by the lipoids is, in any case, exceedingly small compared with that brought about by the proteins.

It is desirable to investigate the phenomenon of inhibition by serum quantitatively, and this can be done by methods devised for the quantitative investigation of problems in haemolysis generally.

Methods.

The following solutions, etc., are required.

1. A standard suspension of cells, preferably human. The standard is made by suspending the thrice washed cells from 1 cc. of blood in 20 cc. of saline (0.85 per cent NaCl).

2. A series of dilutions of saponin in saline, ranging from 1 in 10,000 to 1 in 60,000. Each solution is made 2.5 times as strong as it is labelled - the label then gives the strength of the saponin in actual contact with the cells. This point has been dealt with already.

3. A similar series of taurocholate solutions, from 1 in 1,000 to 1 in 6,000. These should be freshly prepared. Two points should be noted; the salt should be dry and pure, and the weighing should be done in a bottle, for taurocholate is hygroscopic. It is advisable to keep the solution in a concentration of about 1 per cent, and to make the higher dilutions only as required.
Time dilution curves are plotted for (a) taurocholate or saponin acting alone on red cells, and (b) taurocholate or saponin acting in the presence of serum. The serum is introduced into the system exactly as we introduce any other inhibitor.

As in the case of other inhibitors, if \( c_i \) is the concentration of lysin added, and \( c_a \) the concentration apparently acting (as judged from the time taken for complete lysis) then \( (c_i - c_a) \) is equal to \( x \), the quantity neutralised.

The inhibitory power of normal human serum.

Twenty sera were examined, all being obtained from normal persons. The following table expresses the number of milligrammes of lysis neutralised by 0.1 cc of serum diluted 1 in 10. The haemolytic agent was saponin in a dilution of 1 in 25,000; the experiment was carried out at 25°C.

<table>
<thead>
<tr>
<th>Serum</th>
<th>Milligrammes neutralised</th>
<th>Serum</th>
<th>Milligrammes neutralised</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.0423</td>
<td>11</td>
<td>0.0533</td>
</tr>
<tr>
<td>2</td>
<td>0.0510</td>
<td>12</td>
<td>0.0566</td>
</tr>
<tr>
<td>3</td>
<td>0.0467</td>
<td>13</td>
<td>0.0467</td>
</tr>
<tr>
<td>4</td>
<td>0.0455</td>
<td>14</td>
<td>0.0433</td>
</tr>
<tr>
<td>5</td>
<td>0.0464</td>
<td>15</td>
<td>0.0467</td>
</tr>
<tr>
<td>6</td>
<td>0.0625</td>
<td>16</td>
<td>0.0546</td>
</tr>
<tr>
<td>7</td>
<td>0.0616</td>
<td>17</td>
<td>0.0566</td>
</tr>
<tr>
<td>8</td>
<td>0.0650</td>
<td>18</td>
<td>0.0623</td>
</tr>
<tr>
<td>9</td>
<td>0.0616</td>
<td>19</td>
<td>0.0467</td>
</tr>
<tr>
<td>10</td>
<td>0.0555</td>
<td>20</td>
<td>0.0620</td>
</tr>
</tbody>
</table>
It will thus be seen that the inhibitory power of the normal human serum is remarkably constant. The average inhibition is 0.0534 milligrammes per 0.1 cc. of serum diluted 1 in 10.

Although the inhibitory power has been tested in several conditions, no striking variation has been found.

The inhibitory power of the serum of animals.

The table below expresses the results in a similar manner to that used for the expression of the results for human serum.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Cat 1</td>
<td>0.027</td>
<td>Rabbit 1</td>
<td>0.035</td>
</tr>
<tr>
<td>2</td>
<td>0.022</td>
<td>2</td>
<td>0.027</td>
</tr>
<tr>
<td>3</td>
<td>0.030</td>
<td>Guinea-pig</td>
<td>0.037</td>
</tr>
<tr>
<td>4</td>
<td>0.043</td>
<td>Rat 1</td>
<td>0.021</td>
</tr>
<tr>
<td>5</td>
<td>0.032</td>
<td>2</td>
<td>0.023</td>
</tr>
<tr>
<td>6</td>
<td>0.067</td>
<td>3</td>
<td>0.030</td>
</tr>
<tr>
<td>7</td>
<td>0.030</td>
<td>Horse 1</td>
<td>0.043</td>
</tr>
<tr>
<td>8</td>
<td>0.042</td>
<td>2</td>
<td>0.052</td>
</tr>
<tr>
<td>9</td>
<td>0.037</td>
<td>3</td>
<td>0.050</td>
</tr>
<tr>
<td>10</td>
<td>0.039</td>
<td>Mouse</td>
<td>0.032</td>
</tr>
</tbody>
</table>

These figures, which are rather too few to justify the drawing of conclusions, nevertheless indicate that the inhibitory power of the sera of these animals is lower
than that of man. They are nevertheless markedly inhibitory, 0.1 cc of the serum diluted 1 in 10 neutralising 0.02 to 0.05 milligrammes of saponin.

The effect of drying the serum.

In the following table the sera of man and of the cat are compared, in the fresh state and dried by the method of Leers.

<table>
<thead>
<tr>
<th>Serum</th>
<th>Milligrammes neutralised.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fresh</td>
</tr>
<tr>
<td>1</td>
<td>0.0467</td>
</tr>
<tr>
<td>2</td>
<td>0.0443</td>
</tr>
<tr>
<td>3</td>
<td>0.0467</td>
</tr>
<tr>
<td>4</td>
<td>0.0546</td>
</tr>
<tr>
<td>5</td>
<td>0.0566</td>
</tr>
<tr>
<td>6</td>
<td>0.0523</td>
</tr>
</tbody>
</table>

It is very apparent that drying causes a loss of inhibitory power. This occurs even if the sera are dried in vacuo (sera 4, 5, 6.)

Diurnal variation.

In order to see if there is any diurnal variation, or variation from day to day, the sera of four normal persons were examined daily for two weeks. There was no significant variation discovered; such slight fluctuations as occurred might well have been due to experimental error. It is unnecessary to reproduce the figures.
Changes on exposure to air.

It is important to use fresh, and if possible sterile, sera for these tests, for if a serum has been exposed to the air, changes occur in it causing variation in the amount of inhibition produced.

It is unnecessary to produce a mass of figures in support of this almost obvious fact, but we have examined 10 sera on successive days, and come to the following conclusions:

(a) As soon as the serum becomes turbid, there is a loss of inhibitory power.

(b) After a few days, the serum being now thoroughly cloudy, the inhibition appears to increase once more.

(c) Sera kept on ice retain their inhibitory power unchanged for a long time.

Inhibition to saponin and to taurocholate.

Since the degree of inhibition so largely depends on the contained serum proteins, and since these affect the bile salts as well as saponin, it is to be expected that the sera which are most inhibitory to saponin would also be most inhibitory to the bile salts. This is the case; below are the figures for ten sera, all from cats, showing the inhibition produced on saponin and on taurocholate haemolysis respectively. The saponin used was 1 in 25,000; the taurocholate 1 in 2,500. The results are in milligrammes neutralised by 0.1 cc. of serum diluted 1 in 10.
<table>
<thead>
<tr>
<th>Serum</th>
<th>Saponin</th>
<th>Taurocholate</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.0140</td>
<td>0.25</td>
</tr>
<tr>
<td>2</td>
<td>0.0300</td>
<td>0.42</td>
</tr>
<tr>
<td>3</td>
<td>0.0400</td>
<td>0.48</td>
</tr>
<tr>
<td>4</td>
<td>0.0320</td>
<td>0.40</td>
</tr>
<tr>
<td>5</td>
<td>0.0670</td>
<td>0.67</td>
</tr>
<tr>
<td>6</td>
<td>0.0420</td>
<td>0.48</td>
</tr>
<tr>
<td>7</td>
<td>0.0320</td>
<td>0.50</td>
</tr>
<tr>
<td>8</td>
<td>0.0320</td>
<td>0.48</td>
</tr>
<tr>
<td>9</td>
<td>0.0370</td>
<td>0.48</td>
</tr>
<tr>
<td>10</td>
<td>0.0320</td>
<td>0.44</td>
</tr>
</tbody>
</table>

The table shows that the parallelism is not quite complete; occasionally a serum will be found which is less inhibitory to one or the other haemolysins than one would expect.

It is difficult to account for this fact.

The relation of the the quantity of lysin neutralised to the quantity producing lysis.

It has been seen that the effect of serum is to lengthen the time required for complete haemolysis. In the case of the bile salts, the addition of serum causes a slight cloud to appear in the previously clear solutions; this cloud is the more marked the more concentrated the solution of the lysin. It may well be that the cloud indicates the formation of a non-haemolytic
substance; there is no opportunity of testing whether this is so or not, for it is impossible to filter off the fine precipitate, both because of its fineness and also because the bile salts lose their haemolytic power when filtered. It is therefore necessary to determine the amount of lysin neutralised by indirect methods.

In order to do so, the time-dilution curve for the lysin acting in the absence of serum, and the time dilution curve for the lysin acting in the presence of a very small amount of serum, are obtained in the usual way. From the curves, the table following is drawn up, much as described in Section 4 of this part of the thesis.

<table>
<thead>
<tr>
<th>$S_1$</th>
<th>$c_1$</th>
<th>$T$</th>
<th>$S_2$</th>
<th>$c_2$</th>
<th>$x$</th>
<th>$x_{\text{calc.}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1000</td>
<td>2.00</td>
<td>1.2</td>
<td>1400</td>
<td>1.43</td>
<td>0.57</td>
<td>0.57</td>
</tr>
<tr>
<td>2000</td>
<td>1.00</td>
<td>4.2</td>
<td>4300</td>
<td>0.58</td>
<td>0.42</td>
<td>0.41</td>
</tr>
<tr>
<td>2500</td>
<td>0.80</td>
<td>9.0</td>
<td>4900</td>
<td>0.408</td>
<td>0.39</td>
<td>0.35</td>
</tr>
<tr>
<td>3000</td>
<td>0.66</td>
<td>24.0</td>
<td>6000</td>
<td>0.333</td>
<td>0.33</td>
<td>0.33</td>
</tr>
</tbody>
</table>

This is the result of an actual experiment, with sodium taurocholate as the lysin, in which the inhibition was brought about by 0.01 cc of cat serum.

The relation of the amount of lysin neutralised, $x$, and the quantity acting on the cells, $c_2$, is given by the expression

$$x = A \cdot c_2^n$$

In the above experiment $A$ equals 0.5, while $n$ equals 2.66. This expression gives the experimental results with sufficient accuracy, within the limits of experiment.
Proceeding in this way, the following results have been found for various sera.

<table>
<thead>
<tr>
<th>Animal</th>
<th>n</th>
<th>A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Man</td>
<td>2.00</td>
<td>0.26</td>
</tr>
<tr>
<td></td>
<td>1.50</td>
<td>0.414</td>
</tr>
<tr>
<td></td>
<td>1.33</td>
<td>0.445</td>
</tr>
<tr>
<td></td>
<td>1.18</td>
<td>0.315</td>
</tr>
<tr>
<td></td>
<td>1.33</td>
<td>0.455</td>
</tr>
<tr>
<td>Cat</td>
<td>1.54</td>
<td>0.517</td>
</tr>
<tr>
<td></td>
<td>1.125</td>
<td>0.699</td>
</tr>
<tr>
<td>Rabbit</td>
<td>1.125</td>
<td>1.020</td>
</tr>
<tr>
<td>Average</td>
<td>1.391</td>
<td>0.543</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Animal</th>
<th>n</th>
<th>A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Man</td>
<td>2.66</td>
<td>0.500</td>
</tr>
<tr>
<td></td>
<td>2.35</td>
<td>0.538</td>
</tr>
<tr>
<td></td>
<td>1.88</td>
<td>0.594</td>
</tr>
<tr>
<td></td>
<td>2.40</td>
<td>0.668</td>
</tr>
<tr>
<td></td>
<td>3.30</td>
<td>0.525</td>
</tr>
<tr>
<td>Cat</td>
<td>2.26</td>
<td>0.602</td>
</tr>
<tr>
<td></td>
<td>2.50</td>
<td>0.603</td>
</tr>
<tr>
<td>Rabbit</td>
<td>2.20</td>
<td>0.642</td>
</tr>
<tr>
<td>Average</td>
<td>2.44</td>
<td>0.648</td>
</tr>
</tbody>
</table>
We may now deal with the values which these constants assume under various conditions.

The first point to be observed is that the values of the constants vary with the amount of serum used to produce inhibition. This is shown in the following set of tables, giving that data for horse serum used with saponin at 25° C.

<table>
<thead>
<tr>
<th></th>
<th>$\delta_1$</th>
<th>$c_1$</th>
<th>T.</th>
<th>$\delta_2$</th>
<th>$c_2$</th>
<th>x.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saponin plus</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-04 c.c. serum</td>
<td>10,000</td>
<td>0-200</td>
<td>1-8</td>
<td>33,000</td>
<td>0-061</td>
<td>0-139</td>
</tr>
<tr>
<td></td>
<td>13,000</td>
<td>0-154</td>
<td>5-0</td>
<td>55,000</td>
<td>0-036</td>
<td>0-118</td>
</tr>
<tr>
<td></td>
<td>15,000</td>
<td>0-133</td>
<td>12-0</td>
<td>69,000</td>
<td>0-283</td>
<td>0-164</td>
</tr>
<tr>
<td>Saponin plus</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-02 c.c. serum</td>
<td>10,000</td>
<td>0-200</td>
<td>0-7</td>
<td>20,000</td>
<td>0-100</td>
<td>0-100</td>
</tr>
<tr>
<td></td>
<td>15,000</td>
<td>0-133</td>
<td>2-1</td>
<td>36,000</td>
<td>0-050</td>
<td>0-078</td>
</tr>
<tr>
<td></td>
<td>20,000</td>
<td>0-100</td>
<td>5-0</td>
<td>53,000</td>
<td>0-036</td>
<td>0-064</td>
</tr>
<tr>
<td></td>
<td>25,000</td>
<td>0-080</td>
<td>13-0</td>
<td>70,000</td>
<td>0-028</td>
<td>0-042</td>
</tr>
<tr>
<td>Saponin plus</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-016 c.c. serum</td>
<td>15,000</td>
<td>0-133</td>
<td>1-5</td>
<td>30,000</td>
<td>0-066</td>
<td>0-066</td>
</tr>
<tr>
<td></td>
<td>20,000</td>
<td>0-100</td>
<td>3-2</td>
<td>44,000</td>
<td>0-045</td>
<td>0-055</td>
</tr>
<tr>
<td></td>
<td>25,000</td>
<td>0-080</td>
<td>5-3</td>
<td>59,000</td>
<td>0-034</td>
<td>0-046</td>
</tr>
<tr>
<td></td>
<td>30,000</td>
<td>0-066</td>
<td>16-0</td>
<td>71,000</td>
<td>0-028</td>
<td>0-038</td>
</tr>
<tr>
<td>Saponin plus</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-01 c.c. serum</td>
<td>20,000</td>
<td>0-100</td>
<td>2-0</td>
<td>35,000</td>
<td>0-057</td>
<td>0-043</td>
</tr>
<tr>
<td></td>
<td>25,000</td>
<td>0-080</td>
<td>3-2</td>
<td>45,000</td>
<td>0-044</td>
<td>0-036</td>
</tr>
<tr>
<td></td>
<td>30,000</td>
<td>0-066</td>
<td>5-5</td>
<td>57,000</td>
<td>0-035</td>
<td>0-031</td>
</tr>
<tr>
<td></td>
<td>35,000</td>
<td>0-057</td>
<td>10-0</td>
<td>60,000</td>
<td>0-030</td>
<td>0-027</td>
</tr>
<tr>
<td>Saponin plus</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-0083 c.c. serum</td>
<td>20,000</td>
<td>0-100</td>
<td>1-4</td>
<td>32,000</td>
<td>0-062</td>
<td>0-038</td>
</tr>
<tr>
<td></td>
<td>25,000</td>
<td>0-080</td>
<td>2-4</td>
<td>40,000</td>
<td>0-050</td>
<td>0-030</td>
</tr>
<tr>
<td></td>
<td>30,000</td>
<td>0-066</td>
<td>3-8</td>
<td>49,000</td>
<td>0-040</td>
<td>0-026</td>
</tr>
<tr>
<td></td>
<td>35,000</td>
<td>0-057</td>
<td>5-9</td>
<td>56,000</td>
<td>0-035</td>
<td>0-022</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>$\delta_1$</th>
<th>$c_1$</th>
<th>T.</th>
<th>$\delta_2$</th>
<th>$c_2$</th>
<th>x.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saponin plus</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-005 c.c. serum</td>
<td>25,000</td>
<td>0-080</td>
<td>2-1</td>
<td>36,000</td>
<td>0-055</td>
<td>0-025</td>
</tr>
<tr>
<td></td>
<td>30,000</td>
<td>0-066</td>
<td>3-0</td>
<td>44,000</td>
<td>0-045</td>
<td>0-021</td>
</tr>
<tr>
<td></td>
<td>35,000</td>
<td>0-057</td>
<td>4-0</td>
<td>51,000</td>
<td>0-039</td>
<td>0-018</td>
</tr>
<tr>
<td></td>
<td>40,000</td>
<td>0-050</td>
<td>5-2</td>
<td>56,000</td>
<td>0-035</td>
<td>0-015</td>
</tr>
<tr>
<td></td>
<td>50,000</td>
<td>0-040</td>
<td>11-0</td>
<td>67,000</td>
<td>0-029</td>
<td>0-011</td>
</tr>
<tr>
<td>Saponin plus</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-0045 c.c. serum</td>
<td>30,000</td>
<td>0-066</td>
<td>2-4</td>
<td>41,000</td>
<td>0-048</td>
<td>0-018</td>
</tr>
<tr>
<td></td>
<td>35,000</td>
<td>0-057</td>
<td>3-5</td>
<td>46,000</td>
<td>0-043</td>
<td>0-014</td>
</tr>
<tr>
<td></td>
<td>40,000</td>
<td>0-050</td>
<td>4-0</td>
<td>52,000</td>
<td>0-038</td>
<td>0-012</td>
</tr>
<tr>
<td></td>
<td>50,000</td>
<td>0-040</td>
<td>10-0</td>
<td>66,000</td>
<td>0-030</td>
<td>0-010</td>
</tr>
<tr>
<td>Saponin plus</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-0025 c.c. serum</td>
<td>30,000</td>
<td>0-066</td>
<td>2-5</td>
<td>41,000</td>
<td>0-049</td>
<td>0-017</td>
</tr>
<tr>
<td></td>
<td>35,000</td>
<td>0-057</td>
<td>3-5</td>
<td>48,000</td>
<td>0-049</td>
<td>0-016</td>
</tr>
<tr>
<td></td>
<td>40,000</td>
<td>0-050</td>
<td>5-0</td>
<td>55,000</td>
<td>0-036</td>
<td>0-014</td>
</tr>
<tr>
<td></td>
<td>50,000</td>
<td>0-040</td>
<td>10-0</td>
<td>66,000</td>
<td>0-030</td>
<td>0-010</td>
</tr>
<tr>
<td>Saponin plus</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-00125 c.c. serum</td>
<td>40,000</td>
<td>0-050</td>
<td>3-5</td>
<td>48,000</td>
<td>0-041</td>
<td>0-009</td>
</tr>
<tr>
<td></td>
<td>50,000</td>
<td>0-040</td>
<td>6-0</td>
<td>56,000</td>
<td>0-034</td>
<td>0-006</td>
</tr>
<tr>
<td></td>
<td>60,000</td>
<td>0-033</td>
<td>10-0</td>
<td>66,000</td>
<td>0-030</td>
<td>0-003</td>
</tr>
</tbody>
</table>
Fig. 2

$log (c_1 - K)$

0.04 cc.

0.02 cc.

0.01 cc.

0.005 cc.

0.0025 cc.
Taking each quantity of serum by itself, we find that the plotting of log c₂ against log x gives a good enough straight line, but that the values of A and of n are different for every line. Indeed, the lines cross, an impossible state of affairs.

In order to find the meaning of this occurrence, we plot c₂ against x, as in the figure marked 1. Here we have a series of curves, each making an intercept on the c₂ axis. Now selecting any curve, and calling the coordinates of the point where it cuts the c₂ axis, C, K, we can plot log(c₂ - K) against log x.

This procedure gives, for every curve, a straight line. The lines are shown in the next figure. They are all parallel, with a slope of n = 1.75. The taking account of the constant K has thus simplified the whole problem, although its meaning is by no means clear.

Were it not for the constant K, the whole phenomenon of the inhibition by serum might be the result of an adsorption, in which the serum adsorbed saponin according to the well known expression

\[ \frac{x}{m} = A \cdot c_2^n \]

in which m is the mass of the adsorbent, or the quantity of serum, x the amount of lysin neutralised, and c₂ the amount of lysin acting on the cells.
PART I.

SECTION VI.

Development of the fundamental equations.

The fundamental equation.

The time dilution curves.

The percentage haemolysis curves.

The R-notation.

The symbol $x$ representing a quantity of lyric used up in combination with the cell.

2. The available evidence points to cytoplasm, the bile salts, and the other simple lytics, forming compounds with the proteins of the cell wall, the compound being in many respects not unlike an adsorption aggregate.

3. In a hemolytic system in which cells and lyric are interacting, we have a very different state of affairs from that in a true solution. The difference lies in the fact that the reaction cannot take place at all points in the fluid, but only at the cell surfaces. It is the visually incorrect to apply to such a system, in which the reactants must be discontinuous, the same treatment as holds for a system in which there is no discontinuity.

4. As has already been pointed out, there may accompany the primary reaction a number of secondary reactions, producing acceleration or inhibition of the primary reaction.
In developing equations for the action of the simple haemolysins, a number of rather unusual conditions appear; these may first be set down.

1. It has been seen that the curves obtained by plotting percentage haemolysis against time are sigmoid in nature. This form of curve indicates that the cell population varies as regards resistance to the lysin, and that the distribution of the resistances is according to some form of frequency curve - probably an ideal one. We shall take this curve to be of the type

\[ n = A e^{-b x^2} \]

The symbol \( x \) representing a quantity of lysin used up in combination with the cell.

2. The available evidence points to saponin, the bile salts, and the other simple lysins, forming compounds with the proteins of the cell wall, the compound being in many respects not unlike an adsorption compound.

3. In a haemolytic system in which cells and lysin are interacting, we have a very different state of affairs from that in a true solution. The difference lies in the fact that the reaction cannot take place at all points in the fluid, but only at the cell surfaces. It is obviously incorrect to apply to such a system, in which the reaction must be discontinuous, the same treatment as holds for a system in which there is no discontinuity.

5. As had already been pointed out, there may accompany the primary reaction a number of secondary reactions, producing acceleration or inhibition of the primary reaction.
to shall now develop the necessary equations, consider the nature of the reaction at those points in the system where it can occur, that is, at the neighborhood of the cell surface. The equation for the velocity of formation of an adsorption compound, when neglected of terms which have no meaning in this problem, is the well known expression

\[ \frac{dx}{dt} = x(t - c) \]

in which:

- \( x \) is the amount of the adsorption compound,
- \( t \) is the time,
- \( c \) is the concentration of the adsorption compound, and
- the adsorption is constant.

Suppose the surface of formation is not constant. At lysing, it is 5, but as lysing proceeds, liberated cell contents may form an ever-increasing surface of formation. Supposed these liberated contents react with the lysin, we shall take first the simple case where the contents react with the lysin, such as sarcin, and act in such a way as to render it inert.

Suppose that we have a population of cells of various resistances, and that these resistances are distributed according to the curve \( N = N \times e^{-x} \), \( x \) being a number of cells, and if the amount of lysin which requires to be transformed is a reaction with the cell surface in order to diffuse through. Then, as lysing proceeds, the number of cells transformed will be a function of the time.
We shall now develop the necessary equations.

Consider the nature of the reaction at those points in the system where it can occur, that is, at the neighbourhood of the cell surfaces. The equation for the velocity of formation of an adsorption compound, when deprived of terms which have no meaning in this problem, is the well known expression

$$\frac{dx}{dt} = \kappa(c-x)S$$

in which $\kappa$ is a velocity constant, $S$ the surface at which formation of the compound occurs, $x$ the amount of the reacting substance, in this case lysin, which is used up in the formation of the compound, and $c$ the initial concentration of the reacting substance.

To utilise an expression of this sort as a basis for an equation relating to haemolysis, it is to be remembered that the surface of formation is not constant. As lysis begins, it is $S$, but as lysis proceeds, liberated cell contents may form an ever-increasing surface of formation, provided these liberated contents react with the lysin.

We shall take first the simple case where the contents react with the lysin - such as saponin - and act in such a way as to render it inert.

Suppose that we have a population of cells of various resistances, and that these resistances are distributed according to the curve $n = Ae^{-\frac{x}{a}}$, $n$ being a number of cells, and $x$ the amount of lysin which requires to be transformed in a reaction with the cell surface in order to effect lysis. Then, as lysis proceeds, the number of cells haemolysed will be $n' = \int_{0}^{\infty} Ae^{-\frac{x}{a}} dx$. In these
expressions the values of \( x \) in the indices are values referred to the mean, while the limits of the integral refer to absolute values of \( x \).

This integral must be replaced by another expression, for as it stands it leads to insoluble equations. An approximation of sufficient accuracy is \( n' = k'x \), the sigmoid curve which represents the integral being replaced by a straight line through its extremities.

The liberated cell contents are clearly proportional to \( n' \), the cells haemolysed, and so we have the added surface of formation as \( S' = kx \). Note that \( k \) is not necessarily the same as \( k' \), for a unit of cell surface may not be so effective in reacting with the lysin as a unit of the new surface \( S' \).

The lysin may react either with \( S \) or with \( S' \). In the former case it tends to produce lysis, in the latter case it is rendered inert, and has no effect on the cells. If \( x \) is the amount reacting with \( S \) and \( y \) the amount reacting with \( S' \), we have \( x/y = S/S' \), whence \( y = kx^2/S \). The free lysin at any moment is accordingly \((c - x - kx^2/S)\).

Further, as the lysin may react either with \( S \) or with \( S' \), the probability of it reacting with \( S \) is \( S/(S + S') \) or \( S/(S + kx) \). Thus the lysin used up in a reaction with the cells is

\[
\frac{dx}{dt} = k(c - x - kx^2/S) \frac{S^2}{S + kx}
\]

A rather tedious integration gives,

\[
kx = \frac{1}{2S\sqrt{(1 + 4k\alpha/S)}} \log \frac{x[1 - \sqrt{(1 + 4k\alpha/S)}] - 2c}{x[1 + \sqrt{(1 + 4k\alpha/S)}] - 2c} + \frac{1}{2S\log c} \frac{a_{S}}{c - x - kx^2/S} \quad \ldots (1)
\]
When \( k = 0 \), that is when the liberated contents play no part in the reaction, the expression becomes

\[
\kappa t = \frac{1}{S} \log \frac{c}{c-x}
\]

and is similar to the monomolecular reaction formula advocated by Arrhenius and his pupils.

In these expressions, which are the fundamental expressions to be considered, there are four variables possible - \( t \), \( c \), \( x \), and \( S \). If \( t \) and \( c \) are variables the curve given is a time-concentration curve, related to the time dilution curve of experiment. If \( t \) and \( x \) are variables the resulting curve is one which is the basis of the percentage haemolysis curve. If we treat \( x \) and \( S \) as variables, we get relations which determine the behaviour of the lysin to suspensions of different strengths and which will be considered in the next section.

Each of these cases may now be considered.

**Time-dilution curves.**

In (1) when \( c = x + \frac{kx^2}{S} \), \( t \) becomes infinite; the curve therefore approaches an asymptote determined by the value of \( (x + \frac{kx}{S}) \). We take it as fundamental that the lysis of a certain number of cells corresponds to the using up of a certain amount of lysin, and hence it follows that \( x \) must be constant for all concentrations when 100 per cent haemolysis is complete. If, therefore we plot the time for complete haemolysis against \( c \), the initial concentration of lysin, we should get a curve described by (1) in which we have \( k \), \( S \) and \( x \) to determine.
Sodium Ozone
Upon

Saponin at 25 C.

