THE KINETICS OF IN VIVO HEMOLYTIC SYSTEMS

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The purpose of this paper is to explore the possibilities of treating the contents of the blood stream as a hemolytic system in which a more or less steady state is maintained by the production of new red cells to replace those which are destroyed. The attempt will raise both new questions and questions which have been the subject of investigation already, and I realize that it will often do no more than outline the problems still to be solved. In their essentials, these problems are those of the nature of the in vivo lysins, the nature of their accelerators and inhibitors, the rules which apply to the mixtures of lysins, accelerators, and inhibitors which compose the in vivo hemolytic systems, and the kinetics which describe the way in which red cell destruction by the hemolytic mixtures is balanced by the production of new red cells.

1. The Lytic Effect of Mixtures of Lysins, Accelerators, and Inhibitors

In vivo hemolytic systems consist of mixtures of many lysins, accelerators, and inhibitors, and relations must exist between the hemolytic activity of these mixtures and the activity of the lysins acting singly, accelerated or inhibited by individual accelerators and inhibitors. One would expect the effect of the individual components to be approximately additive, although reactions between them might enhance or depress their activities. This is what seems to occur in mixtures of lysins (Ponder, 1933).

Two types of experiment are suitable for studying the combined effects of lysins, accelerators, and inhibitors. In the first the effects are obtained in systems in which lysis occurs in short times, while the second applies to systems more closely resembling in vivo systems, in which the lysin exists in small and nearly asymptotic concentration.

1. A standard time-dilution curve is obtained at 37°C. for systems containing 0.8 ml. of lysin, 0.8 ml. of 1 per cent NaCl (saline), and 0.4 ml. of red cell suspension (the thrice washed cells of 1 ml. of blood suspended in 20 ml. of saline). Systems in which the accelerators or inhibitors are present in various amounts are then prepared e.g., a system might contain 0.8 ml. of lysin, 0.4 ml. of an accelerator, 0.4 ml. of saline, and 0.4 ml. of cell suspension. If cₐ is the concentration of lysin in the standard system which takes the same time for complete hemolysis as a concentration cₐ in the system containing the accelerator, or cᵢ in the system containing the inhibitor, cₐ/cₐ = Rₐ for the accelerator, and cᵢ/cᵢ = Rᵢ for the inhibitor.
Systems containing both the accelerator and the inhibitor can be prepared by taking 0.8 ml. of lysin, 0.4 ml. of the accelerator, 0.4 ml. of the inhibitor, and adding 0.4 ml. of the cell suspension. The effect of the accelerator and the inhibitor acting together is expressed, by reference to the standard time-dilution curve, as $R_{s4}$. Innumerable ways will suggest themselves for combining accelerators and inhibitors in systems which are comparable to standard systems. The general problem is whether $R_{s4}$ is the same as the calculated net effect of the individual effects $R_{s}$ and $R_{i}$, which, because of the way in which $R$ is measured, is equal to $(R_{s})(R_{i})$.

Table I shows the result of a typical experiment at 37°C. in which the lysin is saponin, the accelerator 1 mM/l. indol, and the inhibitor plasma diluted 1 in 100. In this system the correspondence between $R_{si}$ and $(R_{s})(R_{i})$ is quite good, although the component of acceleration seems to be favored, especially in the systems in which lysis is rapid. This may be due to some such effect as the accelerator being more rapidly concentrated at the cell surfaces than the inhibitor. Speaking generally, the results obtained by this method are the same as those obtained by the method next to be described, which measures the values of $R_{s}$, $R_{t}$, and $R_{al}$ at the asymptotes of the respective time-dilution curves.

2. The asymptote of the standard time-dilution curve is found in the usual way for systems containing 0.8 ml. of lysin, 0.8 ml. of saline, and 0.4 ml. of red cell suspension. In a similar way, the asymptotes are found for (a) a system containing 0.8 ml. of lysin in various dilutions, 0.4 ml. of an accelerator, 0.4 ml. of saline, and 0.4 ml. of suspension, and (b) a system containing 0.8 ml. of lysin, 0.4 ml. of inhibitor, 0.4 ml. of saline, and 0.4 