Sodium taurocholate.
Unfortunately there is no way of determining the constants save by trial and error. This involves a long series of computations at the end of which one is by no means sure that one has selected the best values. The constant $S$ may be put at once equal to unity, but the difficulty lies in the fact that the value for the asymptote, determined by $(x + kx^2)$, gives an unlimited number of values of $x$ and $k$. One has to try successively the values 10, 20, 30, etc., for $k$, find the value of $x$ which will give the proper position for the asymptote, and then compute the value of $t$. From the curves so obtained, the best has to be selected. This process leads nowhere, for large variations in $k$ alter the form of the curves but little, provided that the position of the asymptote is kept the same throughout the series.

This consideration, upon which it is not necessary to dwell, leads to the conclusion that for all practical purposes equation (2) is sufficient. In the figures are given a number of curves, with calculated and experimental values - the applicability of the expression (2) will be seen at a glance.

So far as the fundamental reaction goes, we may therefore conclude that it is a monomolecular one, perhaps involving an adsorption process, perhaps a reaction of the first order.
The percentage haemolysis curves give the relation of \( n' \), the number of cells haemolysed, to \( t \), the time, for various concentrations of lysin; \( n' \) is usually expressed as a percentage of the total number.

We have to derive these curves from the relations

\[
n' = f(x) = \int_0^x e^{-tx^2} \, dx.
\]

and

\[
x = f(t)
\]

which is given by making \( x \) and \( t \) variables in (1) or (2).

These two expressions, solved simultaneously, give the form of the percentage haemolysis curve. The entire treatment of the curve is a very difficult matter, but we can readily obtain its especial characteristics.

If the distribution of the cells is a symmetrical one, it will be plain that when half the lysin is used up half the total number of cells will be haemolysed, and so that if in a percentage haemolysis curve we measure \( t_{50} \), the time for lysis of half the cells, and \( t_{100} \), the time for complete lysis, we shall have the ratio

\[
t_{100}/t_{50} = \frac{\log \frac{c}{c-\infty}}{\log \frac{c}{c-0.028}}
\]

if the reaction proceeds according to (2). When it proceeds according to (1) the expression is much more complex, but of no greater use.

Now the ratio \( t_{50}/t_{100} \) is a kind of measure of the skewness of the curve; this will vary with the concentration. Below are a few experimental and observed results, calculated for the asymptote \( x = 0.028 \), which
was the asymptote of the time dilution curve for the lysin in question (saponin).

<table>
<thead>
<tr>
<th>$c$</th>
<th>$t_{x}/t_y$ calc.</th>
<th>$t_{x}/t_y$ exper.</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2</td>
<td>2.06</td>
<td>-</td>
</tr>
<tr>
<td>0.1</td>
<td>2.18</td>
<td>-</td>
</tr>
<tr>
<td>0.05</td>
<td>2.5</td>
<td>2.6</td>
</tr>
<tr>
<td>0.04</td>
<td>2.78</td>
<td>2.9</td>
</tr>
<tr>
<td>0.033</td>
<td>3.39</td>
<td>3.5</td>
</tr>
<tr>
<td>0.030</td>
<td>4.3</td>
<td>4.2</td>
</tr>
</tbody>
</table>

When $t_{x}/t_y = 2$, the percentage haemolysis curve is symmetrical. This condition is found in practice when $c$ is large. With small values of $c$ the skewness becomes more marked, and the skew curves shown in a previous section are encountered.

In the case of sodium taurocholate a very difficult point arises - we have to account for a skewness which results from an apparent acceleration of the reaction. This particular case cannot be treated here, for it involves experiment and theory which fall into a subject of their own.

It must accordingly taken as sufficient to state that experimental investigation shows that the monomolecular reaction, whose equation is developed above, when reacting with cells distributed in resistance according to a symmetrical frequency curve, is sufficient to explain the form of the percentage haemolysis curves.
The R-notation.

In a method described above, two haemolytic systems, each containing the same lysin, were shown to be able to be compared with one another, the comparison being carried out by means of a resistance constant $R$, together with a small empirical constant $k$.

As an example, take two systems, one containing saponin and cells, the other containing saponin, cells, and sucrose, an inhibitory substance.

The time-dilution curve for the saponin-cell system is

$$\kappa t = \log \frac{c_1}{c_2}$$

and that for the inhibited system is

$$\kappa t = \log \frac{c_i}{c_{i'} - x_i}$$

The method is to eliminate $t$ by taking equal values, thus,

$$\log \frac{c_1}{c_2 - x_2} = \log \frac{c_i}{c_{i'} - x_i}$$

Now the saponin-cell system is $R$ times as resistant when sucrose is present as when it is not - that is, $R$ times as much lysin is needed to produce the same effect. So we have

$$\log \frac{c_i}{c_{i'} - Rx_2} = \log \frac{c_1}{c_2 - x_2}$$

from which it will be plain that $c_1 = Rc_2$, the result obtained in so many examples in Section 4.
PART I.

The previous section expressions were developed when held at the surfaces of the cells, where such reactions lyse can occur. The idea of the adjacent continuity of the cell membrane develops further.

SECTION VII.

The theory of the zone of action.

The zone of action and the measurement of its radius.

The zones of different types of erythrocytes.

The volume of the zone of action, and the zone as a concentration gradient.

The zone of action is a region around a cell where the surrounding fluid is altered by the cell. The zone of action is defined by the radius, which is the distance from the cell surface to the edge of the zone where the reaction is no longer detectable.

The zones of different types of erythrocytes are characterized by their unique volume of the zone of action and the zone as a concentration gradient. This gradient is a measure of how much the concentration of a substance changes from the surface of the cell to the interior of the zone of action.

When a new cell is added to the suspension, it may or may not have a zone that overlaps with the zone of another cell. If the volume of the zone of action is small, and the total volume of the fluid is large, the chance of a cell being in close proximity to another cell and the two zones overlapping is low. If the cell does not overlap with the reaction zone, it will not interfere with the reaction. However, if the cell overlaps with the reaction zone, it will establish a zone of its own, and the reaction will continue in the same manner, but with a different zone forming around each cell.
In the previous section expressions were developed which hold at the surfaces of the cells, where such reactions as result in lysis can occur. The idea of the discontinuity of the haemolytic system must now be developed further.

Imagine a system in which there are few cells, and in which each cell is separated from its neighbour by a great distance. In the presence of a considerable amount of lysin, the molecules near each cell will react with the cell component, while those remote from the surfaces will not react. Call the portion of the fluid surrounding each cell and containing the reacting molecules the "zone of action". Each cell will now have its own zone, and within this zone the expression developed in the previous section (1) will apply.

Suppose more cells are added to the suspension. Each new cell may then lie either within one of the zones or outside them. If the volume of the zones be \( v \) and the total volume of the fluid be \( V \), the chance of a cell, surrounded by its zone, entering another zone will be \( v/V \). If it does not enter, it will not interfere with the reaction round the cells initially present, but will establish a zone of its own. It will under these circumstances be haemolysed, but its presence will in no way be detectable, and its addition will make no difference to the course of the reaction. If it enters a zone, the surface of formation will then be increased, and the cell, interfering with another, will be detected as an addition to the suspension.
If then we have two suspensions, the one having half the number of cells in the other, and if the equation for the time-concentration curve of the weaker of the suspensions be

$$\kappa t = \frac{1}{S} \log \frac{c}{c-x}$$

that for the suspension of double the strength will not be

$$\kappa t = \frac{1}{2S} \log \frac{c}{c-2x}$$

but

$$\kappa t = \frac{1}{S+\psi S} \log \frac{c}{c-\psi x-x}$$

where $\psi$ is the probability that the cell occupy positions in the fluid inside the zones of action, and where this probability is equal to $v/V$.

The asymptote of (2) is reached when $c = x$. If, therefore we have a series of time-dilution curves for a lyxin acting on suspensions of various strengths, we can obtain the value of $v/V$, and thence the value of $v$, the volume of the zones of action.

This may be illustrated by an experiment. In the figure are shown a set of curves for amponin acting on suspensions of different strengths. The positions of the asymptotes are marked, and are as in the following table.

<table>
<thead>
<tr>
<th>Suspension in terms of standard</th>
<th>Asymptote, c.</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>0.0350</td>
</tr>
<tr>
<td>2</td>
<td>0.0285</td>
</tr>
<tr>
<td>1 standard</td>
<td>0.0255</td>
</tr>
<tr>
<td>$\frac{1}{2}$</td>
<td>0.0237</td>
</tr>
<tr>
<td>$\frac{1}{4}$</td>
<td>0.0222</td>
</tr>
</tbody>
</table>
Now as the strength of the suspension is double on successive occasions, we have a sample on successive occasions. Y is constant throughout. We calculate the volume for the zone of action.
Now as the strength of the suspension is doubled on successive occasions, we have \( v \) doubled on successive occasions. \( V \) is constant throughout. We calculate the volume for the zones of action,

<table>
<thead>
<tr>
<th>Suspension</th>
<th>( v ), exp.</th>
<th>( v ), calc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \frac{1}{2} )</td>
<td>0.067</td>
<td>0.067</td>
</tr>
<tr>
<td>1</td>
<td>0.148</td>
<td>0.138</td>
</tr>
<tr>
<td>2</td>
<td>0.284</td>
<td>0.268</td>
</tr>
<tr>
<td>4</td>
<td>0.576</td>
<td>0.536</td>
</tr>
</tbody>
</table>

and find the correspondence excellent. In order to show how good it is, we show the results for an extensive experiment for saponin acting on suspensions of various strengths.

\[ \frac{1}{2} \text{ suspension; } \psi = 0, x = 0.0222. \]

<table>
<thead>
<tr>
<th>( c )</th>
<th>( t ) observed</th>
<th>( t ) calculated</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2</td>
<td>0.3</td>
<td>0.25</td>
</tr>
<tr>
<td>0.1</td>
<td>0.7</td>
<td>0.74</td>
</tr>
<tr>
<td>0.05</td>
<td>1.75</td>
<td>1.68</td>
</tr>
<tr>
<td>0.025</td>
<td>7.0</td>
<td>6.30</td>
</tr>
</tbody>
</table>

\[ \frac{1}{2} \text{ suspension; } \psi = 0.07, x = 0.0237. \]

<table>
<thead>
<tr>
<th>( c )</th>
<th>( t ) observed</th>
<th>( t ) calculated</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2</td>
<td>0.35</td>
<td>0.35</td>
</tr>
<tr>
<td>0.1</td>
<td>0.63</td>
<td>0.75</td>
</tr>
<tr>
<td>0.05</td>
<td>1.75</td>
<td>1.63</td>
</tr>
<tr>
<td>0.025</td>
<td>3.7</td>
<td>3.28</td>
</tr>
</tbody>
</table>

\[ 1 \text{ suspension; } \psi = 0.14, x = 0.0253. \]

<table>
<thead>
<tr>
<th>( c )</th>
<th>( t ) observed</th>
<th>( t ) calculated</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2</td>
<td>0.4</td>
<td>0.35</td>
</tr>
<tr>
<td>0.1</td>
<td>0.75</td>
<td>0.76</td>
</tr>
<tr>
<td>0.05</td>
<td>1.9</td>
<td>1.88</td>
</tr>
<tr>
<td>0.04</td>
<td>2.75</td>
<td>2.75</td>
</tr>
<tr>
<td>0.027</td>
<td>3.75</td>
<td>3.75</td>
</tr>
</tbody>
</table>

\[ 2 \text{ suspension; } \psi = 0.28, x = 0.0285. \]

<table>
<thead>
<tr>
<th>( c )</th>
<th>( t ) observed</th>
<th>( t ) calculated</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2</td>
<td>0.42</td>
<td>0.36</td>
</tr>
<tr>
<td>0.1</td>
<td>0.85</td>
<td>0.79</td>
</tr>
<tr>
<td>0.05</td>
<td>2.4</td>
<td>1.99</td>
</tr>
<tr>
<td>0.04</td>
<td>3.3</td>
<td>2.84</td>
</tr>
<tr>
<td>0.03</td>
<td>7.5</td>
<td>7.02</td>
</tr>
</tbody>
</table>

\[ 4 \text{ suspension; } \psi = 0.56, x = 0.0350. \]

<table>
<thead>
<tr>
<th>( c )</th>
<th>( t ) observed</th>
<th>( t ) calculated</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2</td>
<td>0.45</td>
<td>0.37</td>
</tr>
<tr>
<td>0.1</td>
<td>0.9</td>
<td>0.83</td>
</tr>
<tr>
<td>0.05</td>
<td>2.6</td>
<td>2.31</td>
</tr>
<tr>
<td>0.04</td>
<td>4.2</td>
<td>3.9</td>
</tr>
<tr>
<td>0.037</td>
<td>7.0</td>
<td>5.3</td>
</tr>
</tbody>
</table>
To obtain the absolute size of the zone of action, we note that in the standard suspension there are \(10^3\) cells in each tube. The total volume of the system is \(2 \times (10^3)^2 \). For this suspension, as seen in the above tables, \(v/V\) is 0.14 approximately, and hence the radius of the zone of action in the case of these cells and this lysin (human cells and saponin) is about 8.8\(\mu\). In the centre of this lies the cell, of radius, if in the spherical form, of 3\(\mu\); the zone therefore extends outwards from the cell surface into the fluid for a distance of 5.8\(\mu\) in all directions. If the cell is taken as ellipsoidal, the figure requires a slight modification.

Were the strength of the suspension increased to 7 or 8 times that of the standard, \(v/V\) would equal unity. That is, it would be certain that the zones would overlap one another, and after that point the increase in \(S\) would be proportional to the number of cells in the suspension. This fact is in agreement with experiment, but unfortunately experiments with suspensions so concentrated are very difficult to carry out with any degree of satisfaction.

The meaning and the mode of derivation of the zone of action in any particular haemolytic system having been explained, we shall now develop the idea in a somewhat different manner.
As has been shown above, the size of the zones of action in a particular haemolytic system can be obtained in a very simple way. We take the most dilute suspension with which it is possible to work (½ the arbitrary standard used in these studies) plot the time dilution curve for the action of the lysin upon it, and find \( c_{i\infty} \), the concentration of lysin which corresponds to its asymptote. A suspension of known cell content, some four times stronger than this first suspension, is now taken; its time dilution curve is plotted, and \( c_{i\infty} \), the concentration of lysin which corresponds with its asymptote, obtained.

Then

\[
\frac{c_{i\infty}}{c_{i\infty}} - 1 = \psi \quad \ldots \ldots 1.
\]

But \( c_{i\infty}/c_{i\infty} \) is equal to \( R \), the resistance constant of the second suspension compared with the first (see Section 4), and so, calling this constant not \( R \) but \( R_x \), to avoid confusion in what follows, we have,

\[
R_x - 1 = \psi \quad \ldots \ldots 2
\]

Call \( v' \) the volume of a single zone of action, and we get

\[
\frac{v (R_x - 1)}{N} = v' \quad \ldots \ldots 3
\]

where \( N \) is the number of cells in the suspension.

If \( \rho \) is the radius of the zone of action measured from the surface of the cell outwards,

\[
\rho = \sqrt[3]{\frac{3V(R_x - 1)}{4\pi N}} - r \quad \ldots \ldots 4
\]

where \( r \) is the radius of the cell in the spherical form.

The determination of the dimensions of the zone of action is therefore an easy matter, for all that has
action is therefore an easy matter, for all that has to be done is to determine $R_z$ for a suspension of known cell content as compared with the most dilute suspension with which it is possible to work.

Suitable strengths of the suspensions are as follows. (a) A suspension containing in 20 cc of saline the cells, thrice washed, for 1 cc. of blood, and (b) a suspension containing one fourth the number of cells.

We shall illustrate the method by an example.

Lysis of sheep cells by saponin. The strengths of the suspensions were as in (a) and (b).

For suspension (a) the asymptote of the time dilution curve at 25°C was found to be a concentration of lysin of 0.21 milligrammes.

For the suspension (b) the asymptote was found to correspond to a concentration of 0.13 milligrammes.

Dividing the one figure by the other we get $R = 1.63$, and so $(R_z - 1) = 0.63$. This figure is less than 1, as it should be. The number of cells in the system was $2 \times (10^3)$, the volume of the system 2 cc, and the radius of the sheep cell, in the spherical form, $2 \mu$.

Applying equation 4 with these values inserted, we find that the value of $\xi$ works out as $9.5 \mu$. The zone of action therefore extends, in the haemolytic system with which we are dealing, outwards from the cell surface for a distance of $9.5 \mu$.

Applying this method to the cells of various animals, we obtain the following table. In all cases saponin was
The lysin.

<table>
<thead>
<tr>
<th>Animal</th>
<th>$E_z$</th>
<th>$N_1(10^2)$</th>
<th>$r_+/\mu$</th>
<th>$q_+/\mu$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit</td>
<td>1.07</td>
<td>1.0</td>
<td>2.5</td>
<td>4.5</td>
</tr>
<tr>
<td>Man</td>
<td>1.14</td>
<td>1.0</td>
<td>3.0</td>
<td>5.8</td>
</tr>
<tr>
<td>Dog</td>
<td>1.18</td>
<td>1.4</td>
<td>2.5</td>
<td>6.0</td>
</tr>
<tr>
<td>Cat</td>
<td>1.23</td>
<td>1.2</td>
<td>2.3</td>
<td>7.4</td>
</tr>
<tr>
<td>Cx</td>
<td>1.59</td>
<td>1.6</td>
<td>2.3</td>
<td>9.0</td>
</tr>
<tr>
<td>Sheep</td>
<td>1.63</td>
<td>2.0</td>
<td>2.0</td>
<td>9.5</td>
</tr>
</tbody>
</table>

This result is a remarkable one, in that the cells are arranged in the well-known Ryvosh series. The order of the cell arranged according to the size of the zone of action is thus the same as the order of the cells arranged according to their resistance to saponin. The most resistant type of cell - that of the sheep - has the largest zone, while the least resistant type - that of the rabbit - has the smallest zone.

Turning to the idea of the zone of action as a concentration gradient, and regarding its boundary as a point where the gradient becomes negligible, we see at once the meaning of this result. In the case of a cell which is very resistant to the action of saponin, such as that of the sheep, a large amount of lysin has to be used up in order to bring about lysis. As a result the zone of action is large. On the other hand, if we have a cell such as that of the rabbit or of man, which is relatively unresistant to saponin, little saponin requires to be used up, and the zone of action is small.

We may now go further, and attempt to discover the
exact relation between the size of the zone of action and the resistance of the particular cell to the lysin. As has been seen, the resistance of a particular haemolytic system, compared with that of another haemolytic system chosen as a standard, may be expressed as the value of a constant R, and with the value of this constant we shall attempt to compare the sizes of the zones of action.

In using this method it is to be remembered that R refers to an entire haemolytic system. If we wish to use values of R to show the resistance of a particular cell to the lysin - or, what is the same thing, the amount of lysin combining with a single cell - we have to be very careful that the haemolytic systems which are compared are comparable in every respect. They must be comparable as regards the cell content, and as regards the state of the zones of action in the systems. It is no use comparing two systems in one of which the zones overlap and in the other of which they are separate. Further, if R is to refer to the single cell, the systems must be the same in cell content. We have therefore to determine values for R for haemolytic systems in which the zones of action are all separate, and in which the chance of a cell entering a zone of action is small enough to approach zero. Having done this, the systems are to be reduced to the same cell content. A value of R determined in this way may be properly compared with the figures for the dimensions of the zone of action.
With these values of $R/N$ we shall tabulate values of the volume of the zones of action. Call the volume of a zone $Z$; it is obviously $(v' - v'')$ where $v'$ is given by equation 3 and where $v''$ is the volume of the cell supposed to be in its centre.

<table>
<thead>
<tr>
<th>Animal</th>
<th>$R/N, \times 10^5$</th>
<th>$v', \mu^3$</th>
<th>$v'', \mu^3$</th>
<th>$Z, \mu^3$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit</td>
<td>0.46</td>
<td>1400</td>
<td>65</td>
<td>2335</td>
</tr>
<tr>
<td>Man</td>
<td>0.82</td>
<td>2800</td>
<td>110</td>
<td>2690</td>
</tr>
<tr>
<td>Dog</td>
<td>0.71</td>
<td>2540</td>
<td>65</td>
<td>2485</td>
</tr>
<tr>
<td>Cat</td>
<td>1.4</td>
<td>3830</td>
<td>51</td>
<td>3779</td>
</tr>
<tr>
<td>Ox</td>
<td>2.5</td>
<td>7275</td>
<td>51</td>
<td>7224</td>
</tr>
<tr>
<td>Sheep</td>
<td>2.15</td>
<td>6300</td>
<td>33</td>
<td>6267</td>
</tr>
</tbody>
</table>

If the values of $R/N$ are plotted against the corresponding values of $Z$, a fairly good straight line is obtained. The points lie along the line in a very ever way.

The equation of the line is

$$Z = \frac{3}{N} \times 10^5 \cdot R$$

in which $R$ refers to suspensions in which the zones of action are completely separated, and in which $Z$ is given in $\mu^3$.

It will be appreciated that there is room for the extension of these results to other haemolytic systems.
PART II.

THE SIZE OF THE ERYTHROCYTE.

Section 1. Photographic measurements of erythrocytes.

Section 2. The mean size and frequency distribution in man.

Section 3. The effect of drying.

Section 4. The effect of exercise.

Section 5. On Burker's correlation.
PART II.

SECTION I.

Photographic measurement of erythrocytes.

Introductory.

Preparation of the cells.

The photographic process.

1. Limit of resolution.
2. Focus.
4. Exposure.

Measurement of the cells.
Measurements of the diameters of numbers of erythrocytes may be carried out on (a) dried or stained films, (b) red cells suspended in isotonic saline, (c) red cells suspended in serum, or (d) red cells suspended in plasma. When the absolute size is to be considered, stained films cannot be employed, since the process of drying produces changes of unknown extent in the diameter, so that the cells of dried films cannot be compared as regards diameter with cells in the fresh state. If the cells are to be measured without any preliminary fixation, the most suitable medium in which to suspend them is obviously the plasma of the individual from which they are derived. To replace this plasma by saline or serum in a much less satisfactory proceeding; it is difficult to be certain that a saline solution is isotonic with a given sample of blood, and further a saline solution differs so much from plasma as regards physical properties that one cannot assume that erythrocytes do not alter their size when suspended in saline. The use of plasma has other properties to be noted later, and since it is the medium in which the red cells float in the blood stream, the measurement of the cells in plasma is the nearest approach we can make to a measurement of the cells in the condition in which they are in the organism.

To measure a large number of cells in the fresh state by the use of a screw micro-
meter eyepiece (each cell being measured individually) would take a very long time, for the careful measurement of each cell would occupy at least thirty seconds; by the time the measurements were made, so long a time would have elapsed from the making of the first preparation that it would be impossible to be sure that the physical and chemical properties of the plasma had not altered during the time of the measurement, and affected the result. A fresh preparation cannot be kept for hours under the microscope, and still be called fresh. In order to obviate this difficulty, the cells may be photographed and then measured from the plate. Such a procedure has the additional advantage that there is no need to make the measurements rapidly, so that the diameters of the cells may be determined with great accuracy, and, if need be, checked by an independent observer.

The details of the method based on these principles will be discussed under three heads: the preparation of the cells, the photographic process, and the measurements from the plates.

Preparation of the cells.

If we desire to make a preparation of red cells from an individual and to suspend them in the plasma of the same individual in such a way that the cells shall be easily photographed and without introducing any factors which may interfere with the size of the cells, several difficulties arise. The cells cannot be obtained from a single drop of blood which is prevented from clotting,
for the cells in such a drop are far too numerous, and lie so closely packed that it is impossible to obtain photographs in which the diameters are measurable. Nor can the cells be obtained from the blood by centrifuging, since this may introduce a fallacy, the larger cells being thrown down more rapidly than the smaller; to mix the cells again, shaking or mechanical mixing have to be employed, these procedures being best avoided. Both cells and plasma, moreover, have to be kept free as far as possible from contact with the air, for changes in the gas content of the plasma may lead to changes in the diameter of the cells - CO₂, for example, in the plasma tends to increase the cell volume, and therefore to cause changes in the diameter. Lastly, care has to be taken that evaporation does not occur at any time during the procedure.

These difficulties are met by the following very simple method.

The subject, from whom the cells are to be withdrawn, is made to rest for a short time. Three tubes are then prepared to receive the blood - the tubes used are centrifuge tubes of about 1 cm. diameter - to each is added 20 mgs. of potassium oxalate, or better, lithium oxalate. A large needle is inserted into the basilic vein of the subject, and the blood directed so that it flows to the very bottom of the tubes; in this way, only the blood coming from the vein first comes into contact with the air. The tubes are filled full, and into each a rubber stopper is inserted, so that there is
air space above the level of the blood. Two of the tubes are placed in the centrifuge and the plasma separated. The third tube is not centrifuged - from this tube is obtained the cells to be photographed, while from the other two the plasma in which to suspend the cells. The tubes are kept stoppered as much as possible until the experiment is finished.

The only point on which this method is defective is in the use of an anti-coagulant. The addition of oxalate certainly may produce a condition of the plasma not present in the circulating blood. Of this we have no evidence; and when it is considered that most of the oxalate will be precipitated as calcium oxalate, and also that it is necessary to use some anti-coagulant, it may be taken that no great fallacy is introduced by the use of a small amount of oxalate. Faced with the alternative of measuring the cells in serum, or of using oxalate, we have selected the latter alternative as the less fallacious.

Immediately after the cells have been obtained the photography is commenced. A sample of the cells is prepared in the following way.

From the tube containing plasma, about 0.1 cc. is drawn up in a clean capillary pipette; the stopper of the tube is immediately replaced. The pipette is then inserted into the tube containing the cells, and a volume of cells, about one-tenth that of the plasma taken, is drawn up. The contents of the pipette are placed on a slide, rapidly mixed, and drawn
up again. The process is done as rapidly as possible, and takes only a few seconds. From the pipette is now delivered one drop of the mixture of cells and plasma on to a thin clean slide of white glass; the drop is immediately covered with a No. 1 coverglass, the edge of which has been previously smeared with vaseline. The size of the drop should be such that it just fills the space under the coverglass without leaving an air-space. The preparation is now placed under the microscope, and a selected field focussed and photographed. The process just described, although it seems very simple, requires some little practice; it must be carried out very rapidly, the drop must just fill the space under the coverslip, and the dilution of the cells with plasma must be such as to give suitable fields for photography. It is, as a rule, better to dilute the cells too little than too much - the dilution varies from 1 part of cells to 6 parts of plasma to 1 part of cells to 10 parts of plasma.

No more than four fields are photographed from any one preparation; since the photography of one field occupies not more than 2 minutes, the cells are always photographed within eight minutes of the making of the sample on the slide, and usually within a shorter time. A series of preparations are made until a sufficient number of fields have been photographed - the exposure of twenty plates is enough for most purposes. Needless to say, the selection of suitable fields is a very important point; the fields chosen must not be too crowded.
nor yet too thin. Fields with red cells overlapping or in rouleaux are to be avoided for obvious reasons. The formation of rouleaux is a very troublesome factor, and differs markedly in different samples of cells and plasma. It cannot be avoided altogether, but can be diminished by making preparations containing a large proportion - ten times - of plasma to cells, and by photographing the cells as quickly as possible after preparations are made. Before photographing a field, the cells should be allowed to settle; this they do very quickly if the film between the slide and the coverslip is thin.

The photographic process.

In order to obtain reliable photographs of the cells the optical system employed, as well as the conditions regarding exposure under which the photograph is taken, must be carefully studied. The main difficulties are sources of error attached to the photomicrography will therefore be dealt with in detail.

(a) The limit of resolution.

The accuracy with which an object can be measured depends on the limit of resolution of the optical system employed, and uncertainty as to the absolute size of the object must always exist to a degree equal to the limit of resolution. The limit is expressed by the formula

\[ \text{Limit} = \frac{\lambda}{2 \text{ N.A.}} \]

where \( \lambda \) is
the wave length of the light employed, in A.U., and where N.A. is the numerical aperture. The resolving power can, therefore, be increased by increasing the N.A. and by shortening the wave length as much as possible.

In work of this nature, it is doubtful whether the working N.A. can be increased above 1.2, as any greater aperture introduces difficulties as to the flatness of the field, so that it becomes impossible to focus more than one or two cells at a time. By using a blue light filter together with a high power illuminant, it is possible to reduce the predominant wave-length to 4555 A.U. This wave-length is as short as can be used, owing to the difficulty in focussing with shorter wave-lengths, for the sensitivity of the retina falls off rapidly if the wave-length be below this figure. With a N.A. of 1.5 and blue light, the limit of resolution is fixed at 0.2μ. This means that if the erythrocyte is in focus, the edge is not clearly seen, but seen as a blurred band 0.2μ in width. Somewhere in this band is the true margin of the cell.

In the observations hitherto made of the diameter of the red cell, this spurious disc is not commented upon. It has therefore to be concluded that the measurement of the diameter has, in these observations, been made either between two points on the inner margin of the spurious disc, or between two points on the outer margin of the disc, or else between two points situated within the margin of the blurred band. As a result of
this, the error attached to any observation may be \( \pm 0.2 \mu \); it is impossible to say what it actually is, as investigators do not state between which points on the spurious disc the measurements were made. Most of the measurements have been made on stained films, and in such cases the tendency is to measure between the extreme edges of the stained material—that is, between two edges of the disc, which appears stained owing to the great contrast. Such observations would give a value too great by about \( 0.2 \mu \). This error, small though it is, becomes of great importance in estimations of the volume of the cell, and the degree of error introduced is dealt with in another Section of this Part (On Burker's correlation).

Once the existence and importance of the spurious disc is realised, an attempt may be made to find the position of the real edge of the cell, on theoretical grounds—the only way of finding it, for no optical apparatus, however perfect, will perfectly resolve the margin. We know that the margin must be somewhere within the spurious disc. On first principles, we should expect its position to be regulated by the conditions of the two substances, cell and plasma, between which there is an interface. In the case of a line of great fineness, the spurious disc is equally distributed on either side of the line, because the conditions on the two sides are the same. In the case of the cell in plasma, we suggest that the position of the true margin is dependent on the respective refractive indices of cell
and plasma, so that if \( T_1 \) be the thickness of the portion of the disc to the outside of the true edge, and if \( T_2 \) be the thickness to the inside of the edge, the relation

\[
\frac{T_1}{T_2} = \frac{n_1}{n_2}
\]

will hold, \( n_1 \) being the refractive index of the plasma and \( n_2 \) the refractive index of the cell. Since the refractive index of serum is about 1.3 and that of the cell not greater than 1.5, the true margin of the cell will lie very nearly in the middle of the spurious disc, but a little nearer the cell than the midpoint. If the disc be 0.2 \( \mu \) in width, as in the case we are considering, the true edge will lie 0.093 \( \mu \) from the inner margin of the disc. It is to be noted that we are using a monochromatic light, and that we therefore avoid the chromatic effects in diffraction; this formula would probably have to be altered if white light were used.

We can therefore reduce the error imposed by the limit of resolution by taking the margin of the cell as situated in the middle of the spurious band, and by measuring between two points in this position: at the very most, there will be an error of less than 0.2 \( \mu \), while the error will probably be very small indeed if the position of the cell edge is as we have suggested.

However interesting the subject may be theoretically, it is not possible to measure the cells in photographs between two points in the middle of
the spurious disc with an accuracy greater than 0.2µ-
(that it, to 0.1 mm. on the plate) - for it is a very
difficult thing to bisect the spurious disc by eye.
Accordingly if the diameter be measured between two
points in the middle of the disc, as high a degree of
accuracy as practicable will have been attained.

(b) Considerations as to focus.

Since the limit of
resolution imposes an error at the margin of the cell
of ±0.1µ, we cannot be certain of the position of the
margin of the cell to within this amount, even under
the most perfect conditions of focussing. There must,
therefore, always be an error due to faulty focussing of
the edge, for we cannot see that edge in order to focus
it clearly. It will be necessary to find what depth of
focus is necessary to make this error of focussing lie
within the limit of resolution.

Consider a cell as an
ellipsoid of rotation about its minor axis. The depth
of focus which will allow an error due to faulty focussing
to fall within the limit of resolution will be the same
as the length of a line cut off by the curve, at right
angles to the major axis, and situated 0.2µ inside the
outer edge of the blurred band; considering it in this
way makes the depth of focus as great as possible. If
the length of the major axis of the cell be taken as
8µ, and the minor axis as 2µ, the length of such a
line will be found to be 0.63µ. If, then, we use an
optical system whose depth of focus is greater than
this, every point on the margin of the cell will be in focus, and so the edge will be in focus; any blurring which appears at the edge will be that imposed by the limit of resolution. In order to be sure that this error will not affect the results, we have used an optical system of a depth of focus of 0.75 μ. It will be noted that this depth is about half that of the thickness of the average red cell; the rule may therefore be laid down that any cell whose image does not show marked hollowing in the centre is lying in the plane of focus, and that the edge of such a cell is in focus; such a cell is fit for measurement.

(c) The effect of Brownian movement.

It is necessary to consider the effect of Brownian movement, since, if this occur, the measurement of the cells will be subject to error. The formulae for Brownian movement apply to small spheres, and not to ellipsoidal bodies such as the red cell; it will, however, be obvious that the cell will undergo more movement in a horizontal plane than in a vertical one, since the axis major is greater than the axis minor. The movement in the horizontal plane will not be given by inserting in the Smoluchowski formula the value for the semi-axis minor, because the extent of the movement will be lessened by the friction over the large upper and lower surfaces. Further, we gain an advantage by measuring the cells suspended in plasma, for the viscosity of plasma is about 1.5 to 2 times that
of water or saline. This almost halved the movement. The effect of the movement is to produce a further blurring of the edge of the cell, enlarging the blurring due to the spurious disc. The true position of the edge of the cell, relative to the margin of the disc, will not be appreciably altered, so that by bisecting the apparent spurious disc by eye the edge of the cell can be determined. The wider the blurred band, however, the more difficult it is to bisect it by eye, so that in the case of small cells, such as those of the goat, there may be considerable difficulty introduced because of this movement. There is no way of avoiding this.

(d) Exposure.

For obvious reasons it is desirable to use conditions which necessitate as small an exposure as possible. The exposure may be reduced in five ways; by increasing the power of the illuminant, by increasing the N.A., by using no light filter, by using fast plates, and by decreasing the magnification.

We use as an illuminant the Pointolite. The carbon arc is more powerful, but not so reliable when there are many exposures to be made, as the power of the illuminant is apt to alter. Probably an oxyhydrogen lime would be preferable to the Pointolite, but it would require a great deal of attention. The thorium disc light, using dissolved acetylene, has not yet been adapted to microscopical work.

The N.A. used is as large as is consistent with good photography, i.e. one of 1.15.
It is derived from a semi-apochromatic oil immersion, 1/12th. of N.A. 1.35, and a dry condenser working at about 0.93. The working aperture is the mean of these two. The objective is thus working at about 2/3rds. of its full cone, which is as large as we can use to photograph objects so devoid of contrast as the red cell. Such a cone with the objective used is also just sufficient to avoid disturbances in the sharpness of the image due to secondary spherical aberration. ("secondary zones").

It is necessary to use a light filter to decrease the wave-length of the light, thus reducing the limit of resolution. The monochromatic light also serves to remove another cause of blurring of images found with all, lenses, even apochromatics, namely, chromatic differences of spherical aberration. It also removes the secondary spectrum produced by all non-apochromatic objectives. With ordinary orthochromatic plates a blue screen does not call for a longer exposure than a green screen. Accordingly a Wratten C filter is used.

The speed of the plate used is limited by the fineness of the grain; the faster the plate, the larger the grain. Very fast plates cannot be used, since the coarseness of the grain interferes with the measurement of the size of the cells. We use Wellington Orthoprocess plates, which have a fine grain, and which are also relatively fast. The size of the plate is not a matter of much importance, byt quarter plates are most convenient.
Finally, we have to consider the magnification. This we have settled at 500. If a higher magnification be used, such as 1000, the exposure would be four times as long, and, what is more important, there would be a tendency to a spurious accuracy of measurement. When the magnification is 500, 0.2µ is represented by 0.1 mm. on the plate - the finest division which can be read off with a simple lens without enlarging the grain of the plate unduly. It cannot be too strongly emphasised that in work of this nature the actual magnification is a matter of secondary importance; the essentials are accuracy and sharpness of image, which tend to be less with high magnification. At this magnification, under the conditions described above, an ample exposure is 3 seconds.

To determine the magnification, photographs of a stage micrometer are taken at intervals during the photography of the cells. These photographs of the scale may be compared, and thus the possibility of the magnification varying from any accident is guarded against.

Measurement of the cells.

The plates having been developed, (hydroquinone is an excellent developer, as it imparts a good amount of contrast to the cells), fixed, and dried, the measurement of the cells is proceeded with in the following way.

The photographs of the scale are
first examined and the magnification determined. It is to be remembered that, as the field is not absolutely flat, the magnification at the centre of the field may not be that at the periphery; this is determined by comparing different divisions of the scale. It is sufficient to average the two central divisions, and to apply this value to all the cells lying in the central parts of the plate. The values applicable to the periphery of the scale must be used for all cells lying far from the centre.

The plate containing the cells to be measured is covered with a piece of paper with a circular aperture 2 inches in diameter; this is arranged so as to lie concentric with the circle of exposed plate, for all the cells at the edge of the plate are to be excluded from measurement, for there the focus is defective. The plate is then placed on the stage of a dissecting microscope, and the cells examined and measured individually under a low power lens, a strong light being thrown upwards upon them from a plane mirror. The lens should magnify about 4 times, and give a reasonably flat field; each cell is arranged in the centre of this field when being measured. The measurement is made with a scale, 10 mm. divided into 1/10ths, mounted on a slide without a coverglass. (obtained from Julius Rheinberg and Co). The scale is placed over the cell in such a way that the diameter can be read off. The plate being film upwards, and the slide bearing the scale being thin, parallax is negligible. The scale is placed along a diameter
of the cell, the zero placed in the centre of the blurred edge on one side, and the length of the diameter, to the central point of the blurred band on the opposite side, is read off correct to 0.1 mm. It is not possible to read more correctly than this, and since 0.1 mm. corresponds, at a magnification of 500, to 0.2μ, the accuracy of the measurement is the same as the error imposed by the limit of resolution.

Certain cells are on no account to be measured. These are: - cells which show marked hollowing, cells which show crenation, cells which show diffraction rings at the edges, cells which are touching or in rouleaux, and cells which are obviously not circular. It may be mentioned that when cells are photographed in plasma it is very rare to find a crenated cell, and irregular cells, such as abound in films, are almost never seen.

On the average 10 to 20 cells can be obtained from each plate. The diameters are reduced to from the figure for the magnification.

It is necessary to calibrate the scales used if the absolute size of the cells is of importance. Our micrometers and scales are calibrated at Teddington.

The method of obtaining measurements of erythrocytes in the fresh state has been described at some length, since the results obtained differ considerably from those obtained from dried films.
PART II.

SECTION II.

The mean size and normal distribution in the cells of man.

The number of cells to be measured.

The grouping of the cells.

Expression of the frequency distribution.

Results for six normal persons.
As an example of the results obtained by the method just described, we shall consider the mean size and the frequency distribution of the cells of man. Before giving the results, one or two points fall to be considered in detail.

The number of cells to be measured.

This is a point of great importance from the practical aspect, and depends on at least three considerations.

(1) The larger the number of cells to be measured the less will the mean be subject to error. The error of the mean depends on the standard deviation divided by the square root of the number of cells measured; obviously we shall obtain a more accurate mean if we measure a large number of cells. The standard deviation is, however, a small quantity — about 0.5 — so that if about 300 cells be measured, the mean will be subject to little error, the error being in the second place of decimals. The error due to sampling has also to be considered. The greater the numbers in the sample, the smaller will the sampling error be, but in practice it will be found to be very small if about 300 cells are taken.

(2) The number of groups into which we can divide the cells obviously depends, among other things, on the number measured. Sufficient cells should be
obtained to allow of a good frequency curve being constructed. If too few cells are taken parts of the curve, especially near the extremes, will be irregular. The numbers are, however, restricted by the following consideration.

(4) The measurement of a large number of cells is not possible without the introduction of errors due to the fact that the cells may alter on standing. From 10 to 20 cells can be obtained from each plate; to obtain, say, 1000 cells would take a long time. The advantage of having a large number of cells from which to construct a good frequency curve is, therefore, outweighed by the disadvantage that in order to obtain them we have to photograph and measure cells which have been removed from the blood stream for a considerable period of time - a thing which it is the object of the method to avoid. Hence the number of cells to be measured is the greatest number which can be photographed in a reasonably short space of time, say in 30 to 40 minutes. This number is from 300 to 500 cells.

The grouping of the cells.

Since we can measure the cells with an accuracy of 0.1 µ, it is permissible to group them in groups of 0.2 µ. In this way about 15 to 20 groups may be obtained. On the other hand, with a number so small as 300, the groups, and especially those at the extremes, are apt to show irregularities. A more even curve can be obtained by using 0.4 µ groups. Whether
this or the smaller grouping is best in any particular case must be decided by trial and by a consideration of the number of measurements available.

Expression of frequency distribution.

The frequency distribution is expressed in this study by giving the position of the mean ($\bar{M}$) of the median ($\bar{M}_i$) of the quartiles ($Q_1$ and $Q_2$) and of the extremes. The standard deviation ($\sigma$) and the probable error of the mean are obtained in the usual way. For an expression of skewness, the measure suggested by Bowley is used,

$$\kappa = \frac{(Q_2 - \bar{M}_i)(\bar{M}_i - Q_1)}{(Q_2 - \bar{M}_i) + (\bar{M}_i - Q_1)}$$

This measure is very insensitive to the condition of the frequency distribution at the extremes, and is obtained from three values which can be obtained with a high degree of accuracy. A measure of a greater degree of sensiveness is apt to be misleading in a case such as this, where there is considerable (relatively) error on the measurements. The measure used however, would indicate any marked degree of skewness.

In order to find the mean value of the diameter, and the frequency distribution for cells in health, six healthy males were examined, with the result as shown in the following tables.
<table>
<thead>
<tr>
<th>Subject</th>
<th>Plates Giving</th>
<th>Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>18</td>
<td>256</td>
</tr>
<tr>
<td>Mean</td>
<td>8.8</td>
<td>0.0375</td>
</tr>
<tr>
<td>Median</td>
<td>8.8</td>
<td></td>
</tr>
<tr>
<td>Upper quartile</td>
<td>9.2</td>
<td></td>
</tr>
<tr>
<td>Lower quartile</td>
<td>8.4</td>
<td></td>
</tr>
<tr>
<td>Upper limit</td>
<td>10.7</td>
<td></td>
</tr>
<tr>
<td>Lower limit</td>
<td>7.0</td>
<td></td>
</tr>
<tr>
<td>σ</td>
<td>0.6</td>
<td></td>
</tr>
<tr>
<td>κ</td>
<td>0.0</td>
<td></td>
</tr>
</tbody>
</table>

Subject 2. 22 plates giving 324 cells.

| Mean    | 8.8           | 0.030 |
| Median  | 8.7           |       |
| Upper quartile | 9.1   |       |
| Lower quartile | 8.35  |       |
| Upper limit | 10.4  |       |
| Lower limit | 7.4   |       |
| σ       | 0.54          |       |
| κ       | 0.06          |       |

Subject 3. 18 plates giving 289 cells.

| Mean    | 8.8           | 0.0241|
| Median  | 8.85          |       |
| Upper quartile | 9.09  |       |
| Lower quartile | 8.6   |       |
| Upper limit | 10.2  |       |
| Lower limit | 7.6   |       |
| σ       | 0.41          |       |
| κ       | -0.02         |       |
Subject 4. 18 plates giving 400 cells.

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>8.75</td>
<td>0.025</td>
</tr>
<tr>
<td>Median</td>
<td>8.8</td>
<td></td>
</tr>
<tr>
<td>Upper quartile</td>
<td>9.08</td>
<td></td>
</tr>
<tr>
<td>Lower quartile</td>
<td>8.50</td>
<td></td>
</tr>
<tr>
<td>Upper limit</td>
<td>10.3</td>
<td></td>
</tr>
<tr>
<td>Lower limit</td>
<td>7.5</td>
<td></td>
</tr>
<tr>
<td>σ</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>κ</td>
<td>-0.034</td>
<td></td>
</tr>
</tbody>
</table>

Subject 5. 20 plates giving 324 cells.

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>8.8</td>
<td>0.032</td>
</tr>
<tr>
<td>Median</td>
<td>8.8</td>
<td></td>
</tr>
<tr>
<td>Upper quartile</td>
<td>9.1</td>
<td></td>
</tr>
<tr>
<td>Lower quartile</td>
<td>8.5</td>
<td></td>
</tr>
<tr>
<td>Upper limit</td>
<td>10.0</td>
<td></td>
</tr>
<tr>
<td>Lower limit</td>
<td>7.6</td>
<td></td>
</tr>
<tr>
<td>σ</td>
<td>0.58</td>
<td></td>
</tr>
<tr>
<td>κ</td>
<td>0.0</td>
<td></td>
</tr>
</tbody>
</table>

Subject 6. 18 plates giving 324 cells.

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>8.85</td>
<td>0.033</td>
</tr>
<tr>
<td>Median</td>
<td>8.8</td>
<td></td>
</tr>
<tr>
<td>Upper quartile</td>
<td>9.15</td>
<td></td>
</tr>
<tr>
<td>Lower quartile</td>
<td>8.42</td>
<td></td>
</tr>
<tr>
<td>Upper limit</td>
<td>10.4</td>
<td></td>
</tr>
<tr>
<td>Lower limit</td>
<td>7.0</td>
<td></td>
</tr>
<tr>
<td>σ</td>
<td>0.61</td>
<td></td>
</tr>
<tr>
<td>κ</td>
<td>0.041</td>
<td></td>
</tr>
</tbody>
</table>
The mean size of the diameter of the cells may be taken in each case as 8.8 μ. This is a figure 1 μ greater than the usually accepted figure for the diameter of the human erythrocyte. The difference between the two sets of measurements is that in the above experiment the cells were suspended in plasma, whereas former measurements, giving a mean diameter of 7.8 μ, were made with cells in the dried state.

The accompanying microphotographs show the relative sizes of fresh, dried and stained erythrocytes, the cells being in all three cases under the same magnification. The irregularity and slight shrinkage of the cells in the dried state will be noticed readily, and also the very striking shrinkage and distortion in the stained state.

The distribution of the cell sizes appears to be a symmetrical and normal one. The curves are shown in the figures attached, and are of the usual type. The very slight skewnesses observed are probably insignificant, and the result of experimental or sampling errors.
Human cells
Goughian form
(x 500)

Sheep cells
Goughian form
(x 500)
Human cells, w/ plasma.

Some S or C are measurable. (x500)

Same cells, dried. (x500)
SECTION III.

The effect of drying.

Development of expression for shrinkage during drying.

Methods of measurement

Results.

The frequency curve for undried cells.

The value of $K$.

The frequency curve for dried cells.
In the previous section, it has been shown that when human erythrocytes are measured in plasma, the value of the mean diameter is greater than when they are measured in the dried state, the drying causing a shrinkage in the diameter of the cells. Since nearly all hitherto published observations on the diameter of red cells have been made on cells in a dried state, it has been thought important to investigate the extent of the alteration produced by the drying.

It is obviously impossible to measure a large number of cells in a fresh state, and then to re-measure the same cells when dry. The effect of drying may be expressed as the mean diameter of the cells in plasma compared with the mean diameter of the cells when dried; this is, however, insufficient for our present purpose. A special mode of procedure has therefore been adopted, based on the following considerations.

It has been shown in the last Section that the frequency curve obtained by grouping a large number of cells, measured in the fresh state immersed in plasma, according to diameter, closely approximates to the symmetrical type. If the curve be plotted on axes YOX, the abscissa showing the values of the cell diameters and the ordinate showing the number of cells in each group, the formula

\[ y = A e^{-\frac{(x-m)^2}{2}} \]
will apply, \( M \) being the mean diameter of the cells.

Consider the frequency curve obtained by grouping the same number of cells from the same individual, the measurements being in the dried state. We know that the mean diameter will be less than that for the cells measured in plasma. Let the position of this mean for the dried cells, on the same abscissa as used for the first frequency curve, be a point \( m \). The form of the second curve may be one of many, of which two are of special interest.

(a) The frequency curve for the dried cells may be symmetrical, the values of \( A \) and \( b \) being the same as that for the curve giving the distribution of the cells measured in plasma. Referred to the same axes as used in the curve for undried cells, but, to avoid confusion, using \( x \) for the diameter of the dried cells instead of \( X \), which will be used for the diameters of the cells in the undried state, the formula of such a curve will be

\[
Y = Ae^{-b(x-m)^2}
\]

In such a case the difference between the diameter of any cell in the undried and in the dried state will be constant, or \((X - x) = (M - m)\), whether the cell under consideration be of average size or not; that is, all cells will shrink equally when dried.

(b) The cells may not all shrink equally, but each cell lose on drying a constant percentage of the water contained within it, so that the volume of the dried cell bears a constant proportion to the content
of the cell in a moist state. The form of frequency
distribution which would result under these circum-
stances, the distribution of the diameters of the un-
dried cells being symmetrical, is one of special interest,
and may be arrived at in the following way.

Let the volume of the undried cell be $V$. This volume
will be obtained from the expression

$$ V = \frac{4}{3} \pi a^2 b. $$

the cell being treated as an ellipsoid of rotation about
its minor axis, and $a$ and $b$ being the semi-axes major and
minor respectively.

Let the semi-axis major shrink by an extent $da$, and the semi-axis minor by an extent $db$. The volume
of the dried cell will then be

$$ v = \frac{4}{3} \pi (a - da)^2 (b - db). $$

Neglecting unimportant terms, this equals

$$ \frac{4}{3} \pi (a^2 - a^2 db - 2a b da). $$

Since in the case of human red cells there is a relation
between the diameter and the thickness, so that $b = 0.3a$,
and assuming $db = 0.3 da$,

$$ v = \frac{4}{3} \pi (0.3a^2 - 0.9a^2 da) $$

Now in this special case, where the cell loses a constant
percentage of water on drying, and therefore a constant
volume,

$$ v = V/K. $$

$K$ is some constant to be determined. Hence

$$ v = \frac{4/3 \pi a^2 b}{K} $$

or

$$ \frac{4/3 \pi (0.3a^2 - 0.9a^2 da)}{K} = \frac{4/3 \pi a^2 b}{K} $$

From which it appears that

$$ da = \frac{a(K - 1)}{3K} $$

But $2da = (X - x)$ and $2a = X$, therefore
\[(X - x) = \frac{X(k-1)}{3K}\]

If therefore values of \(da\) — that is, values of the extent by which the semi-axis major shrinks on drying — be plotted against values of the semi-axis \(a\) of the cell in the dried state, a straight line through the origin will result.

If the distribution of the population before drying be a curve of the form

\[y = Ae^{-b(x-M)^2}\]

and the relation of the shrinkage of any cell to its diameter before drying be expressed by

\[(X - x) = \frac{X(k-1)}{3K}\]

the form of the frequency curve for dried cells will be

\[y = Ae^{-b\left\{\frac{x + \frac{x(k-1)}{2k+1} - m - \frac{m(k-1)}{2k+1}}{a k^{2n}}\right\}^2}\]

or

\[y = Ae^{-b\left\{\frac{3k(x-M)}{2ak^{2n}}\right\}^2}\]

It is now to be observed that the value of \(k\) can be obtained in a very simple way, for if this is a constant for all cells, its value can be arrived at by a consideration of the sizes of mean cells only. Hence

\[\frac{M - M_0}{M} = \frac{k-1}{3K}\]

These relations have been discussed at some length, for although they are very simple, they are by no means obvious.

By studying the relation of the frequency curve for cells in plasma and that for cells in dried films, information can be obtained regarding the changes which occur on drying; it should be possible to show which of the conditions (a) or (b) is fulfilled, if either.
Methods of measurement.

The diameters of the cells were determined by the photographic method described in the previous Section. The optical system was one giving an N.A. of 1.15, and a magnification of 505. Together with a wave-length of 4555 A.U., this system gives a limit of resolution of 0.2 μ.

The plates used were Wellington Orthoprocess, with an exposure of 1 second.

The manner in which the diameters of the cells were measured was that described in the previous Section. Great care was taken to split the spuricous disc, and great care taken to determine with accuracy the magnification, this being obtained from the measurement of the stage micrometer photographed at the same time as the cells.

The dried cells were measured in a manner similar to those in plasma. Films were taken from time to time from the undried erythrocytes, and dried at room temperature. Suitable fields were then selected and photographed. It is much more difficult to measure the dried cell than it is to measure the cell in a fresh state.

Results.

In order to obtain sufficient cells to give a good frequency curve, 700 cells in plasma, and the same number in the dried state, were photographed and measured
as described above. The number chosen is sufficiently large to give a smooth frequency curve when the cells are divided into groups of $4\mu$, and sufficiently large to give a very small error of sampling; at the same time it is not so large as to introduce errors due to changes in the diameters of the cells as the result of exposure.

In order to meet the criticism that during the time taken to photograph the cells in plasma the mean size might undergo alteration, the cells were measured from the plates, which were numbered in order of exposure, in groups of 100. A comparison between the mean diameter of successive 100's would show whether or not any significant alteration took place on standing. In point of fact, no such change was observed, the mean values for the successive groups never showing significant variation.

The cells were grouped according to the value for the diameter in groups of $0.4\mu$. The limit of resolution being $0.2\mu$, a finer grouping would be permissible, but a much larger number of cells than 700 would be required to give a smooth curve.

We shall now proceed to show that condition (ii) of the introduction satisfies the results obtained.

1. The frequency curve for the cells measured in plasma when referred to axes $YOX$, values of $X$ being values of cell diameters in scale divisions ($5.05$ scale divisions equal to $1\mu$), and values of $Y$ being values for the num-
ber of cells in each group of two scale divisions, is given by the formula

the value of $A$ being 187.5, that of $b$, 0.04, and that of the mean $M$, 43.05.

The extent to which this formula fits the curve is shown in the following table.

<table>
<thead>
<tr>
<th>X</th>
<th>Y exper.</th>
<th>Y calc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>39.05</td>
<td>85.0</td>
<td>99.0</td>
</tr>
<tr>
<td>40.05</td>
<td>132.5</td>
<td>130.8</td>
</tr>
<tr>
<td>41.05</td>
<td>160.0</td>
<td>159.8</td>
</tr>
<tr>
<td>42.05</td>
<td>180.0</td>
<td>180.1</td>
</tr>
<tr>
<td>43.05</td>
<td>187.5</td>
<td>187.5</td>
</tr>
<tr>
<td>44.05</td>
<td>180.0</td>
<td>180.1</td>
</tr>
<tr>
<td>45.05</td>
<td>158.0</td>
<td>159.8</td>
</tr>
<tr>
<td>46.05</td>
<td>130.0</td>
<td>130.8</td>
</tr>
<tr>
<td>47.05</td>
<td>89.0</td>
<td>99.0</td>
</tr>
</tbody>
</table>

There is some divergence between the calculated and the experimental results at the limits of the curve; this is only to be expected, for the number of cells dealt with is comparatively small. Apart from this, the results agree very well.

2. The mean value for the diameter of the cells in the dried state ($m$) is 38.4 scale divisions or 7.6 $\mu$. The frequency curve for the cell in plasma, as has been seen, has a mean at 43.05 scale divisions or 8.52 $\mu$.

Now we have

$$\frac{M - m}{M} = \frac{K - 1}{3K}$$
from which $K$ is found to be equal to 1.479

3. If condition (ii) of the introduction is applicable, the formula of the curve for the dried cells will be

$$y = Ae^{-b \left( \frac{2K(x - m)}{x^2 K + 1} \right)^2}$$

where $A = 187.5$, $b = 0.04$, $K = 1.479$, and $m = 38.4$.

The frequency curve which we obtain for the dried cells passes through the following points -

<table>
<thead>
<tr>
<th>$x$</th>
<th>$y$</th>
</tr>
</thead>
<tbody>
<tr>
<td>32</td>
<td>26</td>
</tr>
<tr>
<td>34</td>
<td>60</td>
</tr>
<tr>
<td>36</td>
<td>140</td>
</tr>
<tr>
<td>38</td>
<td>185</td>
</tr>
<tr>
<td>38.4</td>
<td>187.5</td>
</tr>
<tr>
<td>40</td>
<td>165</td>
</tr>
<tr>
<td>42</td>
<td>92</td>
</tr>
<tr>
<td>44</td>
<td>24</td>
</tr>
</tbody>
</table>

The correspondence between values of $Y$ obtained by the use of the above expression and those obtained from the experimental curve just given, are shown in the table below.

<table>
<thead>
<tr>
<th>$X$</th>
<th>$Y_{\text{exper.}}$</th>
<th>$Y_{\text{calc.}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>34.0</td>
<td>60</td>
<td>71</td>
</tr>
<tr>
<td>35.0</td>
<td>165</td>
<td>103</td>
</tr>
<tr>
<td>36.6</td>
<td>161</td>
<td>159</td>
</tr>
<tr>
<td>38.4</td>
<td>187.5</td>
<td>187.5</td>
</tr>
<tr>
<td>X</td>
<td>Y exper.</td>
<td>Y calc.</td>
</tr>
<tr>
<td>----</td>
<td>---------</td>
<td>--------</td>
</tr>
<tr>
<td>40.0</td>
<td>165</td>
<td>165</td>
</tr>
<tr>
<td>41.1</td>
<td>132</td>
<td>130</td>
</tr>
<tr>
<td>42.0</td>
<td>92</td>
<td>99</td>
</tr>
</tbody>
</table>

Except at the extremes, the correspondence is good.

The equation deduced for the frequency curve for dried cells, on the basis that each cell loses a constant amount of water, accordingly described the results in a satisfactory manner.

4. In a number of other cases similar drying experiments were carried out, comparisons between the dried and undried cells being made by comparing the means, as in the above case. The value of K given was found to vary from 1.45 to 1.65. Different conditions of drying the films will certainly influence the value of this constant, and investigations on this point are being made.

5. Since K is so nearly constant, it follows that the mean diameter of the cells before dying can be approximately obtained by adding to the mean value for the cells in a dried state a quantity varying from 0.15 to 0.115 times the mean value. In the case of human erythrocytes the mean value for dried cells is usually given as 7.8\(\mu\); the mean diameter of the cells in a fresh state may therefore be placed at a value between 8.7\(\mu\) and 8.95\(\mu\). Direct measurement shows the figure to be 8.8\(\mu\). Whether a similar correction can be made for the cells of other animals cannot at present be stated.
PART II.

SECTION IV.

The effect of exercise.

Apparatus required.
The preliminary analyses.
The experiment proper.

The preparation of the chamber.
The preparation of cells and plasma.
The preparation of Operator 1.
Introduction of gases.
Making of the films.

The photomicrography.

Results.
In 1920, Price Jones published the results of certain experiments concerned with the physiological variations in the size of the red cells of man. One of the more important conclusions drawn from the investigation was that the mean diameter of the erythrocyte increases in diameter during exercise, the variation being sometimes as great as 0.5 μ. There are certain fallacies in the experiments, one of which is that it is unpermissible to expose films of blood to air and then to suggest that any alteration in the mean diameter of the cells is due to the fact that the blood after exercise contains more CO₂. Such exposures to the air invalidate the whole experiment, for an equilibrium is rapidly reached between the blood and the air. Since the question of the size of the red cell, and especially its physiological variations, is of considerable importance, we have thought fit to investigate the matter by a new technique.

The principal difficulty is to arrange that the cells shall be measured in a fresh state, floating in plasma, and that the preparation of cells shall be made without exposure to the air, or to any atmosphere other than one whose gaseous content is such as to be in equilibrium with the gases of the blood before and after exercise respectively.

Apparatus required.

In addition to the usual apparatus for
The gas chamber.

Floor of chamber, with table ready for experiment.
the determination of dissociation curves, for gas analysis and for photography, the following special apparatus is needed.

1. A rectangular gas chamber, constructed of metal, and measuring 18" by 12" by 12". In the front and back walls are glass windows, joined by airtight joints to the metal; these windows measure 9 inches by 6 inches. On either side of the front window is a hole of diameter 3½", surrounded by a flange which projects from the metal for a distance of 1½ inches. In the roof of the chamber are eight holes about ½" diameter, fitted with tubulures; six of these are closed with solid rubber stoppers. Into one hole passes a mercury manometer; another holds a thermometer. In one end wall are two similar holes, one near the roof into which is inserted a two way stopcock, and one near the floor, closed with a rubber stopper. Below the front window is a slit through which one can just pass a slide when lying endways and when covered with a coverglass; this slit is closed with a plate faced with leather and held by two thumbscrews.

The whole apparatus stands raised about 4" on supports. Beneath it is a hot plate, and outside the window on the back wall is a light. The inside of the roof is covered with felt, to prevent water condensing and forming drops which fall on the preparations inside.

On the floor of the chamber stands a collapsible table consisting of two strips of glass on wooden
supports which raise the glass above the level of the floor. Both the strips of glass and the supports are of such dimensions that they can be taken out of the chamber through the holes at either side of the front window.

Two collars of thick canvas and rubber material, of such an external diameter that they will fit tightly into the flanges of the holes on the front wall of the chamber are provided. In length they are 2"; they should be able to be passed over the hands without difficulty. With them is provided a couple of pieces of oiled silk, about 12" long and 5" broad, and two pairs of rubber gauntlets which may be obtained by cutting a pair of surgical rubber gloves across at the level of the thumbs. A supply of adhesive plaster, about 1" thick, is required.

A centrifuge tube of about 10 cc. capacity is prepared for the collection of plasma in the following way: A rubber stopper, about 1/4" thick, is selected and cut so that it will fit tightly in the tube about half-way up. In the stopper are bored two holes of about 1 mm. diameter, and into one of them is inserted a piece of capillary tubing; the length of this piece of tube is such that it will just penetrate the stopper when the latter is in position, while its upper end just reaches the top of the centrifuge tube. A thin piece of rubber tubing is needed to connect the capillary with a syringe.

A small water oven is also required. The front of this is filled in with a metal plate, leaving only
a small opening near the floor; into the opening there slides a tray which, when not drawn out, closes the oven completely. The oven has a thermometer.

The preliminary analyses.

As previously observed the slides made from the blood of the subject at rest, and those made from the blood of the subject after exercise, must be prepared in atmospheres appropriate to the gaseous tensions of these bloods respectively. The method used in finding the composition of the proper gas mixture is as follows:

1. Resting blood - The subject is placed in a comfortable position and is left undisturbed for three-quarters of an hour. At the end of this time his jacket is slipped off and his left arm bared to well above the elbow. His left hand and part of the forearm are immersed in a large basin of hot water, as hot as can be borne, and kept there for 15 minutes. This procedure produces a great engorgement of veins, and it is unnecessary to use a tourniquet in withdrawing the blood.

There is then introduced into the median cephalic a No. 18 hypodermic needle, smeared on the outside with liquid paraffin. The needle is specially ground down for the purpose to a length of 20 mm. As soon as it is in position in the vein, as 20 cc. syringe is applied, and the piston cautiously withdrawn as the blood flows in. Coagulation of the blood is prevented by the presence of mixture of 1 part of sodium fluoride to 4 parts of
sodium oxalate in the bottom of the syringes. Of this mixture, 0.05 gm. is used for every 20 cc. of blood collected. One cc. of blood is now transferred to a van Slyke apparatus and the CO₂ estimated; similarly 1 cc. is transferred to a Haldane apparatus and the CO and the O₂ estimated.

The dissociation curves are now plotted for the remainder of the specimen, both the oxygenated and for reduced blood.

Independently there is determined the Hb. content by the Gowers-Haldane haemoglobinometer; the alveolar CO₂ is also determined in the usual way.

2. Blood after Exercise - This specimen of blood must be obtained from a person who is thoroughly exhausted, and the following procedure is carried out. The subject is given a dynamometer to work in the left hand, and is asked to work it continuously. At the same time he is made to run up and down stairs as rapidly as he can till he is almost on the verge of collapse. In the present investigation two steep flights of stairs were ascended and descended ten times; two of the three subjects were unable to stand on their feet when the exercise was finished. Immediately on the cessation of the exercise blood was taken from the cephalic vein, without immersion in hot water, and without the use of a tourniquet. The blood was received into fluoride and oxalate.

Without delay the CO₂ content, the O₂ content and the O₂ capacity are determined in the manner
The data of one of the experiments will be given here to illustrate its nature.

---

**CO₂ Dissociation Curves for Blood**

- **A**: "Resting" Blood
- **B**: Blood after Exercise
- **G**: Reduced Blood
- **X**: Oxygenated Blood

---

**Graph Details**

- **X-axis**: Pressure of CO₂ in mm. Hg
- **Y-axis**: Volume CO₂ in 100 cc of Blood

---

The graph illustrates the dissociation of CO₂ in blood under different conditions.
described above. The remainder of the sample is used to plot the dissociation curve for CO in both oxygenated and reduced blood.

Interpretation of the data - The data of one of the subjects (W.P.B.) will be given here to illustrate the procedure adopted.

1. Resting blood - The analysis gave,

<table>
<thead>
<tr>
<th>CO₂ cc. per cent</th>
<th>O₂ cc. per cent</th>
<th>O₂ capacity cc per cent</th>
<th>Per cent. O₂ saturation.</th>
</tr>
</thead>
<tbody>
<tr>
<td>51.3</td>
<td>18.86</td>
<td>19.3</td>
<td>97.8</td>
</tr>
</tbody>
</table>

Alveolar air: CO₂, 6.17 per cent. O₂, 12.43 per cent.
Barometer, 771 mm.

From the table the CO₂ partial pressure in the alveoli can be calculated in the usual manner;

\[
(771 - 47) \times \frac{6.17}{100} = 44.6 \text{ mm.}
\]

It can thus be inferred that the CO₂ tension in the subject's arterial blood is 44.6 mm., and at this tension its total CO₂ content is 51.3 per cent, as found by direct observation.

2. Reoxygenated blood - The analyses gave,

<table>
<thead>
<tr>
<th>CO₂ cc. per cent.</th>
<th>O₂ cc. per cent</th>
<th>O₂ capacity cc per cent</th>
<th>Per cent. O₂ saturation.</th>
</tr>
</thead>
<tbody>
<tr>
<td>43.7</td>
<td>6.95</td>
<td>24.25</td>
<td>28.6</td>
</tr>
</tbody>
</table>

From the table it can be seen that blood withdrawn immediately after exercise contained 43.7 per cent CO₂. By reference to the dissociation curves, it may be seen that this content is obtained by a pressure of 68 mm. when
the blood is fully reduced and by 82 mm. when the blood is fully oxygenated. From the analysis of the blood as withdrawn, however, it will be seen that it is only saturated to 28.5 per cent of its full O₂ capacity.

Interpolating the dotted curve in the figure to represent this condition of partial O₂ saturation, it will be found that a CO₂ content of 43.7 per cent corresponds to a CO₂ tension of 72.5 mm.

The dissociation curve is now worked out for HbO₂, with the blood under a CO₂ tension of about 73 mm. From the analysis of the curve so obtained it may be determined that the O₂ tension of the venous blood as withdrawn from the vein was in this case 23 mm.

From these data we have the atmosphere in the chamber, to be in equilibrium with the blood after exercise, as having the following partial pressures:

\[
\begin{align*}
\text{CO}_2 &= 72.5 \text{ mm}.
\text{O}_2 &= 23 \text{ mm}.
\end{align*}
\]

Establishing the gas mixture in the chamber, as has already been indicated, in the case of the blood at rest, it is sufficient to prepare the atmosphere of the chamber in which the films are to be made by adding a calculated amount of CO₂ to the chamber. In the case of the blood after exercise the proper atmosphere has to be established by making a mixture of air and nitrogen to which CO₂ has afterwards to be added. In making up this latter mixture, it has to be remembered that the volume of the air nitrogen mixture introduced into the
the chamber has to be increased by the addition of the 
$CO_2$, and that therefore the percentage of $O_2$ has to be  
greater in this mixture than that which is ultimately  
desired in the chamber. If $x$ represents the percentage  
of $O_2$ desired in the chamber in the final mixture, and  
y the final percentage of $CO_2$, then the percentage of  
$O_2$ in the air nitrogen mixture must be 

$$x = xy/(100 - x)$$

The necessary amount of $CO_2$ can be added to the chamber  
from graduated containers; the gas being stored above  
calcium carbonate solution. The volume of the gas to  
be added is found as follows: The volume of the chamber,  
by external measurement, was about 40 litres. The internal  
capacity for gas, however, was appreciably less than  
this figure when the chamber was fitted up for an experi- 
ment, for under these circumstances there must be present  
in it apparatus of all sorts, as well as the hands and  
forearms of one of the operators. The internal volume  
has therefore to be obtained experimentally. The chamber  
is sealed with the operators hands in position (see later) 
a measured volume of $CO_2$ introduced, and, after mixing,  
a sample of the air withdrawn. This was done twice and  
the results of the analyses tabulated.

In making these observations, another  
point has to be noted. The temperature at which the $CO_2$  
is introduced is that of the room (about 15°C) while  
that of the chamber is usually 37°C, as will be seen.  
This means that the volume of $CO_2$ is increased so as to  
equal 311/288, or 1.08 of the amount added; or, what
is of the same moment, that when a gas sample is withdrawn for analysis, the constituents of the gas present in the chamber shrink to 0.925 of their bulk, while the CO₂ so withdrawn returns to the same volume as it has when being introduced into the chamber.

These points have to be carefully observed in making the analyses.

The experiment proper.

For the purposes of the experiment proper at least six persons are required, three operators, responsible for the more technical parts of the experiment, and three assistants.

The preparation of the chamber. Into the chamber, the floor of which is covered with blotting paper, is placed about 300 cc. of water. The heating unit and the light are then turned on. The windows should be rubbed with soap to prevent steaming. On the glass table on the floor of the chamber are then arranged the requisites for the making of films. (1) Some 20 slides of the usual size, but of thin white glass, cleaned and flamed to remove grease; while still hot they are arranged in piles on the near left hand corner of the table. (2) The same number of No. 1 coverslips, smeared along the edges with an equal mixture of bees wax and vaseline. Each coverslip is inserted, standing on its edge, in a shallow tray of this grease. (3) Two flamed watch glasses, two capillary pipettes with teats, and two pairs
of dissecting forceps are placed on the central part of the table, the teats of the pipettes pointing to the right. (4) A special pipette consisting of a glass tube with teat connected to a wide bore hypodermic needle. (5) A mechanical fan to mix the gases in the chamber; and (6) a stand to hold centrifuge tubes, together with a supply of filter paper.

The comparatively great restriction of the movements of the operator's hands when in the chamber makes the prearrangement of the apparatus very important.

The preparation of the cells and plasma. About 10 cc of blood is withdrawn from the patient, who may be either at rest or exhausted by exercise as the case demands in the manner described above. In either case it is essential that the condition of the patient shall be the same as that established for the previous analyses - he must be at rest for the same time or have done the same amount of exercise. The blood is, however, drawn into oxalate alone instead of oxalate and fluoride, for the presence of fluoride gives rise to great trouble through the occurrence of rouleaux, and the appearance of crenations. A few cc of the blood is transferred, without coming into contact with the air, into a small syringe containing a glass bead; a metal cap is then put on the end of the syringe, and the syringe placed in the chamber on the right hand side of the table.
The special centrifuge tube already described is then flushed out with the mixture of gases which is to constitute the atmosphere in the chamber, or with the alveolar air of the subject if the measurements are to be made of cells during rest. A rubber tube is next attached to the capillary of the tube, and through this blood is introduced until all the space beneath the stopper is filled. The capillary is pulled out, and the centrifuge tube centrifuged till clear plasma makes its appearance under the stopper; it is then placed in the stand in the chamber, its mouth closed by a loosely fitting cork. The cork serves to prevent water condensing above the rubber stopper.

The preparation of Operator 1.

During the centrifugalisation of the blood, Operator 1 prepares to be sealed into the chamber. He draws the first pair of rubber gauntlets on his arms until their upper edges reach just below the elbows. Round the lower part of the forearms are then wrapped the pieces of oiled silk, so that their lower edges lie half an inch above the styloid processes of the radii. These lower borders are secured to the skin with strips of plaster. Over the oiled silk are placed the second pair of gauntlets, in such a way that their lower edges lie just below those of the silk; these edges are likewise strapped to the skin by bits of adhesive plaster.

The operator then slips the rubber collars over his wrists; he inserts his hands into the chamber,
and his assistant forces the rubber collars into the flanges which surround the openings in the chamber for the arms. The operator arranges his hands in a comfortable position, and his assistant turns back the upper edges of the lower pair of gauntlets so that they lie over the flanges. He secures them there with plaster. The upper edges of the oiled silk are next turned back in the same way, and secured also; then upper pair of gauntlets are then pulled down the arms so that their lower edges lie over the flanges, while their upper edges are secured to the skin of the arms about 3 inches below the elbows.

This method of sealing provides an air-tight and CO tight seal which allows of a considerable degree of movement.

Introduction of the gases. As soon as Operator 1 is sealed inside the chamber, he begins to fan the air with the fan, and continues till the air has reached 37° C. The required gases are then introduced into the chamber.

When cells of the blood during rest are under consideration it is necessary to add 0.04% CO₂ to the air of the chamber, so that the content of CO₂ may be equal to that in the patient's alveolar air when he is at rest. This quantity is about 2.5 litres, and may be introduced into the chamber from syphon bottles. During the introduction Operator 1 fans the air of the chamber of secure mixing.

When measurements of the blood cells after exer-
cise are to be made, it is necessary to adjust the atmosphere of the chamber for both $O_2$ and $CO_2$. The most convenient way is to mix the calculated volumes of air and nitrogen is a bag of about 100 litres capacity; the necessary amounts of air and nitrogen are passed through a meter. The gases are mixed, and the mixture passed into the chamber by one of the holes near the floor. At the same time some of the stoppers in the holes in the roof of the chamber are removed; as the air of the chamber is warm and the mixture cold, the former is displaced with but little diffusion. The gas bag is filled a second time, and the chamber flushed out once more. The stoppers are put back in the holes, and the air mixed with the fan. To this air is then added $CO$ as before. The result of this procedure is to establish in the chamber an atmosphere in equilibrium with the gases in the blood of the exhausted subject.

The fillings, of course, result in a rise of pressure in the chamber. The fanning is stopped when the fillings are complete; Operator 2 takes a sample of the air in the chamber and analyses it; if the quantities in the air are in accordance with calculation; as they should be, the chamber is connected with the outside for a moment so that the pressure falls, and the experiment is proceeded with.

Making of the films. Operator 1 now brings the blood and plasma into contact with the air of the chamber. A little blood is transferred to one watch glass, and,
with the metal tipped pipette, some plasma is placed in the other watch glass.

At the end of a short period, during which the blood and plasma are allowed to come into equilibrium with the air of the chamber, the first film is made. On a clean slide a small drop of plasma and a smaller drop of blood are placed and mixed. With forceps a coverslip is lifted from the tray, and placed on the drops; it is gently pressed down so that the grease adheres on all sides. The assistant opens the slit under the window, the operator passes the slide out, and the slit is closed. The slide is received on the tray of the oven previously mentioned, which is heated to 37 °; it is carried up to the dark room for photography. Blood films are made in this way till the material is exhausted.

The principal difficulties are the following -

1. The cells may show crenation. If this occurs, the experiment has to be abandoned, and this constitutes the principal difficulty.

2. Rouleaux formation may occur. It may be reduced by keeping the films thin, and above all by avoiding movements of the coverslip.

3. Unless the grease is in contact with the slide all the way round, the cells will not settle. This is most important, and necessitates careful and even greasing of the coverslips. The completeness of the film of grease is essential for another reason - that the gaseous content of the preparation
shall not alter during the photography. If the grease seal is complete, the blood is essentially in a sealed chamber.

When all the films have been made and the experiment is over, Operator 2 takes a second sample from the chamber, in order to find the composition of the atmosphere at the end of the experiment.

Operator 1 then leaves the chamber.

The photomicrography.

This is conducted exactly as described in an earlier section, by the method therein detailed. The only difference is that the stage of the microscope is warmed to above 30°c. by a radiator.

One aims at measuring 100 cells - rarely more; this entails the exposure of some fifteen to twenty plates, together with the plates giving the magnification by means of photographs of the micrometer.

Results.

The results are best set forth in tabular form, the data for three subjects being given in full.

(Tables follow here)
### BLOOD DURING REST.

<table>
<thead>
<tr>
<th>Subject</th>
<th>J. M. L.</th>
<th>W. P. B.</th>
<th>R. W. P.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alveolar CO₂ per cent.</td>
<td>6.5</td>
<td>6.17</td>
<td>6.45</td>
</tr>
<tr>
<td>Partial pressure, CO₂ in lungs, mm.</td>
<td>44.8</td>
<td>44.5</td>
<td>45.7</td>
</tr>
<tr>
<td>O₂ capacity, per cent. (18.5 c.c. per cent. =100)</td>
<td>104</td>
<td>104.2</td>
<td>100</td>
</tr>
<tr>
<td>Per cent. O₂ saturation</td>
<td>83.5</td>
<td>87.6</td>
<td>87.6</td>
</tr>
<tr>
<td>CO₂ content, per cent.</td>
<td>56.1</td>
<td>51.3</td>
<td>49.4</td>
</tr>
<tr>
<td>CO₂ tension, mm.</td>
<td>45.3</td>
<td>44.0</td>
<td>45.65</td>
</tr>
<tr>
<td>*pH</td>
<td>7.39</td>
<td>7.36</td>
<td>7.33</td>
</tr>
</tbody>
</table>

**In chamber.**

| CO₂ per cent. at beginning of experiment | 6.8 | 6.38 | 6.5 |
| CO₂ per cent. at end | 6.39 | 6.03 | 6.2 |
| Barometer, mm. | 749 | 770 | 759 |
| Mean partial pressure of CO₂, mm. | 49.1 | 44.7 | 45.05 |
| Measurements: | | | |
| Mean diameter in µ | 8.60 | 8.63 | 8.55 |
| Probable error, µ | ±0.041 | ±0.052 | ±0.043 |

### BLOOD AFTER EXERCISE.

<table>
<thead>
<tr>
<th>Subject</th>
<th>J. M. L.</th>
<th>W. P. B.</th>
<th>R. W. P.</th>
</tr>
</thead>
<tbody>
<tr>
<td>O₂ capacity, per cent. (18.5 c.c. per cent. =100)</td>
<td>105</td>
<td>120</td>
<td>120</td>
</tr>
<tr>
<td>O₂ saturation, per cent.</td>
<td>42.2</td>
<td>28.6</td>
<td>21.4</td>
</tr>
<tr>
<td>CO₂ content, per cent.</td>
<td>51.3</td>
<td>43.7</td>
<td>45.1</td>
</tr>
<tr>
<td>CO₂ tension, mm.</td>
<td>83</td>
<td>72</td>
<td>93</td>
</tr>
<tr>
<td>*pH</td>
<td>7.07</td>
<td>7.06</td>
<td>7.05</td>
</tr>
</tbody>
</table>

**In chamber.**

| CO₂ per cent. at beginning of experiment | 11.8 | 11.6 | 13.5 |
| CO₂ per cent. at end | 10.5 | 11.17 | 11.0 |
| O₂ per cent. at beginning | 3.91 | 4.35 | 3.62 |
| O₂ per cent. at end | 3.76 | 5.82 | 6.64 |
| Mean CO₂ partial pressure, mm. | 80 | 79.2 | 87 |
| Mean O₂ partial pressure, mm. | 34.6 | 35.4 | 36.5 |
| Barometer | 760 | 745 | 760 |
| Measurements: | | | |
| Mean diameter in µ | 8.51 | 8.57 | 8.50 |
| Probable error, µ | ±0.048 | ±0.044 | ±0.041 |
From these tables it will be seen that the differences between the mean diameter of the cells from the blood before and after exercise respectively was in the three cases:

<table>
<thead>
<tr>
<th></th>
<th>Difference (μ)</th>
</tr>
</thead>
<tbody>
<tr>
<td>J. McL.</td>
<td>0.09μ</td>
</tr>
<tr>
<td>W.P.B</td>
<td>0.06μ</td>
</tr>
<tr>
<td>R.W.P</td>
<td>0.05μ</td>
</tr>
</tbody>
</table>

These differences are quite insignificant. We are therefore entitled to conclude that the red cells of man do not alter in diameter after exercise, to any measurable extent.

Reference.

PART II.

SECTION V.

**On Burker's correlation.**

Burker's relation between Hb. and red cell area.
Formulae for area and volume.
Calculation of thickness.
Tables of areas, volumes, and haemoglobin contents.
The values of the constants.
Errors, and their effects.
Conclusion.
In connection with the dimensions of the red cell and the methods of ascertaining them, there arises a very interesting point - the correctness or otherwise of what is known as Bürker's correlation.

Bürker has pointed out that there is a relation between the surface area of the red cell and the amount of haemoglobin contained in the cell, the quantity of pigment per square \( \mu \) of surface being constant in all mammals investigated. This constancy he illustrates in nine different animals. The correlation is expressed by

\[
\frac{\text{Haemoglobin in one red cell}}{\text{Surface area}} = \text{Constant},
\]

the value of the constant being 32.1 "grammes per square \( \mu \) of area.

As soon as we inspect this relation closely, a difficulty of great magnitude arises. It is obvious that any expression for the area or the volume of the cell will involve values for the diameter and for the thickness of the cell. The measurement of the diameter is fairly easily carried out within certain limits of error, but it is a very difficult matter to measure the thickness of a red cell of average size. Measurements made by attempting to focus the upper and lower surfaces of the cell are valueless, for the depth of focus of any lens system is of the same order as that of the thickness to be measured. Measure-
ments by micrometer have to be conducted either on stained films, or in fresh preparations; in the former case, few cells are seen on edge, in the latter, they tend to roll over. Only by photography can any estimate be made, and even then the technical difficulties in obtaining a good value for the thickness are almost insuperable.

Setting this difficulty aside for the moment, we shall consider the correlation of haemoglobin content and area.

The red cell, being circular on horizontal section and biconvave on vertical section, may be regarded with very little error, as an ellipsoid of rotation about the minor axis, from which two little ellipsoids, each equal in volume to twice the volume of the concavity, are subtracted. Treating the cell in this way, the expressions for area and volume are easily found.

We have

\[ \text{Area} = 2\pi A^2 + 2\pi AB \frac{\sinh^{-1} e}{e} \]

where \( e \) is the eccentricity about the minor axis, and equal to

\( A \) is the semi-axis major of the ellipsoid, and \( B \) the semi-axis minor.

Also we have

\[ \text{Volume} = \frac{4\pi A^2 B}{3} - 2 \left( \frac{4\pi a^2 b}{3} \right) \]

where \( A \) and \( B \) have the meaning above, and where \( a \) and \( b \) are the semi-axes of the little ellipsoids to be subtracted.
Burker's formula for the area is

\[ \text{Area} = \frac{D^2 \pi}{4} \]

where D is the diameter of the cell, and equal to 2A.

It is necessary, in order to calculate with any degree of accuracy the volume and area of various cells of various diameters, to have values for B, which values can only be obtained from measurements of the thickness of the cells. Looking up the best tables for the diameters of the cells of the mammalia—those of Gulliver—we find few values for the thickness of cells, but by determining the relation which is in general true for the values for diameter and thickness, we can find the thickness of cells of any diameter, assuming the relation to be followed. Taking the values for the diameters from Gulliver's tables, and plotting these against the values for thickness, it will be seen that the thickness becomes less as the diameter becomes less, the relation apparently being

\[ \text{Thickness} = \frac{\text{Diameter}}{3.3} \]

Such a line passes very evenly among the points, and may be taken as as good a relation as is available.

Taking Burker's values for the diameters of the cells of the various animals, the thickness has been calculated from this relation; thereafter, we have calculated the areas and volumes of the different cells. To do this, we take A as equal to 0.5 the diameter, B as equal to 0.67 the thickness, and a as 0.25 the diameter,
and b as 0.125 the thickness. These figures are selected as the result of the examination of microphotographs of the cells of various animals, as being the best values obtainable. Even if a and b are judged a little wrongly, scarcely any effect is produced on the volume, in which these terms are unimportant, and no effect on the area, which does not contain a and b.

In the following table the results are expressed. After the name of the animal in the first column is given the diameter of the average red cell, according to Burker; the next column contains the corresponding thickness. The volume, first without the subtraction of the little ellipsoids representing the bi-concavities, and then corrected by their subtraction, is shown in the following columns; these values are followed by the figure for area, calculated from the formula for the area of an ellipsoid. The area, calculated according to Burker's formula, and the haemoglobin content, complete the table.

<table>
<thead>
<tr>
<th>Animal</th>
<th>Diam. in µ</th>
<th>Thickness in µ</th>
<th>Volume uncorr. in µ³</th>
<th>Volume corr. in µ³</th>
<th>Area in µ²</th>
<th>Area Burker, in µ²</th>
<th>Hb. 10⁷ grm.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dog</td>
<td>7.26</td>
<td>2.15</td>
<td>72.1</td>
<td>64.7</td>
<td>100.9</td>
<td>82.7</td>
<td>24</td>
</tr>
<tr>
<td>Pig</td>
<td>6.60</td>
<td>2.0</td>
<td>56.8</td>
<td>51.3</td>
<td>85.5</td>
<td>68.4</td>
<td>22</td>
</tr>
<tr>
<td>Rabbit</td>
<td>6.60</td>
<td>2.0</td>
<td>56.8</td>
<td>51.3</td>
<td>85.5</td>
<td>68.4</td>
<td>20</td>
</tr>
<tr>
<td>Calf</td>
<td>5.94</td>
<td>1.8</td>
<td>41.4</td>
<td>37.2</td>
<td>69.4</td>
<td>55.4</td>
<td>19</td>
</tr>
<tr>
<td>Horse</td>
<td>5.94</td>
<td>1.8</td>
<td>41.4</td>
<td>37.2</td>
<td>69.4</td>
<td>55.4</td>
<td>18</td>
</tr>
<tr>
<td>Cat</td>
<td>5.77</td>
<td>1.6</td>
<td>30.4</td>
<td>27.2</td>
<td>57.7</td>
<td>...</td>
<td>15</td>
</tr>
<tr>
<td>Sheep</td>
<td>4.62</td>
<td>1.45</td>
<td>20.0</td>
<td>18.0</td>
<td>42.3</td>
<td>33.6</td>
<td>11</td>
</tr>
<tr>
<td>Goat</td>
<td>4.00</td>
<td>1.25</td>
<td>13.4</td>
<td>12.2</td>
<td>31.7</td>
<td>25.1</td>
<td>8</td>
</tr>
</tbody>
</table>
To these we shall add a table of figures showing the constance of haemoglobin content (a) per unit of volume, (b) per unit of area, and (c) per unit of area as calculated by Burker's formula.

<table>
<thead>
<tr>
<th>Animal</th>
<th>Volume</th>
<th>Constancy of Hb. per unit of</th>
<th>Area</th>
<th>Area (Burker)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dog</td>
<td>37.1</td>
<td>23.8</td>
<td>29</td>
<td></td>
</tr>
<tr>
<td>Pig</td>
<td>42.9</td>
<td>25.7</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td>Rabbit</td>
<td>39</td>
<td>23.6</td>
<td>29</td>
<td></td>
</tr>
<tr>
<td>Calf</td>
<td>51</td>
<td>21.6</td>
<td>34</td>
<td></td>
</tr>
<tr>
<td>Horse</td>
<td>49.4</td>
<td>20.2</td>
<td>33</td>
<td></td>
</tr>
<tr>
<td>Cat</td>
<td>55.5</td>
<td>26</td>
<td>..</td>
<td></td>
</tr>
<tr>
<td>Sheep</td>
<td>61.1</td>
<td>26</td>
<td>33</td>
<td></td>
</tr>
<tr>
<td>Goat</td>
<td>65.6</td>
<td>25.2</td>
<td>32</td>
<td></td>
</tr>
</tbody>
</table>

The animals in these table are those dealt with by Burker, with the exception of the cat, which has been added.

Examining this last table, it will be seen that the figures in the last column are the most constant. The mean value is 31.7; the highest value is 34, and the lowest 29 - the difference between the extremes is therefore 5. With so few figures as these, a good way to express the variation is to give half the difference between the extremes as a percentage of the mean; in this case there is a variation about the mean of ± 7.5 per cent.

In the second column the variation is greater; the mean is 24, and the variation ± 12 per cent.

In the first column, the mean is 50, and the vari-
ation as great as ± 28 per cent.

It must therefore be conceded that, with this method of procedure, the correlation between the haemoglobin content and the cell area as calculated by Burker's formula is the best, and that between the haemoglobin content and the true area only a little less good. There is not a good simple relation between haemoglobin content and volume.

At this point it may be observed that if the haemoglobin content divided by the area as obtained by Burker's formula be a constant, it is not possible for the haemoglobin divided by the volume to be a constant too, unless the thickness of the cells of all animals is constant. For the area is

\[
\frac{\pi D^2}{2} \text{ or } \frac{4A^2\pi}{2h}
\]

and the volume (uncorrected) is

\[
\frac{4\pi A_B^2}{3}
\]

and if both, when divided into the same thing - the haemoglobin content - are to give a constant, \(2h/3\) must be constant, which is impossible unless all cells have the same thickness. It is therefore not surprising to find no simple relation between haemoglobin content and volume, if Burker's figures are to be taken as correct.

It will now be shown that no significance can be attached to the fact that haemoglobin divided by area gives a constant, and that we are possibly dealing with a spurious correlation.
Burker’s formula for the area, while it gives a good enough approximate result, is obviously not a formula applicable to a solid body, for it contains no third dimension; it is the formula for the area of an infinitely thin disc. As soon as we take an expression for the area, one component of which is the thickness of the cell, the simple relation obtained by the use of the other formula becomes subject to a greater variation. It is, accordingly, necessary, before coming to the conclusion that the ratio

$$\frac{\text{Haemoglobin}}{\text{area}} = \text{Constant}$$

is real, to consider the accuracy of the measurement of the cell, upon which all the calculations are based. In other words, we have to see if the function to which the haemoglobin content is related is sensitive to small errors or not. In order to make a study of this, we shall assume that the cell is measured under ideal conditions of microscopy.

The limit of resolution of even the most perfect optical system is not less than 0.2µ as has been indicated in Section 1 of this part. We must therefore attach a possible error of ±0.1µ to any measurement of red cells hitherto recorded - an estimate which is very charitable, as rarely have cells been measured under a system with so small a limit. Attaching the error of ±0.1µ to each semi-axis, we proceed to find the error involved in calculations of area.

Taking a horse erythrocyte, of diameter 5.52µ,
and of thickness 1.89µ, the area of the surface works out at 61.4µ². If the semi-axes are increased by 0.1µ, the area becomes 66.6µ², and if they are diminished by the same amount, the area becomes 56.4µ². The error due to the limit of resolution may therefore introduce an error of ±8 per cent on the area.

Taking next a small erythrocyte - that of the goat - with a diameter of 4µ and a thickness of 1.25µ, the area is 31.7µ². Increasing the semi-axes by 0.1µ makes the area 35.3µ², and diminishing them by 0.1µ makes the area fall to 28.9µ². The variation is therefore about ±10 per cent.

Now let us consider the effect of the same error on the volume. Uncorrected volumes will be given for simplicity. Taking again the horse erythrocyte, the volume is 37.3µ³, with a diameter of 5.52µ and a thickness of 1.89µ. An increase of 0.1µ in the axes makes the volume 43.7µ³; a decrease of the same amount reduces the volume to 32µ³. The variation makes ±16 per cent difference in the volume.

Taking the goat cell, the volume is 13.4µ³. An increase of 0.1µ in the semi-axes gives a volume of 16.3µ³, and a decrease of the same amount a volume of 10.2µ³. The variation in the semi-axes therefore results in an error of ±23 per cent.

We may now consider the effects of these variations on Burker's measure for area; to these we shall add figures for human cells (diameter 7.3µ), and shall
collect all the results together into a table.

<table>
<thead>
<tr>
<th></th>
<th>Area</th>
<th>Area</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Burker, in μ²</td>
<td>in μ²</td>
<td>in μ³</td>
</tr>
<tr>
<td>Man</td>
<td>95.5 ± 5</td>
<td>115.8 ± 6</td>
<td>79.0 ± 10</td>
</tr>
<tr>
<td>Horse</td>
<td>47.1 ± 3.5</td>
<td>61.4 ± 5.1</td>
<td>37.3 ± 5.8</td>
</tr>
<tr>
<td>Goat</td>
<td>2.51 ± 2.5</td>
<td>31.7 ± 3.2</td>
<td>13.4 ± 3.0</td>
</tr>
</tbody>
</table>

In plotting the values for area or for volume, we must now plot, not the area and volume obtained by the use of the respective formulae, but these values plus and minus the errors attached, for, because of the limits imposed by the optical system, the true area or volume may vary within the limits defined by the attached error. Considering this, it will be seen that the relation

\[
\frac{\text{Haemoglobin}}{\text{Volume}} = \text{Constant}
\]

may be quite a good one, and that the relation

\[
\frac{\text{Haemoglobin}}{\text{Area}} = \text{Constant}
\]

may be less accurate than appears at first sight. The point cannot be decided because of the great errors attached to the initial measurements, and because the volume is so sensitive to small differences in the values of the diameter and the thickness.

Certain further observations may now be made.

1. When dried films are used (the measurements used by Burker, and those given by Gulliver, are on dried Gells) there is, because of the great contrast, a tend-
ency to measure between points on the outside of the spurious disc. These errors will not cancel out, for they are not errors of judgment, but constant errors. Although each error is constant and all the errors of the same magnitude, they will produce very different effects on the area and volume of large and small cells respectively, as has been seen above.

2. The optical systems used in this type of work rarely have so small a limit as 0.2μ. Most of them could not show a limit of less than twice this figure.

3. The most careful measurements of thickness do not approach in accuracy those of diameter, and so the errors which may occur in the measurements of thickness are even greater than we have allowed for.

4. The measurements on dried cells, such as are used by Burker, are not even approximately the same as those for fresh cells; it is obvious that accurate areas and volumes cannot be calculated from the existing figures.

The errors which affect the measurements on which Burker’s correlation is based are therefore many and serious, but even if we allow no error except that of necessity imposed by the limit of resolution, it will be clear that we cannot insist on a linear relation between area and haemoglobin content. As soon as the errors are attached to the figures for volume, we get, instead of a line, straight or curved, passing through the points, a broad band, opening out like a trumpet towards the cells of large volume, and within which a straight line could quite well be laid down.
From the existing measurements Burker's correlation could accordingly never be established with any degree of certainty. A linear relation between haemoglobin content and volume is as possible as one between haemoglobin content and area.

It is to be carefully noted that we cannot conclude that Burker is wrong, any more than Burker could claim that he is right. The best relation must be left undetermined, for it cannot be decided from the existing figures, and probably not even from the best figures which it would be possible to obtain. The value of this study of the matter lies in the showing of the immense importance of accuracy in measurement, the magnitude of the errors which creep in when bodies of the same order as the wave-length of light are being measured, and the danger of coming to conclusions from defective figures.

Papers referred to:
PART III.

THE ERYTHROCYTE AS A BALLOON-LIKE BODY.

Changes in form with changes in volume.

The changes in a spheroidal balloon.

The changes in the mammalian cell.

Photographic measurements.

Diffraction measurements

A theory to explain the biconcave discoid shape.
It is well known that there are two principal views regarding the structure of the red cells of mammals. According to the first view, advocated by Rollett, the cell is made up of a more or less dense sponge work in which the haemoglobin is held contained. According to the second view, originally held by Schwann, the red cell is composed of a membrane containing a fluid or a semi-fluid.

In this Section it is not proposed to discuss the evidence, but to treat the problem in a direct way. It will be shown that (i) a body possessing a stroma such as Rollett postulates will behave in a certain way when the volume is increased. Thereafter (ii) it will be shown that a balloon-like body of the shape of the red cell undergoes definite changes of form when distended, the changes being quite different from those met with under (i). Finally, it will be shown that the changes which occur in the form of the mammalian red cell when fluid passes into it and distends it are such as can only be explained by the assumption that the cell is a balloon-like body.

(i) Consider a spheroidal body composed of a sponge-like network, when its volume is increased by the passage of fluid from without. Without going into detail, we may say that the change of form accompanying an increase in volume will be an increase in both axes of the spheroid. This is obviously the case.
(11) Now consider a spheroidal balloon possessing an elastic membrane, and the changes of form which will occur when it is distended.

The problem is rather difficult, and may be approached in the following way.

Let us calculate the energy in stretching a small rectangle of membrane. In the deformation the perpendicular lines will remain perpendicular and as others. Take these planes. Then the ratio 
etended length \over \text{old length} = \text{the extension ratio}. Call his two 
etended lengths $s_1, s_2$. The energy of stretching can be

$$W = \frac{1}{2} \lambda \left( s_1^2 + s_2^2 \right) - \mu s_1 s_2$$

per unit area, where $\lambda$ and $\mu$ are elastic constants. To find them, take 1st case. In membrane and stretch it without supporting the 
sides. The tension is

$$T = \frac{\partial W}{\partial s} = 2\lambda s_1 - \mu s_2$$

and if there is zero cross force $2\lambda s_1 - \mu s_2 = 0$

$$T = \frac{1}{2} \lambda \left( s_1^2 + s_2^2 \right)$$

and $\lambda = \frac{\mu}{2\lambda}$. Prove elastic modulus as in Hook's law. Further $s_2/s_1 = \mu/2\lambda$ is

Poisson's ratio.

In the case of the balloon the principal areas of strain will be the meridians and the circumferences of latitude by symmetry. Let us describe a point by $S$, the length of a generator origin $O$. Then when the membrane is not extended we have any point as $\text{d}x$, $\text{d}y$, functions $S$. On distortion let these go to $x + \text{d}x$, $y + \text{d}y$, where $\text{d}x$ and $\text{d}y$ are also functions of $S$. Then $x + \text{d}x$ is, $y + \text{d}y$ goes to

$$x + \frac{\text{d}x}{\text{d}s} \left( \text{d}x + \text{d}s \right) \text{d}s, y + \frac{\text{d}y}{\text{d}s} \left( \text{d}y + \text{d}s \right) \text{d}s$$

writing $\text{d}s = \text{d}x/\text{d}s \text{d}s$, and

$$\text{d}s = \text{d}x/\text{d}s \text{d}s.$$
approximately: so the extinction ratio is \(\alpha \gamma + \frac{v}{\gamma}\).

In the case perpendicular to this the radius increases from \(\gamma\) to \((\gamma + \eta)\); the extinction ratio is \(\eta / \gamma\).

The wing corresponding to this has unscrewed area \(2\pi \eta d\). The potential energy of stretching is therefore

\[ W_i = \frac{\pi}{2} \gamma \eta d \left[ (\alpha \gamma + \frac{v}{\gamma})^2 + \frac{\eta^2}{\gamma^2} \right] - 2\mu (\alpha \gamma + \frac{v}{\gamma}) \gamma \eta \]

as works done in distending the balloon have to be subtracted.

The volume is

\[ V = \pi \int (\gamma + \eta + \ldots) (\alpha \gamma + \frac{v}{\gamma}) d\eta. \]

As \(\eta\) is small, only terms \(\eta\) of first order are considered.

We require the minimum

\[ W/\pi = \int d\eta \left[ \frac{\pi}{2} \left( (\alpha \gamma + \frac{v}{\gamma})^2 + \frac{\eta^2}{\gamma^2} \right) - 2\mu (\alpha \gamma + \frac{v}{\gamma}) \gamma \eta \right]. \]

Vary \(W\), and put for short

\[ W/\pi = \int F(\frac{v}{\gamma}, \eta, \gamma) d\eta. \]

\[ SW/\pi = \int \frac{\partial F}{\partial \frac{v}{\gamma}} \frac{dx}{\partial \eta} + \frac{\partial F}{\partial \eta} d\eta. \]

\[ = \frac{\partial F}{\partial \frac{v}{\gamma}} \frac{dx}{\partial \eta} + \frac{\partial F}{\partial \eta} d\eta - \int \frac{\partial F}{\partial \frac{v}{\gamma}} \frac{dx}{\partial \frac{v}{\gamma}} + \frac{\partial F}{\partial \frac{v}{\gamma}} \frac{dx}{\partial \frac{v}{\gamma}} - \frac{\partial F}{\partial \eta} d\eta = 0 \]

The lower limit has \(\frac{v}{\gamma}, \gamma = 0\). The upper limit has \(\eta = 0\), but as lengthening may occur, \(\frac{v}{\gamma}\) is not necessarily zero. We require \(\partial F/\partial \frac{v}{\gamma}\) to be zero at this end, and at all points along.

\[ \frac{d}{d\gamma} \frac{\partial F}{\partial \frac{v}{\gamma}} = 0 \]

\[ \frac{d}{d\gamma} \left( \frac{\partial F}{\partial \frac{v}{\gamma}} - \frac{\partial F}{\partial \eta} \right) = 0 \]

and everywhere

\[ \frac{\partial F}{\partial \frac{v}{\gamma}} = 0 \]

Then,

\[ 2 \mu (\alpha \gamma + \frac{v}{\gamma}) \frac{dx}{\partial \gamma} - 2\mu \frac{dx}{\partial \gamma} - \mu \frac{dx}{\partial \gamma} = 0 \]
\[
\begin{align*}
\frac{d}{dx} \left( 2 \lambda (x \phi + y \eta) y - 2 \mu \eta \right) &= \frac{2 \lambda}{\eta} \eta - 2 (x \phi + y \eta) - 2 \phi y \\
\text{Substituting for } x \phi + y \eta \text{ out } y \text{ have just } \phi x
\end{align*}
\]
\[
\frac{d}{dx} \left( \frac{\phi y}{2} \right) = \frac{2 \lambda}{\eta} \eta - 2 \mu \phi y^2 + 2 \mu y \phi x - 2 \phi y.
\]
Therefore
\[
\eta \left\{ \frac{2 \lambda}{\phi} - \frac{2 \phi y^2}{\eta} \right\} = 2 \phi y + \left( \frac{\lambda}{\phi} \right) \phi \left( \frac{\phi y}{2} \right) + \frac{\eta}{\phi} \left( \frac{\phi y}{2} \right)
\]
From which
\[
\eta = \frac{1}{2} \frac{\lambda}{\phi} \cdot \frac{\eta}{\phi} \left\{ 2 \phi y + \mu y + \phi \cdot \frac{\phi y}{2} \right\}
\]
Whether there is contraction or expansion \( \eta \) has same sign depends on the signs of the bracket in its expression
\[
\eta = \frac{1}{2} \frac{\lambda}{\phi} \cdot \frac{\eta}{\phi} \left\{ \lambda \left( 2 \phi y + \frac{\phi y}{2} \right) + \mu y \right\}
\]
Hence
\[
\eta = \frac{1}{2} \frac{\lambda}{\phi} \cdot \frac{\eta}{\phi} \left\{ \lambda \left( 2 \phi y + \frac{\phi y}{2} \right) + \mu y \right\}
\]
Changing \( y \) to \( \xi \) as independent variable
\[
\frac{d}{d\xi} \left( \phi y \right) = \frac{1}{2} \frac{\lambda}{\phi} \cdot \frac{\eta}{\phi} \left\{ \phi \frac{d}{d\xi} \left( \phi y \right) + (2 + \frac{\phi y}{2})(\frac{d\phi}{d\xi})^2 \right\}
\]
\[
\frac{d}{d\xi} \left( \phi y \right) = \frac{1}{2} \frac{\lambda}{\phi} \cdot \frac{\eta}{\phi} \left\{ \phi \frac{d}{d\xi} \left( \phi y \right) + (2 + \frac{\phi y}{2})(\frac{d\phi}{d\xi})^2 \right\}
\]
Now \( y \xi \) is the radius \( \rho \)-coordinate
\[
-\frac{\rho'}{\rho} = \frac{d\phi}{d\xi} \left( \frac{d\phi}{d\xi} \right)^2 + \frac{1}{\phi} \frac{d\phi}{d\xi} = \left( \frac{d\phi}{d\xi} \right)^2 + \frac{1}{\phi} \frac{d\phi}{d\xi}
\]
Thus, taking its negative sign,
\[
\eta = \frac{1}{2} \frac{\lambda}{\phi} \cdot \frac{\eta}{\phi} \left\{ -\frac{\rho'}{\rho} \left(1 + (\frac{d\phi}{d\xi})^2 \right)^2 + (2 + \frac{\phi y}{2})(1 + (\frac{d\phi}{d\xi})^2) \right\}
\]
\[
\eta = \frac{1}{2} \frac{\lambda}{\phi} \cdot \frac{\eta}{\phi} \left\{ \left(2 + \frac{\phi y}{2}\right) - \frac{\sqrt{(1 + (\frac{d\phi}{d\xi})^2)}}{\phi} \right\}
\]
But the normal at the point \( n, \phi, \rho \) is
Relation of Drawell's cells to Tonicity.
\[ \sqrt{1 + \left( \frac{dy}{dx} \right)^2} \]

so that for contraction to occur at any point we must have:

\[ \frac{\text{Radius of curvature}}{\text{Normal}} > 2 + \mu/\lambda \]

Poisson's ratio, and equal to about 0.3. If we apply his result to the Redwood we have the condition for shrinking of the equatorial axis as:

\[ \frac{a^2}{b^2} > 2 + \mu/\lambda \]

If, then, the ratio equatorial axis / polar axis is greater than about 1.6, a slight distension of the balloon will be followed by a diminution in the length of the equatorial axis. The diminution will continue to take place till the ratio of the axes is about 1 to 1.6, after which both axes will increase, as the volume further increases.

(iii) We now pass to the third consideration, and shall show that the mammalian erythrocyte behaves like a balloon in that, as its volume initially increases, its equatorial axis decreases in length.

This may be done by giving the results of two sets of experiments.

1. Photographic.

Taking the cells of man, these were immersed in solutions of saline, with 10 per cent serum added to preserve the form of the cell, of varying tonicities. From each solution 100 cells were measured by the photographic methods of the next section. The results are shown graphically - as the tonicity falls
the volume of the cell increases, but the diameter becomes less. The turning point, where the axes reach the ratio, 1.6, is not reached in this experiment.

2. Diffraction.

By a method not discussed in this thesis, millions of red cells can be measured at once, in a relatively accurate manner, by the diffraction spectrum which they produce when light falls upon them normally.

The expression for the diameter is

\[ 2a = z/\pi \cdot \lambda \cdot \csc \theta, \]

where the value of \( z/\pi \) is obtained from the tables of Bessel functions, and varies according to the order of the spectrum, where \( \lambda \) is the wave length of the diffracted light, and where \( \csc \theta \) is the cosecant of the diffraction angle.

For the spectrum of the second order, \( z/\pi \) has the value 2.68 for a maximum intensity.

If a series of dilutions of citrate-plasma mixture, (1.5 per cent citrate plus 10 per cent plasma) are prepared, and to each human red cells added, the following values are obtained for the cells from the different tonicities of medium.

<table>
<thead>
<tr>
<th>Tonicity, plasma = 1</th>
<th>( \theta )</th>
<th>( \csc \theta )</th>
<th>2a</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 to 0.61</td>
<td>9</td>
<td>9.996</td>
<td>10.2</td>
</tr>
<tr>
<td>0.50</td>
<td>9.45</td>
<td>5.905</td>
<td>10.0</td>
</tr>
<tr>
<td>0.44</td>
<td>9.956</td>
<td>5.797</td>
<td>9.8</td>
</tr>
<tr>
<td>0.33</td>
<td>10.30</td>
<td>5.487</td>
<td>9.3</td>
</tr>
</tbody>
</table>
A progressive fall in diameter takes place as the tonicity falls. This confirms the photographic measurements, and the assumption that the red cell is a balloon-like body is thus further supported.

It is to be concluded that the suggestion that the red cell is a balloon-like body is the only one which is in keeping with the changes in form observed in it when its volume undergoes an increase. The changes are not those of a body containing a sponge work, and thus the view of Rollett becomes open to considerable doubt.

Because of the importance of the subject, we propose here to put forward a hypothesis to explain the special form of the mammalian erythrocyte. It must be clearly understood from the beginning that we are not endeavoring to prove this hypothesis, but only to present it as a possible explanation of the observed facts.
The one essential difficulty in the way of the balloon hypothesis is that it fails to explain the special form of the red cells of the mammalia. One would expect a body composed of a thin wall and containing fluid to assume a spherical form in fluid, instead of the typical biconcave form of the erythrocyte. This difficulty was pointed out by Hewson, and has been made much of by physiologists ever since.

Although there have been many explanations of the shape of the red cell on the stroma hypothesis, there has only been one serious attempt to explain the shape on the balloon hypothesis. Norris, in 1882, observed that droplets of myelin do not remain spherical when suspended in a fluid, but tend to enlarge their surface and to spread into flattened films, or bodies of annular form. Since lecithin is known to be a component of the red cell membrane, Norris suggested that the discoidal form of the cell is due to similar causes to those which bring about the myelin forms, and is to be sought in the operation of surface forces. These myelin forms have been recently studied extensively by Leathes; there is no doubt that under certain circumstances the droplets assume flattened and even biconcave forms.

Because of the importance of the subject, we propose here to put forward a hypothesis to explain the special form of the mammalian erythrocyte. It must be clearly understood from the beginning that we do not consider this hypothesis to be proven: such proof as
we shall offer contains certain fallacies, which will be indicated below. We believe, however, that the faulty proof could be made rigid with certain modifications, and shall therefore give it for what it is worth.

We make certain assumptions.

1. It will be assumed that the red cell is a balloon like body of a certain area and volume.

2. We shall assume that the membrane is a complex one, containing at least two components. To make the matter clear, we shall assume that the outer side of the membrane is made up of a layer of protein, with a positive surface tension. This causes the layer to tend to a minimal surface, or to contract. On the inside of the membrane we shall assume that there is a layer of lecithin tending to expand, and with a negative surface tension. This is in keeping with Leathes' work on lecithin films. We shall imagine that the layers oppose one another, and come to a species of equilibrium.

3. This equilibrium will be a hydrostatic one, and given by the condition

$$\rho - \rho = T \left( \frac{1}{\rho_1} + \frac{1}{\rho_2} \right)$$

in which $\rho$ and $\rho$ are the pressures on the two sides of the membrane, $T$ the tension, and $\rho_1$ and $\rho_2$ the radii of curvature of the element considered.

Given these conditions, we have to find the shape of the body formed. We shall take it as a solid of revolution.
We require
\[ \int \sqrt{y^2 + a^2} \, dx, \quad \text{a constant.} \]
and
\[ 2a \gamma \sqrt{1 + y^2} \, \text{a constant.} \]

Also,
\[ \int \left( \frac{1}{x^2} + \frac{1}{y^2} \right) \, dx \, \text{a minimum for the term.} \]

Converting \( p_1 \) and \( q_1 \) into Cartesian, and varying \( y \), we have
\[ \frac{d}{dx} \left( \frac{y}{1 + y^2} \right)^2 + \gamma y^2 + \mu \gamma \sqrt{1 + y^2} \right) \, dx. \]
\[ = \left[ \frac{y}{1 + y^2} - \frac{y^2}{(1 + y^2)^2} \right] \, dx \, \text{a constant.} \]

The differential eqn is
\[ \frac{d}{dx} \left\{ \frac{y}{1 + y^2} - \frac{y^2}{(1 + y^2)^2} \right\} + \frac{\gamma}{1 + y^2} + \frac{\mu}{\sqrt{1 + y^2}} \, dx \, \text{a constant.} \]

\( y = 0 \) when \( x = x_0, x \), the boundary term vanish.

The differential eqn is
\[ \frac{2y}{1 + y^2} \left( \frac{2y}{1 + y^2} \right) + 2\gamma y + \mu \sqrt{1 + y^2} - \frac{y^2}{\sqrt{1 + y^2}} \left( \frac{2y}{1 + y^2} \right) = 0 \]

or,
\[ \frac{2y}{(1 + y^2)^2} + 2\gamma y + \frac{\mu}{\sqrt{1 + y^2}} - \frac{y^2}{(1 + y^2)^2} \left( \frac{2y}{1 + y^2} \right) = 0 \]

Put \( 1 + y^2 = \omega^2 \)
\[ \frac{2y}{\omega^2} \, dx = -2\omega^3 \, dy \]
and
\[ y = -\omega^3 \, dy. \]
The eqn is:

\[
\frac{-2\mu^3 \frac{d\phi}{dx} + 2\frac{dy}{dx} + \mu \omega + \mu \omega^3 \frac{d\phi}{dx}}{\phi - 4} = 0
\]

\[
(\mu \omega - 2\mu) \frac{d\phi}{dx} + (\mu \omega + 2\frac{dy}{dx}) = 0
\]

Put \( \phi = cy \)

\[
(\mu + cy \frac{dy}{dx})(\mu + 2\mu) + (\mu \omega + 2\frac{dy}{dx}) = 0
\]

\[
\frac{d\mu}{dy} (2\mu - cy) = 2\mu \omega + 2\frac{dy}{dx} - 2\mu^2
\]

\[
\frac{d\mu (2\mu - cy)}{\mu^2 - \mu \omega - \phi} + 2 \frac{dy}{dy} = 0
\]

\[
\omega^2 - 2\mu \omega y = 2y^2 = \text{a constant}
\]

Call this constant \(-r\).

The differential eqn is

\[
2y^2 (1 + y^2) + \mu \sqrt{1 + y^2} = 2(1 + y^2) + 1
\]

This, when solved, will give the form required.

If at \( x_0, \phi_0, y = 0, \phi = \infty \), then \( r = 0 \), and the solution is a sphere. This form is precluded by the initial assumptions.

The other possibility is that \( y \) is finite at \( x_0, \phi_0 \). Suppose that it

is equal to \( \frac{r}{2} \) at \( x_0, \phi_0 \). Then we have a body with points

at each end.

We have

\[
x^2 = -1 - \frac{r}{\phi}
\]

For \( x^2 > 0, r < 0 \), and \( - \frac{r}{\phi} > 1 \); so \( r \) lies between 0 and -1.

If \( y \) is real then \( y \) is real

\[
(\mu^2 + 1) y^2 - r > 0
\]

or

\[
y^2 > \frac{r}{\mu^2 + 1}
\]

For a real form we thus require \( \mu^2 > 1 \).

Further when \( y = \infty, y^2 = \frac{r}{\phi} \); for this to have real roots \( r \)

must be negative, since \( r \) is negative.
The values of the constants being finite limited, the differential equations may be solved in the following way. Arbitrary values within the proper limits are given to \( x, z, \) and \( y \); values of \( y \) are then inserted in the differential eqns. and \( y \) solved for.

We can then tabulate

\[ y = f(y) \]

and can obtain

\[ x = f(y) \]

Values of \( x \) and \( y \) are then plotted, and a curve of the type shown in Figure 1 obtained.

Now since we have

\[ \frac{dx}{dy} = f(y) \]

we can obtain

\[ x = \int f(y) \, dy \]

by integrating

the curve between suitable limits of \( y \). This integration may be done by Simpson's rule, or some other rule for evaluating area.

The complete solution will be seen in Figure 2, for the values of the constants

\[ \lambda = -0.5, \quad \mu = 0.72, \quad \nu = -0.8 \]

This curve, if rotated round its minor axis, gives a solid of revolution which is of the form required by the initial conditions.

The form will be recognised to be very suggestive of that of the red cell. From the erythrocyte it differs in one very important manner, however—in the appearance of the points on the minor axis—and these points cannot unfortunately be ignored.

Two things must now be noted about the above derivation of the form of the solid.
\[ \lambda = -\frac{i}{2}, \quad \mu = 0.72, \quad \nu = -0.8 \]

Fig. 2.

Points.

Fig. 3.
1. It is possible to make the points appear other than on the minor axis; they may, for example, appear as in Figure 3, but points there must be somewhere. Even a figure such as 3 is suggestive of the form of the cell, however - all that is required, as in the case of Figure 2, is that the points should be rounded off.

2. It is unfortunately unpermissible to round the points off, for, if this is done, the whole proof falls to the ground from a mathematical point of view. From a physical point of view, however, we suggest that it does not. If we could imagine a body whose wall possesses real elastic constants subjected to the same forces as have been assumed to act on this ideal membrane, we should have an approximation to the form in Figures 2 and 3, although not that form exactly - but there would be no points, for points imply infinite tensions, which are impossible in a real membrane.

We should, however, in the case of a balloon with a wall possessing real elastic constants, obtain under the forces which we have postulated a form not unlike that of the red cell of the mammalia. This form would be a stable one, for it represents an equilibrium; it would be a form under physical forces which we know to exist in the cell, and dependent on nothing but these forces alone. The hypothesis put forward, since it leads to such an interesting conclusion, is well worth consideration.
PART IV.

THE SHAPE OF THE ERYTHROCYTE AND ITS RESPIRATORY FUNCTION.

Equipotential lines to two sinks.

Equipotential lines to a ring sink.
I. THE SHAPE AND RESPIRATORY FUNCTION.

In a note on the shape of the mammalian erythrocyte, Hartridge (1919) has suggested that the red cell has a form intermediate between that of a sphere and an infinitely thin disc. Gas diffusing from the surface of either of these figures would reach the central regions in the same time, irrespective of the point on the surface at which the diffusion started. The sphere, however, offers a small surface compared to its volume, while the thin disc would require the surface layers forming the envelope to be greatly increased at the expense of the contents. In the disc, moreover, gas would gain access too readily at the ends, so that the peripheral regions would be reached first. A body of the shape of the normal red cell meets the difficulties of the problem, for not only is the surface comparatively large for the volume, but the ends are thickened, so that gas diffusing from the surface reaches the central regions in a uniform manner.

The red cell being greatly concerned with gas transportation, it is obviously important to consider whether its special shape offers any special advantages. This we propose to do, thus following up the suggestion of Hartridge.

Consider two equal and isolated sinks, \( S_1 \) and \( S_2 \). They will set up lines of flow,
Supposing that a gas starting from a line of equal velocity potential gains under a line of flow a velocity which changes only because of the external pressure, we shall be able to obtain the equation of the line of equal velocity potential if we have the line of flow.

Such an equation gives, for various values of \( \mu \), a series of curves known as the curves of Cauchy, who first described them in 1827. In our case, the strength of the sinks is equal, or \( \mu = \mu \). Taking a constant strength that of 14 units, and placing the sinks 10 units apart, we can plot these equipotential lines for various values of \( \mu \), the velocity potential.

This is done in the figure attached, lines for \( \mu = \mu \) values of \( \psi \) being shown. The method of plotting the lines is similar to that used for obtaining lines of equal potential due to two similar plates, in the figure, lines above the abscissa lines are shown, the lines below being identical with those above.
\[
\cos \theta + \cos \theta' = \text{Constant}, \quad \ldots \quad (1)
\]

and lines of equal velocity potential,

\[
\frac{1}{r} + \frac{1}{r'} = \text{Constant}. \quad \ldots \quad (2)
\]

Supposing that a gas starting from a line of equal velocity potential passes along a line of flow, it will pass at right angles to all the lines of equal velocity potential which it traverses, and finally reach one of the sinks.

If the strength of one of the sinks be \( m_1 \), and that of the other be \( m_2 \), then, if the sinks are apart by a distance \( a \), the lines of equal velocity potential will be

\[
\frac{m_1}{r} + \frac{m_2}{r'} = \frac{K}{a} \quad \ldots \quad (3)
\]

Such an equation gives, for various values of \( k \), a series of curves known as the curves of Cayley, who first described them in 1857. In our case, the strength of the sinks is equal, or \( m_1 = m_2 \). Taking a convenient strength that of 24 units, and placing the sinks 12 units = \( a \), apart, we can plot these equipotential lines for various values of \( \psi \), the velocity potential. This is done in the figure attached, lines for a series of values of \( \psi \) being shown. The method of plotting the lines is similar to that used for obtaining lines of equal potential due to two similar poles; in the figure, lines above the abscissa alone are shown, the lines below being identical with those above.
It will be seen that, as $\psi$ decreases, the lines representing equal values of $\psi$ pass from an oval, (not shown in the figure) to a curve possessing a concavity; as $\psi$ continues to decrease, the concavity becomes deeper, and ultimately becomes a double point on a curve with two loops. A further decrease in causes the curve to assume the form of two ovals, one lying about each sink, and finally, when the velocity potential is zero, the curve becomes the two points, $S_1$ and $S_2$. From the equation for the curve (3), it will be seen that when the sinks are equal in strength,

1. When $k > 4m$, the curve is a large oval, with or without a concavity.

2. When $k = 4m$, the curve has a double point.

3. When $k < 4m$, the curve consists of two ovals.

It will be further seen that the series of lines for equal values of $\psi$ may be regarded as a wave surface converging on $S_1$ and $S_2$, as $\psi$ decreases, and hence particles of gas commencing to move towards the sinks from any one line of equal velocity potential will arrive at the sinks at the same moment.

Apart from the theoretical aspect of the case, curves very similar to these equipotential curves of Cayley can be produced by a very simple experimental device, in which the conditions are analagous to those which we are considering. A uniform ground of gelatin containing potassium chromate is formed
In the first part a drop is placed above, in the second, two drops a little below each other. The lines which represent the equal potential surfaces are shown in the accompanying photographs.

In the accompanying photographs:

*potential lines to two sources.*
on a glass plate, and on this is placed either one or
two drops of 4N. silver nitrate, the drops being made
as small as possible. The whole preparation is then
kept in a moist condition. As the silver salt diffuses
out of the droplet into the gelatin, a series of rings
appears round the droplet or droplets. These are shown
in the accompanying photographs.

In the first case a single drop was used;
in the second, two drops a small distance apart. The
lines which appear etched on the ground are lines of
equal salt concentration, analogous, in a general way,
to lines of equal velocity potential. It will be seen
that in the case of the single drops these lines are
circles, but that where there are two drops, which we
can look upon as two equal and similar sources, the
lines are similar to the equipotential lines mentioned
above. Reversing the process, and making the sources
become sinks will plainly give the same form of lines
around the sinks as occur round the sources. This ex-
periment must, of course, be taken merely as an illus-
tration - it shows the general result very well.

Selecting the curve \( \psi = 7.5 \), and rotating
it round its minor axis, we obtain a solid in general
form similar to the red cell. In the process, the
sinks \( S_1 \) and \( S_2 \) become a ring, and the equipotential
line becomes the surface of the solid body. This sur-
face is very nearly, but not quite, a surface of equal
velocity potential to the ring source or sink; in order
to find the exact form of such an equipotential surface to a ring, we have to proceed in a somewhat different manner.

Suppose that we require to find the equipotential surface to a circular ring of radius $r$. In the figure, let $PB = r$, the distance of a point $P$ on the vertical surface from the origin $O$. Let $AB$ be the width of the ring at any angle $\theta$. By simple trigonometry we have

The surface to be equipotential to the ring must have

\[ \psi = 0.153 \]

This last integral is a well-known form, being the first complete elliptic integral of the modulus $k$. 
to find the exact form of such an equipotential surface to a ring, we have to proceed in a somewhat different manner.

Suppose that we require to find the equipotential surface to a circular sink, of radius $a$. In the figure, let $PB = r'$, the distance of a point $P$ on the required surface from a point on the ring. Let the distance from the centre of the ring, $O$, to $P$, be $r$. Let the plane $PAO$ be perpendicular to the plane of the ring, and let $OA$ and $OB$ make an angle $\theta$, while $OA$ and $OP$ make an angle $\phi$.

We first require $PB$ in terms of $\theta$ and $\phi$. By simple trigonometry we find that

$$PB = r^2 + a^2 - 2ar \cos \theta \cos \phi.$$ 

The surface to be equipotential to the ring, must have

$$P = m \int_0^{2\pi} \frac{d\phi}{r'},$$

or since $ds = a \, d\phi$,

$$P = 2m \int_0^{2\pi} \frac{a \, d\phi}{\sqrt{(a^2 + r^2 - 2ar \cos \theta \cos \phi)^2}}.$$ 

Here $m$ is some constant which can in the meantime be ignored. Continuing with the integral, and putting $\psi = 2\psi$, $\psi = \frac{T_2}{2} - \chi$,

$$P = \frac{2a}{\sqrt{2} \int_0^{T_2} \frac{dx}{\sqrt{(a^2 + r^2 - 2ar \cos \theta \cdot 2 \sin^2 \chi - 1)}}}.$$

where

$$K^2 = \frac{4 \ar \cos \theta}{a^2 + r^2 + 2ar \cos \theta}$$

This last integral is a well known form, being the first complete elliptic integral of the modulus $k$. 
The necessary condition for the equipotential surface is, therefore, that

\[ \frac{K(k)}{\sqrt{(a^2 + x^2 + 2ax \cos \theta)}} = \psi \]

This is a very awkward expression to deal with, and admits of no further simplification. The best way to obtain the equipotential surfaces which it described is to assign certain values to \( r \) and to \( \theta \), and thus to arrive at the form of the surfaces in a semi-graphical manner. We proceed as follows.

First we assign a convenient value to \( a \), the radius of the ring which is the circular source - an easily worked value is 10 units. Next we proceed to introduce various values for \( r \) and \( \theta \), into (4) and (5), and to evaluate the constant in each case. To facilitate calculation we first take cases where \( \theta = 0 \) and where \( \theta = 90 \), and obtain the following values for \( \psi \).

\[
\begin{array}{cccc}
\text{r} & \theta = 0. & \theta = 90. \\
1.0 & 0.157 & 0.156 \\
2.0 & 0.158 & 0.154 \\
2.3 & - & 0.153 \\
3.0 & - & 0.150 \\
5.0 & 0.166 & 0.140 \\
12.0 & 0.177 & 0.100 \\
12.7 & 0.153 & - \\
13.0 & 0.148 & - \\
15.0 & 0.122 & 0.087 \\
\end{array}
\]

Now the value of \( r \) when \( \theta = 90 \) gives us half the least...
thickness of the cell, and the value of \( r \) when \( \theta = 0 \) gives the semi-diameter. We know the ratio of these two measurements from examination of microphotographs, and that it is approximately 1 : 5.5, we have, therefore, to select from the values of \( \psi \) in this table one which will give this ratio between \( r \) when \( \theta = 90 \) and \( r \) when \( \theta = 0 \). The value 0.153 is such a one, for it gives value for \( r \) of 2.3 and 12.7 respectively. The surface \( \psi = 0.153 \) is therefore one which will fit the red cell as regards its two axes, and is accordingly to be worked out in full.

We obtain the following results.

<table>
<thead>
<tr>
<th>( \theta )</th>
<th>0</th>
<th>10</th>
<th>20</th>
<th>30</th>
<th>45</th>
<th>60</th>
<th>90</th>
</tr>
</thead>
<tbody>
<tr>
<td>( r )</td>
<td>12.7</td>
<td>12</td>
<td>10.6</td>
<td>8</td>
<td>4.5</td>
<td>3</td>
<td>2.3</td>
</tr>
</tbody>
</table>

The curve is shown in the figure adjacent, in which the position of the ring, which is, of course, seen as two points in the section, is indicated by \( S \) in the quadrant shown. \( OS \), therefore, is equal to \( a \).

It now remains to be seen to what extent the curve obtained above fits the form of the red cell, for the better the fit, the better will the cell be adapted to the flow of gases within it. The general form appears satisfactory, and, by construction, the axes are correctly proportioned, but the crucial test lies in comparing the volume under the rotated curve with that of the erythrocyte.

By Pappus' theorem we find that the volume enclosed by this equipotential
surface works out at \(144\mu^3\), if the major axis of the curve be reduced to a scale so that it equals \(3.8\mu\), the mean diameter of the human red cell. The volume of the human erythrocyte is known to be \(110\mu^3\), so the volume enclosed by the equipotential surface is about 45 per cent too large.

It may be mentioned that the best curve of Cayley, given by equation (3), and fitting the red cell as nearly as can be arranged, gives an enclosed volume of \(196\mu^3\), or about 85 per cent too much.

From these figures one concludes that the surface of the red cell is not exactly an equipotential surface to a simple source or sink, but that it approximates to one, a fact of interest and importance in connection with its respiratory function. The resemblance which does in fact exist is such as to make the efficiency of the cell vary great, compared with that of most other figures of the same volume, and very much greater than that of a spherical form, for, in order to obtain the same rapidity of gas diffusion for the same volume distributed in spheres we would require approximately nine spheres of one-ninth the volume.
PART V.

SECTION 1

ON SEDIMENTATION AND ROULEAUX FORMATION.

Section 1.

Sedimentation of erythrocytes in saline.

Section 2.

Rouleaux formation.

Theoretical considerations.

Results.

1. Sedimentation of superimposed cells.
2. Sedimentation of elliptical cells.
5. Concentration of suspension.

N.B. referred to.
PART V.

SECTION I.

Sedimentation of erythrocytes in saline.

Equations applicable to

1. A spherical body falling in a fluid.
2. A flat elliptic disc falling in a fluid.
3. A flat circular disc falling in a fluid.

Methods.

1. Preparation of cells.
2. Sedimentation rate.
4. Viscosity determinations.
5. Density.

Theoretical considerations.

Results.

1. Sedimentation of spherical cells.
2. Sedimentation of elliptical cells.
3. Sedimentation of cells of circular discoid form.
5. Concentration of suspension.

Papers referred to.
In this Section we propose to show the extent to which the phenomenon of sedimentation of red cells can be explained on simple physical principles.

In order to do so, we shall consider first the expressions which are applicable to the fall of small bodies in a fluid.

1. A spherical body falling in a fluid.

Let the radius to the body be $R$, and understood to be of small dimensions, and let the density of the body be $s_1$. If the body falls through a fluid of viscosity $\eta$, and of density $s_2$, with a velocity $V$, we have a balance between the forces tending to bring about the fall and those tending to resist it.

$$\frac{4}{3} \pi R^3 \cdot \frac{g}{(s_1 - s_2) R^2} = 6 \pi \eta V,$$

where $g$ is the gravitation constant, and all the quantities being expressed in the C.G.S. system. This balance of forces gives us the well-known formula of Stokes

$$V = \frac{2 (s_1 - s_2) R^2 \cdot \frac{g}{\eta}}{\eta}$$

which provides us with the constant velocity of the fall of a small sphere.

2. A flat elliptic disc falling in a fluid.

Such a body falls in the most stable position, which is that of the most resistance, or broadside on. Let the axes of the body be $a$, $b$, and $c$, and let the direction of fall be along the $a$
axis. As regards the resistance to its fall, the disc will behave like a sphere of radius

$$R = \frac{8abc}{\pi a_0^2}$$

where

$$\chi_0 = abc \int_0^a \frac{d\theta}{\sqrt{1 - \lambda}}$$

and

$$a_0 = abc \int_0^a \frac{d\lambda}{\sqrt{(a^2 + \lambda)(b^2 + \lambda)(c^2 + \lambda)}}$$

$$\chi$$ being one of the roots of the triple orthogonal system of quadrics

$$\frac{x^2}{\alpha^2 + \theta} + \frac{y^2}{\beta^2 + \theta} + \frac{z^2}{\gamma^2 + \theta} = 1$$

a system very generally used in hydrodynamics.

To evaluate $$\chi_0$$, write it out in full as

$$\chi_0 = \int_0^a \frac{d\lambda}{\sqrt{(a^2 + \lambda)(b^2 + \lambda)(c^2 + \lambda)}}$$

and put $$\lambda = \cos \alpha = b/c$$. Integration gives the value as

$$\frac{2}{c} K\left(\frac{\sqrt{c^2 - \beta^2}}{c}\right)$$

where $$K$$ is the elliptic integral corresponding to $$\cos \alpha = b/c$$. The value of $$R$$ can thus be readily obtained, preferable in terms of $$c$$. This value, substituted in the expression $$\pi \eta RV$$, gives the resistance to the fall of the body.

There is no need to put $$a = 0$$ in the part of the equation giving the downward pull on the body, which is equal to

$$\frac{4}{3} \pi abc (S_1 - S_2). g.$$

So we have the body falling with a velocity

$$v = \frac{2abc(S_1 - S_2). g}{9 \pi R} \ldots (2)$$

3. A flat circular disc falling in a fluid.

This is a special case of the above,
where \( a = 0 \) and \( b = c \). We have \( \cos \alpha = 1 \), and \( K = \frac{1}{2} \).

The disc therefore meets with resistance \( 6\eta V(0.85c) \).

The force pulling it down is
\[
\frac{2}{3} \pi ac^2 (S_1 - S_2) g.
\]

and the velocity of fall is
\[
V = \frac{2ac (S_1 - S_2) g}{\gamma \cdot 65 \eta}.
\] 

...(3)

These fundamental expressions give the velocity of fall of bodies of various forms. It now remains to find whether the expressions can be used to describe in a satisfactory manner the fall of erythrocytes of different sizes and shapes through fluids possessing various physical properties.

METHODS.

Preparation of cells.

Except where otherwise stated, the following procedure was carried out. The blood from the living or freshly killed animal was received into a considerable volume of 0.85 per cent NaCl, in which was dissolved sufficient potassium oxalate to prevent coagulation. Drawing the blood into citrate solutions was in general avoided, for the red cells of certain animals, e.g. the sheep, are haemolysed by citrate solutions which are isotonic for the cells of man. The cells, suspended in the saline, were thrice washed with 0.85 per cent NaCl, and then suspended in sufficient of this fluid to give a suspension containing the cells form 1 cc of blood in 80 cc. of saline.
This strength of suspension is a very satisfactory one for the tests described below.

Sedimentation rate.

This was measured by allowing the cells to sediment in a tube such as shown in the figure below. The tubes are made of plate glass, and are rectangular in section, the dimensions being 1 in. by ½ in. by 12 in. Such a tube holds 80 cc. easily. The rectangular form is a great advantage over a circular, as in the former the upper edge of a sedimenting suspension appears much sharper than in the latter.

Across the face of the sedimentation tube moves, on a double rack and pinion, a neutral-tinted wedge formed by a hollow glass cell of plate glass and of prismatic shape. The dimensions of this cell are approximately 3 in. by 3 in., with an inside measurement of ½ in. at one end and 0 in. at the other. It is filled before use with a solution containing 0.25 per cent nigrosin and 0.002 per cent tropeolin, a mixture which can, however, be varied to suit the particular observer's conception of neutrality. The object of the prism is to enable us to examine the opacity, and therefore the cell concentration, at any level of the sedimenting suspension. It will easily be seen that if we have in the sedimentation tube a series of different cell concentrations, C₁, C₂, C₃, etc., at different levels and each greater than the one preceding it, that the light which will pass through the suspension at the level of C, will be able to pen-
rate through a greater depth of the neutral-tinted wedge than will the light passing through the suspension at C₃, where there are more cells, and accordingly less transmitted light. Similarly, the light from C₂ will traverse a greater depth of the prism than will the light from the denser region at the level C₅.

The result is that the difference of concentration of cells in the sedimentation tube gives rise to an unequal illumination of the prism, part of the prism transmitting light, and part being left in darkness. The margin of the illuminated part will appear as a curve passing across the tube, the shape of the curve being determined by the way in which the cell concentration alters as the level alters. If the cell suspension is uniform over the area of the tube covered by the prism, the margin of the illuminated part will appear as a straight line.

The position of the prism on the face of the sedimentation tube, the lighting, and even the concentration of the dyes contained in the prism, have to be adjusted in order to show up these illuminated and unilluminated portions to the best advantage; this is a matter of experience.

In order that the back of the tube shall be equally illuminated at all points, there is placed behind the tube a long frame holding ground glass, lighted behind by a tube-shaped lamp. In order to guard against changes of temperature occurring or changes which may set up local convection currents, it
is convenient to immerse the sedimentation tube in a thermostat of suitable shape.

The arrangement of the apparatus is illustrated in the following diagrams.

FIG. 1.  
Measurement of the cells. The measurement of the diameters of the cells in the experiments to be recorded were made in the manner described in Part II of this Thesis. In each experiment, 100 to 200 cells were photographed, and the mean diameter obtained; in many cases the cells were measured both before the sedimentation experiment commenced and after its completion. Rarely was any significant difference found.

Viscosity determinations. These were carried out by the Ostwald method in the usual way, and the results reduced to C.G.S. units.

Density. The densities of the suspension media were made in the customary way with the pyknometer. The densities of the cells were taken from the expression

$$\frac{100 \times \text{s.g. blood} - \text{s.g. serum} (S)}{C}$$

where C is the percentage of cells per unit volume of blood, and S the percentage of serum. The density of the whole blood and the density of the serum can be estimated by simple enough methods, as can also the percentage of cells and serum present. (Millar, 1925)

THEORETICAL CONSIDERATIONS

Let us consider the extent to which we may expect Equations 1, 2, and 3, which have been seen to
describe the motion of a single body through a fluid, to be applicable to the fall of bodies in suspensions, where the bodies are of different sizes and where a large number of bodies are present.

We shall take it that the suspension, when placed in the sedimentation tube, contains cells of different sizes distributed in the tube in a uniform manner, and distributed with respect to size according to a symmetrical frequency curve. The first of these assumptions is a very obvious one, and the second is based on good evidence, adduced in Part II of this Thesis.

Suppose that we have a cell $A_0$ in the figure attached, this cell being at the surface of the fluid in the sedimentation tube, and suppose that we have two other cells, one $B_0$ half way down the tube, and another $C_0$ near the bottom of the tube. Let these cells occupy these positions at the beginning of the experiment, or at time $t_0$. Let $A_0$, $B_0$, and $C_0$, be all of the same dimensions, and let them fall, as in fact they will, through an equal distance $D$ in time $t_1 - t_0$. After this interval of time, $A_0$ will be found at $A_1$, $B_0$ at $B_1$, and $C_0$ on the bottom of the tube, provided that $D$ is greater than the distance from the bottom to where $C_0$ was initially situated. Being at the bottom, $C_0$ can be disregarded, and will not influence the movement of $B_0$ and $A_0$. In due course $B_0$ will also arrive at the bottom of the tube, and may then be disregarded, so far as the cells above it are concerned. If, then, the suspension were made up of cells of uniform dimen
sions, each cell would fall through the fluid uninfuenced by the cells below it, until it came to near
the bottom of the tube, when a certain amount of pack-
ing would influence its further movement. In such a
 case of a uniform suspension of cells of uniform size,
we should find no variation in density at different
levels of the suspension as sedimentation proceeded,
even after sedimentation has gone on for a long time,
except that the bottom of the tube would be covered
with a closely packed layer, while the upper part of
the tube would be free of cells altogether.

Now consider a suspension in which the
cells are not all of uniform size, but are distributed
with respect to size according to a symmetrical fre-
quency curve. In the figure attached, take a narrow
strip of the fluid at \(L_o\), at right angles to the length
of the tube. In this strip there will be cells of all
sizes, and, since the number of cells is very large,
these cells will be distributed according to a symmet-
trical frequency curve. Suppose that there are a num-
ber of cells of small size \(a\), a number of larger size
\(b\), \(c\), etc., and a number of largest size \(k\), at the
level \(L_o\) at time \(t_o\). After time \(t\), the cells will
be all found at a level lower than \(L_o\), each cell having
fallen in that time through a distance proportional to
its dimensions, the largest cells furthest, and the
smallest cells least far. The cells of small dimen-
sions \(a\) will be found at \(a\), the cells \(b\) at \(b\), etc.,
and the largest cells \(k\) at \(k\).
Now take a strip across the tube at \( L_i \), the level reached by the cells \( k_i \). This will contain the cells \( k_i \), fallen from \( L_0 \), but the largest cells originally at \( L_i \) will be no longer there, but will be found at a level below \( L_i \). The smallest cells, \( a_o \) from \( L_0 \), will not yet have arrived at \( L_i \), but there will be in \( L_i \) small cells from a level below \( L_0 \), say from \( L_2 \). In like manner \( L_i \) will contain cells of size \( b, c, \) etc., from levels intermediate between \( L_o \) and \( L_i \), the cells of size \( b, c, \) etc., originally at \( L_i \), being now at lower levels.

In general the cells at \( L_i \) will be as numerous, and distributed in the same manner as regards size, as the cells originally at \( L_0 \), at \( L_2 \), or at any other level. At all levels between \( L_i \) and a level near the bottom of the tube, the suspension will accordingly be of unvarying density, at whatever stage the sedimentation may have arrived.

But this does not hold for the surface layers above the level of \( L_i \). Suppose that, as before, there is at the surface of the fluid, when \( t = 0 \), a layer \( L_0 \) containing small cells \( a_o \), larger cells, \( b_o \), \( c_o \), etc., and cells of the largest size, \( k_o \). Let these all fall through a distance proportional to their dimensions in a time \( t, = t_o \); then at \( t \) the small cells \( a_o \) will be found at \( a_o \), a level which we shall call \( L_i \). Above this level no cell can be found, for there are no cells above \( L_0 \) and no cells smaller than \( a_o \); further, cells \( b_o \), from the level \( L_0 \), will be found below \( L_i \).
so that $L_0$ can contain cells of size $a_0$ only.
The layer immediately below $L_0$ will contain cells $b_0$ from $L_0$, and cells $a_0$ from a level immediately below $L_0$. In like manner the layer $L_1$ will contain cells $a_0$, $b_0$, and $c_0$, while the layer $L_2$ will contain cells $a_0$, $b_0$, $c_0$, and $d_0$, and so on until we arrive at the layer $L_{2n}$ containing the large cells $k_0$ from $L_0$, and also containing cells of every other class.

Now if the cells are distributed according to a curve of the form

$$y = Ae^{-b^2x^2}$$

it is plain that if we plot the number of cells between $L_0$ and $L_n$ after time $t$, against the position of the level, we shall obtain a curve of the type

$$y = \int Ae^{-b^2x^2}dx.$$  

to a near approximation. The approximation lies in the fact that the cells descend, not according to their radius, but according to its square; this will distort the integral curve to a slight extent.

We now assume that, in the presence of an enormous number of cells whose variation from a mean size is very small, the light-stopping power of any layer will depend on the numbers of cells present. This being granted, it is clear that on examining the surface layers of a sedimenting suspension with the extinction prism described above, we should have the margin between the illuminated and the unilluminated portions of the prism appearing as a curve of nearly the form
but turned round along the long axis of the sedimentation tube - provided, of course, that the prism is properly adjusted. The upper limit of this curve will be at the level which contains the smallest cells fallen from L, the level of the fluid in the tube, and the lower limit at the level to which the largest cells from the surface layer have fallen during the sedimentation. The position of the cells of mean size which have fallen from the surface layer will be, for practical purposes, midway between the upper and the lower limits of the curve.

If the extinction prism can be so arranged that this integral curve appears clearly as a margin of illumination, the observation of the position of the upper limit, the middle, and the lower limit of this curve will provide information as to the velocity of fall of the smallest cells, the cells of mean size, and the largest cells found in the particular suspension.

RESULTS.

In order to reduce the sedimentation experiments to their simplest form, we shall give results for the fall of cells suspended in saline. Here we may expect the hydrodynamical equations to apply, for the conditions of experiment are such that no rouleaux occur in the sedimenting suspensions.

1. Sedimentation of spherical cells. Under certain circumstances notably when blood is withdrawn into
isotonic saline, the cells of the mammalia lose their discoid shape and become spherical. These changes have been described by Gough (1924), and are alluded to in another part of this Thesis.

As stated there, the altered cells are perfectly spherical in form, and of the same volume as that possessed by the cell when in its normal discoid form. Cells in the Goughian form might be expected to sediment according to the well known Stokes' Law, expressed in Equation 1 of this Section, and below we record the results of experiments on this point.

The cells were ascertained by microscopic examination and photographic measurement to be spherical in form and unaltered in volume, both before, during, and after each experiment. The sedimentation rate is expressed in millimetres per hour, and is the result of observations made every two hours for the first ten hours, with a final observation after 18 hours. During this period we observed no significant difference in the sedimentation rate, nor would one expect anything but constancy from theoretical considerations. The rate of sedimentation was in all cases found by dividing the length from the surface of the fluid to the middle point of the curve seen on the extinction prism by the time during which sedimentation had proceeded, and reducing the results to millimetres per hour.

The results, and comparison between observed
and calculated values, are shown in the following table.

<table>
<thead>
<tr>
<th>Animal</th>
<th>Radius of cells, μm</th>
<th>Observed rate, mm. per hour</th>
<th>Calculated rate, mm. per hour</th>
</tr>
</thead>
<tbody>
<tr>
<td>Man</td>
<td>2.7</td>
<td>4.9</td>
<td>5.25</td>
</tr>
<tr>
<td>do</td>
<td>2.6</td>
<td>4.8</td>
<td>5.20</td>
</tr>
<tr>
<td>do</td>
<td>2.7</td>
<td>5.0</td>
<td>5.04</td>
</tr>
<tr>
<td>do</td>
<td>2.8</td>
<td>5.3</td>
<td>5.42</td>
</tr>
<tr>
<td>Ox</td>
<td>2.3</td>
<td>3.4</td>
<td>2.82</td>
</tr>
<tr>
<td>do</td>
<td>2.2</td>
<td>3.6</td>
<td>3.90</td>
</tr>
<tr>
<td>Sheep</td>
<td>1.9</td>
<td>2.4</td>
<td>2.7</td>
</tr>
<tr>
<td>do</td>
<td>1.8</td>
<td>2.3</td>
<td>2.8</td>
</tr>
<tr>
<td>Rabbit</td>
<td>2.5</td>
<td>4.0</td>
<td>4.1</td>
</tr>
<tr>
<td>do</td>
<td>2.6</td>
<td>4.5</td>
<td>4.8</td>
</tr>
</tbody>
</table>

These experiments were all carried out at a temperature in the neighbourhood of 20°C, although the actual temperature was not the same in all cases. In each case the proper value of \( \eta \) corresponding to the exact temperature was inserted in Equation 1 when finding the calculated results. The results agree very well with theory, but one small point requires notice. The observed results are uniformly too low by a small amount. This may be due to slight disturbances in the fluid, Brownian movement of the cells, or a small variation from the perfectly spherical Goughian form; the difference is, however, so small that there can be little doubt that Equation 1 applies to sedimentation of cells in the spherical form.
During these experiments frequent examination with the extinction prism showed that, except for the surface layers and the layers near the bottom of the tube, the suspension remained homogeneous, as required by theory; inspection of the surface layers showed the appearance of a frequency curve in the integral form, as stated in the previous discussion.

2. Sedimentation of elliptical cells. This is the form met with in birds, reptiles, amphibia, and fishes. As in the previous experiments, the cells were washed thrice and suspended in saline. To this saline was added a small quantity of ammonium oxalate, in order to avoid the appearance of what corresponds to the Coughlan form in the elliptical cell, and the persistence of the elliptical form was ascertained by frequent examination.

The results are shown in the following table.

<table>
<thead>
<tr>
<th>Animal</th>
<th>Value of $b$, in $\mu$</th>
<th>Value of $c$, in $\mu$</th>
<th>Observed rate, mm. per hour</th>
<th>Calculated rate, mm. per hour</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pigeon</td>
<td>4.2</td>
<td>6.5</td>
<td>9.0</td>
<td>9.9</td>
</tr>
<tr>
<td>do</td>
<td>4.1</td>
<td>6.6</td>
<td>9.1</td>
<td>9.3</td>
</tr>
<tr>
<td>Frog</td>
<td>7.5</td>
<td>12.5</td>
<td>16.6</td>
<td>17.6</td>
</tr>
<tr>
<td>do</td>
<td>7.0</td>
<td>11.8</td>
<td>16.1</td>
<td>16.8</td>
</tr>
<tr>
<td>Skate</td>
<td>9.0</td>
<td>14.2</td>
<td>23.0</td>
<td>25.6</td>
</tr>
<tr>
<td>Dogfish</td>
<td>8.2</td>
<td>12.2</td>
<td>17.5</td>
<td>17.9</td>
</tr>
</tbody>
</table>
These results are enough to show that Equation 2 is applicable. As before, the calculated results are too high; the reason for this may be one of the reasons stated before, or it may be that, in putting \( a = 0 \) in the theoretical equation, we have introduced an error of observable magnitude. There is also a considerable difficulty in estimating the value of \( a \) for insertion in the part of the expression which gives the downward force, and this, too, may contribute to the errors.

3. Sedimentation of cells of circular discoid form. This is the form maintained by mammalian erythrocytes when immersed in saline containing serum, plasma, or ammonium oxalate. The addition of the first-named substances causes rouleaux formation, but the addition of oxalate does not; the cells for these experiments were therefore suspended in 0.85 per cent NaCl containing 1 per cent ammonium oxalate, and the discoid form checked by examination and measurement.

The following table shows the results of the experiments, all carried out at temperatures in the neighbourhood of 18°C. The calculated results were obtained by inserting the proper values for the viscosity, density, radius, etc., in Equation 3 of this Section.

The excellent correspondence between calculated and experimental results will be seen at once.

We should expect that, since a rise of temperature lessens the value of $\gamma$, that sedimentation would be more rapid at high temperatures than at low ones, and that the relation between sedimentation rate and temperature would be the same as that between values of $\gamma$ and temperature, for changes of temperature affect the cell dimensions and the density of the suspending fluid to a negligible extent.

The following table shows the variation, calculated and observed, of sedimentation rate with rise of temperature. The cells were the cells of man in the Goughian form, and with a mean radius of 2.7 $\mu$.

The calculated results are obtained from Equation 1.
The correspondence between the theoretical and observed results forms an excellent check on the applicability of the Equation.

5. Concentration of suspension. We have not found that the concentration of the red cell suspension affects the results to any appreciable extent. Experiments have been carried out with suspensions of from half the strength of that described above, to four times as strong as that described; no significant difference in the sedimentation times was observed. Very weak suspensions are, however, to be avoided, for their upper margin becomes indistinct as sedimentation proceeds; at the same time very concentrated suspensions are unsatisfactory, as the cells may become so numerous, especially in the lower layers, as to interfere with each other’s fall.

6. Length and position of Sedimentation tube. In view of certain statements made in the literature that, when sedimentation takes place in serum, the length of the tube affects the rate of sedimentation, we have investigated this point with tubes of different length and
form. We have been unable to find that the length of tube has any effect. Cells fall as rapidly in tubes 3 cm. long as is tubes a metre long as in tubes a metre long, as is to be expected from theoretical considerations. We have also tried tubes of various diameters. Except for very narrow tubes, the diameter makes no difference to the rate of fall.

We have now to deal with the position of the tube. Boycott (1920) records that a greater percentage of cell-free fluid makes its appearance, after the same time of sedimentation, in inclined tubes than in vertical ones. Berceller and Wastl (1923) also record a similar finding; they observe that the vertical distance of descent of the surface of the suspension is greater in inclined tubes than in vertical ones. Both these observers are at a loss for an explanation.

The fact recorded by Berceller and Wastl is easily observed, and it will be found that the rate of sedimentation, judged by the rate of descent of the upper margin of the suspension, is sometimes as much as four times as great in inclined tubes as in vertical ones; the rate depends, moreover, on the inclination.

The explanation is simple as soon as it is realised that sedimentation is the fall of bodies in a fluid, under the action of gravity. For the occurrence there are three reasons, which, taken together, account for the happening in the simplest possible way.

Suppose that, as in the figure, we have vertical and inclined tubes of equal dimensions. In the vertical tube the fluid surface will be a cross-section
of the tube; in the inclined one it will be parallel to the ground. From these surfaces let cells of equal dimensions, \( a_1, a_2, a_3, \) etc., fall through a distance \( h \). The cells will then arrive at \( a_1', a_2', a_3', \) etc. The volume of clear fluid above them will plainly be 

\[ \text{Volume} = Ah \cdot \csc \theta, \]

where \( A \) is their area of cross-section of the tube and \( \theta \) the angle of its inclination. Since \( \csc \theta \) is always greater than unity, the volume of clear fluid in the inclined tube must always be greater for the same amount of sedimentation, than the volume in the inclined tube. The more the tube is inclined, the greater will be the increased volume. This accounts of Boycott's observation.

It does not, however, dispose of that of Berceller and Wastl, since their observation concerns not volume of clear fluid, but vertical descent of the surface of the suspension. To find the reason for this occurrence, consider a tube inclined to the horizontal and filled with suspension, as in the figure. Cells \( a, b, \) and \( c \) will fall from the surface layers in a given time, and arrive at a certain position. After they have attained the positions \( a_1, b_2, c_3 \), etc., the volume of clear fluid at the surface will be 

\[ \text{Volume} = Ah \cdot \csc \theta, \]

where \( h \) is the height of vertical fall and \( \theta \) the angle of inclination to the horizontal; \( A \) is, as before, the cross-sectional area of the tube. But cells \( e, f, g, \) etc., situated at the side of the tube, also fall through the same distance in the same time, to take up new positions \( e_1, f_2, g_3 \), etc. The space above the line passing
through these new positions of the cells will be occupied by clear fluid, provided that no cell moves in any direction other than that of the action of gravity. Call the volume of the clear fluid $V$.

Now, in point of fact, this condition is never seen. The space which should be occupied by clear fluid is, in practice, occupied by cell suspension. It is clear that, in order that cells may make their appearance in this area, some cells must move in a direction other than vertically downwards - in other words, in a direction other than that of the action of gravity. Such motion can be accounted for only by a current in the fluid, causing the cells of the suspension to move into the area which should be occupied by clear fluid, and which causes the clear fluid to pass upwards along the inclined upper wall of the tube, to augment the clear fluid at the surface.

The result is that the surface layer of clear fluid is of volume $(A \cdot h \cdot \cosec \theta + V)$, and not of volume $A \cdot h \cdot \cosec \theta$, as it would be if only the surface layers underwent sedimentation. The result will be that the surface of the suspension falls further in the inclined tube than in the vertical one, and further than a theoretical consideration of the velocity of sedimentation would warrant.

It will also be seen that the extent of this apparent increase depends both on the real sedimentation rate and on the length of the side of the fluid column $OB$, since, the greater either of these factors, the greater will be the volume $V$ to be added.
These considerations are open to experimental verification. We can find the real rate of sedimentation in vertical tubes, and can therefore calculate the volume of fluid which should be rendered clear of cells by the fall of cells from the surface and from the side of a tube inclined at any angle. The added volumes should agree with the volume of fluid found cell-free at the surface of the inclined tube in experiment. This we have done on several occasions, and found the results to agree very well indeed.

Some results are found in the following table.

<table>
<thead>
<tr>
<th>Sedimentation rate, mm per hour.</th>
<th>Fluid volume observed, cc.</th>
<th>Fluid volume calculated, cc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.5</td>
<td>30</td>
<td>19.3</td>
</tr>
<tr>
<td>4.2</td>
<td>40</td>
<td>17.2</td>
</tr>
<tr>
<td>5.0</td>
<td>45</td>
<td>23.5</td>
</tr>
<tr>
<td>3.9</td>
<td>50</td>
<td>16.8</td>
</tr>
<tr>
<td>6.2</td>
<td>60</td>
<td>21.3</td>
</tr>
</tbody>
</table>

In calculating the amount of theoretically cell-free fluid, the length of time of sedimentation, the quantity of suspension in the tube, the dimensions of the tube, and especially the length of the side of the fluid column are factors which vary in each experiment - have to be taken into consideration.

It will be observed that the calculated results are uniformly too low - that is, the descent of the surface of the suspension is not wholly accounted
for by adding the volume of the cell-free fluid produced by the falling of the cells in contact with the side of the tube to the volume liberated by the fall of the cells from the surface. There is, however, an additional and third factor to consider. If the fluid in the neighbourhood of the side OB, on becoming cell-free, moves upwards, it follows that a current must be set up, moving downwards along the lower side of the tube, which region is occupied by cells. Such a current will tend to increase the rate of sedimentation, to an amount which we cannot calculate.

It remains to be explained why the cell-free fluid should move upwards. The explanation lies in the fact that in this fluid there are no currents produced by falling cells, whereas in those portions of fluid occupied by cells innumerable small currents are set up, all tending in the same direction. The exact study of these movements of fluid is too complex a matter to be studied here, and is also somewhat immaterial, for it is sufficient for us to have shown that currents do, in fact exist in inclined tubes and not in vertical ones, and that their general nature explains the more rapid sedimentation sufficiently well for our purpose. These currents do not, of course, interfere with the fall of the cells along the lower side of the inclined tube, for it is to be remembered that the cell-free fluid along the side of the fluid column OB never exists in appreciable amount except in theory, but is continually passing upwards to the surface in a thin sheet. Its place
is taken by cell suspension, the cells of which, nearest to the walls of the tube, sediment, leaving another thin sheet of cell-free fluid to move upwards, and so on indefinitely, the only effect on the cells in the other parts of the tube being the acceleration of their fall by reason of the necessarily accompanying downward current in the lower side of the tube, as explained above.

Except for noting the fact that Abderhalden (1922) finds that cells from the different layers of a centrifugalised deposit sediment at different rates, and that Ohno (1923) finds a relation between sedimentation rate and the surface area of cells - both of which observations suggest the effect of the cell-dimensions - we do not intend to comment on the statements made in the literature on this subject, for most of the observations are concerned with the fall of cells in serum or plasma.
Papers referred to.

Abderhalden (1922) Pfluger's Archiv. cciii, 236.
Onno (1923) Pfluger's Archiv. ccii, 376.
PART V.

SECTION II.

Rouleaux formation.

Chance contact.

1. The equations.
3. Results.
4. Mean size of rouleaux.

Electrical forces.

1. Methods.
2. Results for cells in saline, plasma, and heated plasma.
3. The charge on a single cell.
4. The repulsive forces between cells.

The cohesive forces.

The surface forces.

Papers referred to.
The process of polymerization is a reaction where initially the cells are all separate. A single cell may then coalesce with another to form a colony of cells which for convenience can be called a cluster. This realization of the fact that all the cells with a single cell or with several groups of two, and the day became visible a nucleus of either of two or four, and so the process can be followed sequentially.

As such to examine the behavior of single cells, realizations of one or two of these, and as the which will be done after it can be a regime of fluid containing a type of cells. Each of these is discrete when it can.

To complete examination of the type these assumptions provide that the two are done, and a response position as needed to be done completely.
The phenomenon of rouleaux formation is a very complex one, into which many factors enter. Each of these we propose to study separately.

I. CHANCE CONTACT.

It will be obvious that two cells cannot cohere to form a rouleaux or part of a rouleaux unless they first collide. We have therefore to consider the chance of collisions taking place among the individuals of a large number of cells, given, to begin with, that no factor save chance enters. We may imagine that initially the cells are all discrete; a single cell may then collide with another to form a group of two, which for convenience we shall call a rouleaux of two. This rouleaux of two may then collide either with a single cell or with another rouleaux of two, and thus may become either a rouleaux of three or a rouleaux of four, and so the process may go on indefinitely.

We wish to calculate the number of single cells, rouleaux of two, rouleaux of three, and so on, which will be found after any time \( t \) in a volume of fluid containing a very large number of cells, all discrete when \( t = 0 \).

To simplify matters, we may make three assumptions. (1) Every contact is to result in a cohesion, provided that the contact takes place in a suitable position, as required by the next assumption. (2) The
surface by which a rouleau can add on another cell or another rouleau is the same as that by which a cell can add on another single cell. (3) Single cells and rouleaux, irrespective of their size, are carried about in the fluid with equal velocities.

The second of these assumptions can be seen to be true by reference to the adjacent figure. We take it that the contact of the cell b on the rouleau a will result in the addition to the length of a only if b strikes a in the region of its ends, so the surface of a, so far as rouleaux formation is concerned, is no greater than that of b.

The third assumption will be true if the fluid containing the cells is in turbulent motion - a condition easily fulfilled in experiment.

Given these simplifying assumptions, we treat the case in the following manner, of v. Smoluchowski, and obtain his final equation,

$$w_2 = \frac{M(\ell + M)}{M(\ell + M)^2 + 1}$$

where \(w_2\) is the number of rouleaux \(q\) r. cells, \(M\) the number of cells under consideration, \(\ell\) a constant, and \(t\) the time after which \(w_2\) is ascertained.
The final equation presents some very interesting points.

1. The constant 1, under ordinary circumstances, must be small, so that the term $\frac{1}{2}\ln \frac{M}{2}$ shall be in the neighbourhood of unity. Under these circumstances, when $t$ is small, we have $w_1 > w_2 > w_3$, etc., that is, there are more single cells than rouleaux of two, more rouleaux of two than rouleaux of three, and so on.

2. When $t$ becomes great we approach the condition $w_1 = w_2 = w_3$ etc., that is, there are as many single cells as there are rouleaux of any particular size. This result is rather surprising at first sight, for one would expect that with increasing time all the single cells would disappear in favour of the longer rouleaux. A little thought will show that this is not the case, and that the result of the equation is in no way at variance with experience, for in a preparation which shows many rouleaux, it may certainly be difficult to find a single cell, but it may be equally difficult to find a rouleau of, say, exactly seventeen or twenty-nine.

3. The equation, however, has this limitation, that it places no limit to the length of the rouleaux formed, and, if $t$ become large, a figure may be given for the number of rouleaux containing, say, a thousand or ten thousand cells. Rouleaux of this length do not occur in practice, and therefore we must use the expression up to a certain limit only — to rouleaux of about 20 cells.
In view of this last consideration, it would be desirable to have a reversible equation which would take account of the fact that, as the length of rouleau increases, the tendency to break up increases also. We may take it that the chance of a fracture occurring is directly proportional to the length of the rouleau, but even on this assumption we have failed to develop a reversible equation, and must therefore content ourselves with the one given above, and with the experience that, until we come to rouleaux of over 20 cells, fracture rarely occurs even if the aggregates are carried about rapidly in the fluid.

It remains to test the validity of the equation developed above; this may be done by the following methods.

**METHODS.**

Blood is withdrawn from the arm vein of a subject and is received into a syringe containing a small amount of anticoagulant. This blood is centrifuged and the plasma removed; the plasma is again centrifuged to free it of all cells. About 1 cc. of plasma is then placed in each of a series of tubes, kept at whatever temperature is decided upon for the experiment. If the initial stages of rouleaux formation are to be studied, this temperature should be low (15°C), while if rapid rouleaux formation is desired, it should be higher (37°C).
As soon as the clear plasma has been obtained, a small quantity of blood, about 0.05 cc., is withdrawn from the subject's ear, and mixed with the plasma. The tube containing the mixture is then shaken to and fro, much as one shakes a boiling tube, for a length of time previously decided upon. During this time the temperature of the experiment is maintained, and rouleaux formation occurs. Just before the end of the prearranged time, a drop of the mixture is placed on a clean slide and covered with a coverslip the edges of which are greased with vasaline. The cells quickly settle if the vasaline seal is complete, and may then be placed under a magnification of about 300, and photographed under critical lighting, some 20 or 30 fields being recorded. The necessary exposure being only about half a second when good lighting is used, this number of fields can be photographed within a couple of minutes. The plates are developed, and the number of rouleaux of two, rouleaux of three, and so on, as well as the number of single cells, can be found by examination under a low power microscope. It is, of course, important that the fields should be taken at random, and not selected either for single cells or for rouleaux.

The simplest way to record the count is to count out 1000 beans into a dish, 1000 being a convenient number for M, the cells to be observed. A distributing box containing ten or twelve divisions is then taken, the first division labelled 1, the second 2, and so on up to 12. When a single cell is seen under
The curves are shown in the present figure, but are better to be plotted on a smaller, larger scale.

Taking first the values of \( w_1 \), the value of the factor which corresponds to this curve on the \( w_1 \) curve is read off and taken over from the number of \( w_1 \) in taken, and the corresponding value of \( 1/w_1 \) on the \( w_1 \) curve read off. Similarly, the value of the factors for each of the other observed numbers is read off the proper curve. The values of the factors are then averaged; the result is the best value of \( 1/w_1 \). Given \( l \), the value of \( 1/w_1 \) is obtained. There, as in case of the curve, there are two values for the factor.
the microscope, one bean is put into division 1; when a rouleau of two is seen, two beans are put into division 2, or when a rouleau of eight appears, eight beans are put into division 8. So on till the exhaustion of the 1000 beans. The number of beans in each division is then counted; that in division 2, if divided by 2, gives the number of rouleaux of two, that in division 8, if divided by 8, gives the number of rouleaux of eight, and so on. By this method 1000 cells are distributed exactly without difficulty.

We have now to find the value of the constant 1. The easiest way to do this is to plot a series of curves for the values of \( w_1, w_2, w_3, \) etc., up to \( w_r \), for values of the factor \( \frac{1}{2}tM \), assigning to this factor values of 0.5, 1, 2, 3, 4, and 5. These values cover the range of ordinary experiment.

The curves are shown in the adjacent figure, but are better to be plotted on a somewhat larger scale.

Taking first the number of \( w_1 \), the value of the factor which corresponds to this number on the \( w \) curve is read off and noted down. Next the number of \( w_2 \) is taken, and the corresponding value of \( \frac{1}{2}tM \) on the \( w_2 \) curve read off. Similarly the value of the factor for each of the other observed numbers is read off the proper curve. The values of the factors are then averaged; the result is the best value of \( \frac{1}{2}tM \). Since \( t \), the time, is known, and since \( M \) is 1000, the proper value of 1 is readily obtained. Where, as in some of the curves, there are two values for the factor corres-
ponding to the observed number of rouleaux, that value nearest the one obtained for the $w$ curve is taken, for reasons which will appear as soon as the method is put into practice.

The curves seen in the figure are interesting ones; the curves for $w$ and for rouleaux containing a greater number of cells than two have a maximum, while that for the $w$ has none. Further, this former set of curves passes through the origin, indicating that, when $l$ or $t$ is zero, there are no rouleaux present, while the curve for $w$, cuts the ordinate at 1000, indicating that under these circumstances, when $l$ or $t$ is zero, there are only single cells present.

In order to make the procedure clear, we shall select an worked example as the first of the record of results.

**RESULTS.**

Experiment 1. Cells were allowed to form rouleaux in plasma for 10 minutes, so \( t = 10 \).

Thereafter the number of rouleaux of each length was ascertained, and the following figures obtained.

<table>
<thead>
<tr>
<th>Rouleaux of</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>223</td>
<td>110</td>
<td>63</td>
<td>27</td>
<td>15</td>
<td>12</td>
<td>15</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
</tbody>
</table>

Referring to the graph of the values of the factor \( lW/2 \), we find that the value of the factor on the $w$ curve, and corresponding to the figure 223, is 1.1
On the $w_2$ curve the value 1.35 corresponds to 110.
On the $w_3$ curve the value 1.0 corresponds to 63.
On the $w_4$ curve the value 0.85 corresponds to 27.
On the $w_5$ curve the value 1.0 corresponds to 15.

Adding these values and averaging, we get the best value of $l t M/2$ as 1.06.

Since $t$ is 10 and $M$ is 1000, the proper value of $l$ is 2.12 ($10^{-7}$). The fact that the values of $l t M/2$ obtained from the different curves agree so closely is strong evidence of the applicability of the equation.

It will be noted that the values of $w$ and rouleaux longer than this are omitted in the calculation of the value of $l t M/2$. The reason for the omission is that these values are too liable to error.

If the equation which has been developed is correct, we should be able to predict that number of rouleaux of any particular size when $t = 20$; that is, after twice the time considered in this experiment. In this case, $l t M/2 = 2.12$, and the distribution of rouleaux which we should expect would be,

Rouleaux of  1  2  3  4  5  6  7  8  9  10
           102  70  46  33  21  15  10  6  4  3

The results obtained by experiment were

Rouleaux of  1  2  3  4  5  6  7  8  9  10
           113  74  47  32  24  20  13  5  5  2

Considering experimental difficulties, the correspondence is very good. The low value of $l$ is this experi-
iment is an indication that rouleaux formation was proceeding slowly, an occurrence principally due to the low temperature of the experiment, (14°C).

Experiment 2.
Rouleaux formation at 15°C, t = 20 mins.

<table>
<thead>
<tr>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
</tr>
</thead>
</table>
Rouleaux observed | 117 | 87 | 42 | 33 | 24 | 13 | 16 | 6 | 3 | 3 | .. | 3 |
Rouleaux calculated | 111 | 74 | 49 | 33 | 22 | 15 | 10 | 6 | 4 | 3 | 2 | 1 |

The calculated results were taken from the value \( l_tM/2 = 2 \).

Experiment 3.
Rouleaux formation at 37°C, t = 7 mins.

<table>
<thead>
<tr>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
</tr>
</thead>
</table>
Rouleaux observed | 49 | 30 | 27 | 20 | 16 | 12 | 12 | 9 | 6 | 6 | 3 | 5 |
Rouleaux calculated | 33 | 27 | 22 | 18 | 14 | 12 | 10 | 8 | 6 | 5 | 4 | 3 |

The calculated results were taken from the value \( l_tM/2 = 4.5 \).

Experiment 4.
Rouleaux formation at 37°C, t = 3 mins.

<table>
<thead>
<tr>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
</tr>
</thead>
</table>
Rouleaux observed | 73 | 55 | 40 | 22 | 19 | 17 | 10 | 10 | 6 | 5 | 2 |
Rouleaux calculated | 62 | 47 | 35 | 26 | 20 | 15 | 11 | 8 | 6 | 5 | 3 | 2 |

The calculated results were taken from the value \( l_tM/2 = 3 \).

Experiment 5.
Rouleaux formation at 15°C, t = 2 mins.

<table>
<thead>
<tr>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
</tr>
</thead>
</table>
Rouleaux observed | 462 | 120 | 60 | 14 | 10 | 2 | 1 | 1 | .. |
Rouleaux calculated | 444 | 118 | 50 | 16 | 6 | 2 | 1 | .. | .. |
The calculated values were taken from $ltM/2 = 0.5$

Experiment 6.

Rouleaux formation at 37°C. $t = 4$ mins.

<table>
<thead>
<tr>
<th>Rouleaux observed</th>
<th>130</th>
<th>81</th>
<th>40</th>
<th>37</th>
<th>16</th>
<th>14</th>
<th>12</th>
<th>10</th>
<th>5</th>
<th>5</th>
<th>2</th>
</tr>
</thead>
</table>

Rouleaux calculated | 111 | 74 | 49 | 33 | 22 | 15 | 10 | 6 | 4 | 3 | 2 | 1

The calculated values were taken from $ltM/2 = 2$.

In some of these experiments, the observed number of cells will be found to total to less than 1000; in such cases the remaining cells were in rouleaux of more than 12. Further, when $ltM/2$ becomes greater than about 2, as it does in cases where long rouleaux appear, the sum of the figures obtained by multiplying the number of rouleaux by the number of cells in each falls short of 1000, the total number for calculation; this is principally because calculation gives a fractional number for the longer rouleaux - we cannot take account of 0.5 rouleaux of twelve, for instance, although this figure contributes six cells to the total.

Such difficulties with the rouleaux of great length does not, however, matter much in experiment, for we are rarely concerned with very marked rouleaux formation, and, at any rate, would not select such a condition for the purpose of checking an equation or for the purpose of comparing calculated and experimental results.
THE MEAN SIZE OF ROULEAUX.

To find the mean length of rouleaux present in a suspension such as we have been considering, we multiply the number of each size of rouleaux by the cells in it, and divide the sum of the figures obtained by the number of rouleaux.

The mean size of rouleau thus is

$$\frac{\sum y w _ i}{\sum w _ i}$$

or, as $\sum y w _ i$ is $M$, and $\sum w _ i$ is $M/2 + 1$

the mean is

$$\frac{C M + 1}{2}$$

This is a useful result for the mean can be calculated from the experimental observations and from the value of $l t M / 2$ by adding to it unity. For example, if $l t M / 2$ were 3, the mean size of rouleaux would be a rouleau of 4 cells.

The result is even more useful, however, than appears at first sight, for, if we can find the mean size of rouleau by any method, we can get the value of $l t M / 2$ and thence the value of 1. Now it happens that the most convenient way of finding the mean size of rouleaux is not to count 1000 cells and to distribute them according to their grouping, but to allow an unlimited number of cells to sediment in a fluid, and to calculate from the rate at which they fail the size of the mean rouleau. The constant 1 can then be found from the data.
II. ELECTRICAL FORCES.

The researches of Northrop and Freund (1924) and of Oliver and Barnard (1924) have established that the stability of a red cell suspension is largely dependent on the difference of potential which exists between the cells and the suspending medium. If this potential fall below a certain point the cells tend to agglutinate; the value of this critical potential seems to depend on factors at present unknown. The potential difference, amounting to similar charges on the cells is regarded as keeping them apart; any force acting in this way and tending to preserve the discreteness of a cell suspension must clearly be taken into account in connection with rouleaux formation.

The potential difference between the red cells and the surrounding medium is ascertained by some method of electrophoresis, the migration of the cells being measured in μ per second, and from this migration rate the potential difference calculated from the Lamb-Helmholtz equation. The methods employed in this type of investigation are principally two - that recommended by Michaelis, and used with certain modifications by Oliver and Barnard, and that described by Northrop; the methods do not differ in principle, but only in the form of electrophoretic cell employed.

In the case of suspensions such as those of which this paper treats, and in which a considerable
degree of rouleaux formation is present, the difficulty arises that the rouleaux, owing to their size and peculiar shape, tend to fall out of the plane of focus when the measurements are being made; it takes but a very short time for most of them to collect on the bottom of the measurement chamber, where, of course, they are useless for the purpose of the experiment. This fact, together with the difficulty in cleaning the electrophoretic cells when solutions containing proteins are used, had caused us to abandon the microscopic method, and to fall back on a macroscopic one, which, although by no means free from fallacies, has given better results.

METHOD.

The apparatus used consists of a U-tube of about 5 mm. in cross section, the sides of which are about 10 cm. in length. Into the bottom of the U-tube there is sealed in a glass tube of narrower bore, which expends into a small reservoir with a stopcock by means of which fluid can be passed into the U-tube. Two KCl-agar syphons lead from the limbs of the U-tube to zinc-zinc sulphate electrodes, which are connected with a 230 volt circuit through a voltmeter and reversing switch. A reading microscope with a 3-inch lens is arranged so that it can be focussed on the limbs of the U-tube; the vertical movement of the microscope can be read off on a scale, and the finer movements on a micrometer attached to the eyepiece.
If the reservoir is filled with a suspension of cells in plasma, saline, or serum, and the U-tube filled with the same fluid, but free from cells, it is quite easy to allow the suspension to enter the bottom of the U-tube, displacing the contents thereof, so that clean interfaces are formed in each limb about half-way up. To make these interfaces sharper, the cells may be allowed to sediment for about five minutes, or the current may be allowed to pass for a minute or two in each direction. To make the reading, the level of one interface is first observed on the micrometer in the eyepiece of the microscope, the current is then turned on, and the level of the interface read again after either 100 or 200 seconds. Care should be taken to observe the interface which proceeds away from the electrode on the same side of the U-tube when the current is passing, and not the interface which rises in the limb. The average of three readings may be taken as the final result, and the movement may be checked by reversing the current and observing the alteration in level of the other interface.

The velocity of sedimentation is now to be subtracted from the velocity of movement of the interface; the result is the electrophoretic velocity from which the potential difference between cell and fluid can be calculated. The velocity of sedimentation must be found by measuring the rate of fall of one of the interfaces when no current is passing; this sedimentation velocity is usually much less than the electrophoretic. In connection with it an interesting point arises. The vel-
The practical difficulty attending on this fact is that, since the calculated and observed velocities are not the same, there is an error introduced into the method, and an error, moreover, which we cannot allow for. The taking of the sedimentation velocity in the U-tube when filled ready for the experiment is, however, sufficiently satisfactory for all ordinary purposes.

The cell constant of the tube is easily found, as the cross section of the tube is the same at all points. In view of the fact that the continued passage of a current causes a rise of temperature in the contacts...
ocity of sedimentation in a U-tube such a we describe
will always be found to be greater than that calculated
from the appropriate equation, and greater than that
observed in a straight tube with the same suspension.
The explanation of this will be seen from an inspection
of the figure attached. Cells a and b fall in a given
time to positions a' and b', but at the same time a
cell c, situated initially at the bend of the tube,
falls to c'. This results in clear fluid being de
veloped in contact with the upper wall of the bend of
the tube; such clear fluid never appears in practice,
however, but is replaced by cell suspension, while the
clear fluid passes upwards to augment the clear fluid
above the interfaces. This fluid always appears greater
than the calculated amount in consequence. This con-
dition of affairs in very similar to that observed in a
sloping tube, and dealt with in Section 1 of this Part.

The practical difficulty attendant on this
fact is that, since the calculated and observed veloci-
ties are not the same, there is an error introduced
into the method, and an error, moreover, which we can-
not allow for. The taking of the sedimentation velocity
in the U-tube when filled ready for the experiment is,
however, sufficiently satisfactory for all ordinary pur-
poses.

The cell constant of the tube is easily found,
as the cross section of the tube is the same at all points.
In view of the fact that the continued passage of a cur-
rent causes a rise of temperature in the contents of the
U-tube, as well as to avoid diffusion currents due to local temperature alteration, the apparatus is immersed in a small thermostat, in which may also be kept the tubes for the necessary viscosity determinations.

The Lamb-Helmholtz equation for the calculation of the potential difference between the cells and the surrounding fluid is

\[ V \frac{d}{a} = \frac{\mu \eta \nu}{K \chi} \]

where \( V \) is the potential difference, \( l \) a measure of the slip, \( d \) the distance between the plates of an air condenser equivalent to that which may be supposed to exist between the surface of the cell and the fluid, \( K \) the specific inductive capacity, \( \nu \) the velocity of migration, and \( \chi \) the strength of the electric field. All measurements are electrostatic. Of the variables in the equation, \( l \) and \( d \) are unobtainable, and \( K \) is difficult to determine; we therefore follow Winslow, Falk, and Caufield (1924) in recognising that it is impossible to calculate the potential difference with any accuracy. We shall therefore give, with a claim to accuracy, the value of \( \nu \eta / \chi \), \( \nu \) being observed in \( \mu \) per second, and shall use 14.1 \( \times 10^2 \) times this figure to indicate what the potential difference in millivolts might be taken to be if \( l/d \) were unity and \( K \) were 81. The values of \( l/d \) and of \( K \) cannot vary much from these figures.

The values for the potential difference between erythrocytes and surrounding fluid have hitherto been obtained principally from observations on cells in
hypotonic sugar, with or without the addition of electrolytes. For the purpose of this study, we propose to investigate the migration of human cells in three media; in 0.85 per cent NaCl, in which rouleaux do not form, in plasma, in which rouleaux form to a moderate extent in the cold, and in heated plasma in which rouleaux form rapidly and extensively. The plasma required to be heated to 56°C. for 15 minutes. (Sellards, 1968)

The cells and plasma were obtained by venous puncture, the blood being collected over a small quantity of potassium oxalate. The electrophoretic velocity was measured as described above, the strength of the field being taken in each case - a very necessary precaution - and the viscosity of the suspending fluid being measured immediately after the conclusion of the experiment. In all cases the temperature at which the U-tube was kept was 15°C., this temperature being selected because at higher temperatures the formation of rouleaux is inconveniently rapid. We have been careful to carry out the determinations of the electrophoretic velocity as soon after withdrawal of the blood as possible, in view of some of the findings of Eggerth, (1924).

RESULTS.

These may be given in tabular form, as in the following summarise of experiments. In all cases the cells were those of man, and, where the cells are stated to be suspended in plasma, that plasma was obtained at
the same time as the cells themselves.

1. Cells suspended in 0.85 NaCl.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>vq/x</th>
<th>Approximate P.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.0052</td>
<td>7.3</td>
</tr>
<tr>
<td>2</td>
<td>0.0048</td>
<td>6.7</td>
</tr>
<tr>
<td>3</td>
<td>0.0062</td>
<td>8.7</td>
</tr>
<tr>
<td>4</td>
<td>0.0046</td>
<td>6.5</td>
</tr>
<tr>
<td>5</td>
<td>0.0060</td>
<td>8.4</td>
</tr>
<tr>
<td>6</td>
<td>0.0055</td>
<td>7.7</td>
</tr>
<tr>
<td>7</td>
<td>0.0063</td>
<td>8.8</td>
</tr>
<tr>
<td>8</td>
<td>0.0052</td>
<td>7.3</td>
</tr>
<tr>
<td>9</td>
<td>0.0049</td>
<td>6.8</td>
</tr>
<tr>
<td>10</td>
<td>0.0065</td>
<td>9.1</td>
</tr>
</tbody>
</table>

2. Cells suspended in plasma.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>vq/x</th>
<th>Approximate P.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.0076</td>
<td>10.6</td>
</tr>
<tr>
<td>2</td>
<td>0.0047</td>
<td>6.6</td>
</tr>
<tr>
<td>3</td>
<td>0.0080</td>
<td>11.2</td>
</tr>
<tr>
<td>4</td>
<td>0.0070</td>
<td>9.2</td>
</tr>
<tr>
<td>5</td>
<td>0.0062</td>
<td>8.8</td>
</tr>
<tr>
<td>6</td>
<td>0.0057</td>
<td>8.0</td>
</tr>
</tbody>
</table>
3. Cells suspended in heated plasma.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>(v7/X)</th>
<th>Approximate P.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.0075</td>
<td>10.5</td>
</tr>
<tr>
<td>2</td>
<td>0.0085</td>
<td>11.9</td>
</tr>
<tr>
<td>3</td>
<td>0.0060</td>
<td>8.4</td>
</tr>
<tr>
<td>4</td>
<td>0.0070</td>
<td>9.8</td>
</tr>
<tr>
<td>5</td>
<td>0.0065</td>
<td>9.1</td>
</tr>
<tr>
<td>6</td>
<td>0.0081</td>
<td>11.3</td>
</tr>
</tbody>
</table>

The average P.D's of the cells in the various suspension media thus are:

- Cells in saline: 7.7 millivolts.
- Cells in plasma: 9.2 millivolts.
- Cells in heated plasma: 10.2 millivolts.

The difference between the last two figures may not be significant, as an experimental error of 1 millivolt is quite allowable, but we are inclined to look upon the difference between the P.D. of cells in saline and that of cells in plasma as significant. It may be mentioned that the difference is not a striking one in experiment, the rate of migration being about the same in each case; introduction of the proper value for the viscosity, however, makes the higher charge on the cells in plasma appear at once. It is, of course, to be borne in mind that these values are only correct on the assumption that \(1/d = \text{unity}\), and that \(K = 81\), conditions which may not be fulfilled for both saline
and plasma.

Allowing for all sources of error, we have to conclude from these results that

(1) rouleaux formation will occur even if there is a comparatively great potential difference between cells and surrounding fluid,

(2) the failure of cells to form rouleaux in saline is not due to the repulsive forces between them being unusually great, and

(3) the increased tendency to form rouleaux in heated plasma is not due to the repulsive forces being diminished.

The results of this part of the investigation are accordingly negative.

**THE CHARGE ON A SINGLE CELL.**

This may be found in an approximate manner from the migration rate by a modification of the Stokes equation, in which the force moving the cell and the resistance keeping it back are balanced against one another. We have two important cases, (1) where the cell is a sphere, as in the Coughlan form, and (2) where the cell is in the normal form of the biconcave disc.

(1) When the cell is a sphere, we have the resistance to its movement as $6\pi\eta r$. The velocity in proper units is $v/X$, and so the force acting on the cell is, in e.s.u., $e = 6\pi\eta rv/X$. Here $r$ is the radius
of the cell, while \( v, X, \) and \( \eta \) have the meaning as above.

The charge on a single cell works out as a very small quantity. Taking a cell of radius \( 3\mu \), and assuming that it moves in a fluid of viscosity \( 0.01 \) with a migration rate of \( v/X = 1 \), the charge is approximately \( 1.7 \times (10^{-7}) \) e.s. units. This corresponds to a potential difference of about 1.4 milivolts, as observed by electrophoresis.

(2) If the cell is in the discoid form it will meet with a different resistance as it passes through the fluid, which it does broadside on. The resistance is that met with by a sphere of radius 0.85 \( c \), where \( c \) is the radius of the disc, and so we have the force as \( e = 6\eta(0.85 c)v/X \). This gives approximately a charge of \( 2.1 \times (10^{-7}) \) e.s. units for a cell under the same conditions as above.

**THE REPULSIVE FORCES BETWEEN CELLS.**

It would be of great interest to determine the magnitude of the repulsive forces which exist between two similarly charged cells. Unfortunately it is almost impossible to estimate these forces with any accuracy. The reason for the difficulty is that, although we can estimate approximately the charge on a single cell, this charge is due to a double layer at cell surface - indeed, as the cell is a balloon like body, to two double layers, one at each side of the
membrane - and as such cannot be treated as if it were a charge such as resides on a pith-ball. The repulsive forces, if we are to judge from analogous cases of double layers round colloid particles, only manifest themselves when two cells approach within a very small distance of one another, and occur as the result of a redistribution of the charges as a result of the opposition. The repulsive forces differ in another way from those due to fixed charges, for they do not vary with the square of the distance of the charges from one another but with a higher power, so that when the cells are more than a small distance apart the repulsive forces between them are negligible.

It is, however, quite instructive to treat the charges as though they were fixed, always remembering that no argument can be based on such results alone.

In the case of spherical cells we can locate the charges at the centres, in which case we have the repulsive force between two cells each charged with 1.7 e.s. units, and whose centres are 10 \( \mu \) apart, as \( 3.6 \times (10^{-2}) \) dynes - a very small quantity.

When it is remembered that the erythrocyte is normally in the form of a biconcave disc, the difficulties of forming an estimate of the repulsive forces becomes even greater, for two cells can approach one another in a number of ways, either broadside on, edge on, or in any intermediate position. For each position there will be a corresponding alteration in the charge-distribution, and a corresponding repulsion will develop.
1. Equatorial planes coplanar.
2. Equatorial planes at right angles.
3. Equatorial planes parallel.

Fig. 4.

1. Equatorial planes coplanar.
2. Equatorial planes at right angles.
3. Equatorial planes parallel.
If we treat the charges as fixed, some interesting results appear.

Since the surface of the cell in its normal form approaches very closely to the equipotential surface of a charged ring (see Part IV of this Thesis), we may locate the charge borne by the surface on this hypothetical ring, whose diameter is about five-sixths that of the cell. In order to facilitate computation we may replace this ring by the inscribed hexagon, and locate one-sixth of the total charge at each corner of that figure.

We can now consider the three principal positions in which two cells can come together; they may lie with their equatorial planes in one plane, with their equatorial planes at right angles, or with their equatorial planes parallel.

(1) Equatorial planes coplanar.

For the purpose of computation, take two hexagons inscribed in circles of unit radius, whose centres are 4 units apart. If the hexagons lie so that their sides are parallel, the sides nearest one another are 2 units apart. Let each point of the hexagon bear unit charge. The repulsive force between the hexagons is the found approximately by joining point 1 of the first hexagon to all the points of the second in rotation, measuring the lengths of the lines so formed, and summing the squares of their reciprocals; thereafter point 2 of the first hexagon is joined to all points of the second, and the sum of
the reciprocals of the squares of these lengths obtained. The procedure is continued until all the points of the first figure have been joined to all the points of the second; the sum of the reciprocals of the squares of all thirty-six distances gives the repulsive force in arbitrary units.

In the case of hexagons whose equatorial planes are coplanar, the force is 2.8 units.

(2) Equatorial planes at right angles. A similar summation, this time in three dimensions, gives for hexagons of the same size as in (1), whose centres are 3 units apart and whose edges are 2 units apart, a repulsive force of 3.2 units.

(3) Equatorial planes parallel. If the planes are 2 units from each other, and the hexagons the same as in (1), the force between them is 6.3 units.

Treating the charges in this way, we find the forces greatest when the cells approach broadside on, and least when they are edge on, the distance between two opposing edges being the same. If, accordingly, two cells were to come together to form a rouleau, the greatest work would be done in moving them in the former position.

Slightly different results are obtained if the hexagons are taken with a side of one opposite a point of the other, but the general nature of the result is not affected. Nor is the general nature affected by assuming that the repulsive force varies with the cube
with the fourth power of the distance, instead of with the square, as is most probable in fact. Whether it would be obtained from a rigidly correct treatment of the charges due to the double layer we are not in a position to say at present. The point is perhaps of not very great importance, for the currents which cause the cells to collide are quite capable of overcoming the small electrical repulsions, and, moreover, these currents tend to move the cells broadside on.

III. THE COHESIVE FORCES.

Some of the most important observations in connection with rouleaux formation are those of Sellards (1906). This investigator records that (1) normal human serum, when heated to 60°C. for fifteen minutes, develops the property of causing human erythrocytes to collect in rouleaux; (2) this property is due to an increase of a normal property of the serum; (3) the property of causing rouleaux formation is rapidly lost at room temperature, and less rapidly in the cold, the loss being more marked in unheated than in heated serum; and that (4) rouleaux break up spontaneously after some hours, the necessary time being dependent on the temperature. He also records the fact that rouleaux formation is dependent on the number of cells in the suspension, being more marked when many cells are present, as might be expected from the foregoing considerations of chance contact.
We can confirm Sellard's results without exception, having carried out his experiments, using both serum and plasma. Especially noticeable is the effect of heating serum or plasma.

One of the most important conclusions is that serum possesses a normal property of producing rouleaux formation. Plasma, according to our finding, has also this property. This is in keeping with the fact that cells will form rouleaux in the vessel of an animal if the circulation is arrested for a few moments, as may be well seen in the mesenteric vessels of the mouse. The formation of rouleaux is by no means a phenomenon observed in shed blood only, as sometimes stated or implied.

We have shown above that the difference between the power of unheated and heated plasma in forming rouleaux is not to be explained by the former fluid having a higher potential difference with the cells than the latter. We are inclined to consider that the effect of heating the suspending medium is to cause a change in the surface stickiness of the cells suspended in it, or, in other words, to cause a change in what Northrop, Oliver, and Barnard call the "cohesive forces" between the cells. It is known that the stickiness of the red cell surface can be altered by the addition to the suspending medium of various substances (Fenn, 1922), and both Northrop and Oliver and Barnard employ such a conception to explain the effect of NaCl, BaCl₂, and CaCl₂ on the cells.
The more sticky the surface of the cells, the greater will be the chance of a collision being effective and resulting in a permanent contact. Moreover, such a conception goes to explain why, in typical rouleaux formation, end-contacts are permanent and side-contacts are not, for the former type of contact involves large surfaces over which these cohesive forces act, while the latter type involves small surfaces, and accordingly small cohesive forces. We are, indeed, inclined to think that the stickiness of the cell surface provides the key to the whole problem, and that variations in the potential difference between the cells and the suspending fluid play a comparatively small part.

Unfortunately there is not at present any satisfactory method of investigating these cohesive forces, and a definite statement on the point is therefore impossible.

IV. THE SURFACE FORCES.

Quite apart from the question of stickiness of the surfaces of cells, surface forces play a part of great importance in the formation and stabilization of rouleaux.

Then two cells come into contact, a certain reduction of surface takes place; if they are culled apart again, ther must be produced an enlargement of surface which entails the expenditure of work, quite apart from the work done in overcoming the cohesive
forces which cause the surfaces to stick to one another. This fact tends to stabilise rouleaux and maintain the contact of cells; it will be clear that the mere reduction of surface which occurs as the result of contact in a particular position, the more stable will the contact be.

When two cells collide broadside on, the resulting reduction of surface is as great as can be, and the collision results in a very stable contact, for the separation of the cells involves an increase of surface energy of the order of $10^{-6}$ ergs. Cells which collide with rouleaux at other points than in the neighbourhood of their ends can, however, be easily caused to break away, for as the result of such a collision there is little diminution of surface and therefore little stability associated with the position of contact. Thus it comes about that collisions in the region of the ends of rouleaux result in permanent contacts being formed, while collisions in other regions are practically ineffective - an assumption made in developing the equation in this Section. In the former case there is resistance to separation, for such separation means an increase in surface energy; in the latter case there is no such resistance, and separation takes place under any slight applied force, whether it be that of the currents in the fluid or that associated with the electrical repulsion which occurs between the cells.

The existence of these surface forces is shown in an interesting way in the deformation of the cells.
which build up a rouleau. Since the surfaces of these components will be deformed so as to produce minimal surface, provided that they are deformable, the cells of a rouleau are pressed together, considerable seduction of surface energy being effected in this way.

Further, the whole rouleau is compressed, for the surface forces tend to shorten it until its height is equal to its breadth - the condition for a cylinder of minimum surface for its volume. Such a complete deformation is not possible, for the cells are not sufficiently deformable under the forces acting on them, but an approach to it is seen in the decreased thickness of the cells which compose the rouleau, and also in their increased diameter. This may be shown in the following manner.

A preparation of cells suspended in their own plasma, is made in the way described earlier in this Section, the conditions being such that a moderate degree of rouleaux formation is present. A series of microphotographs are taken of the preparation, with all the precautions set forth in Part II of this Thesis; the fields are selected at random so that some show a number of single cells and others show a number of rouleaux of moderate length. The diameters of the cells are then measured from the plates - first the diameter of the single cells, then that of the cells composing the rouleaux. The following table shows typical results from five experiments based on the blood of man,
each experiment being based on the cells of a different individual.

Mean diameters are shown.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Single cells, diameter in μ</th>
<th>Cells in rouleaux, diameter in μ</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8.72</td>
<td>8.96</td>
</tr>
<tr>
<td>2</td>
<td>8.80</td>
<td>9.11</td>
</tr>
<tr>
<td>3</td>
<td>8.88</td>
<td>9.05</td>
</tr>
<tr>
<td>4</td>
<td>8.75</td>
<td>9.10</td>
</tr>
<tr>
<td>5</td>
<td>8.81</td>
<td>9.24</td>
</tr>
</tbody>
</table>

In all cases the diameter of the cells in rouleaux is greater than that of the cells when discrete. Setting aside the scarcely possible explanation that the rouleaux were formed principally from the larger cells of the preparation, we are left with the conclusion that the cell diameter is increased in the rouleaux - evidence of the compressive force which we should expect on theoretical grounds. The practical result of this finding is that it is not permissible to include cells in rouleaux when obtaining the mean diameter of cells in a sample of blood - a fact insisted upon in Part II of this Thesis.

Similar experiments to the above bring out a fact which might have been foreseen - in rouleaux whose height is equal to their base, and in rouleaux shorter than these, the compressive forces are not in evidence. There is no measurable increase in the diameter of the component cells. Under certain circumstances, the cells in such a rouleaux form am
exception to the rule just laid down, and may be included in determinations of the mean diameter of the sample. If this is done, as is sometimes convenient and occasionally necessary, the measurements of the cells in the rouleaux should be kept separate from those of the single cells of the sample, and included with them only if suitable statistical tests show that there is no significant difference in the diameters of the cells from the two sources. We have frequently shown this to be a safe procedure, if employed with caution.

Papers referred to.

Winslow, Falk, and Caufield (1924) Jour, Gen. Physiol. vi, 177.
Eggerth (1924) Jour, Gen. Physiol. vi, 587.

The paper by Winslow, Falk, and Caufield contains an extensive summary of the bibliography on potential difference.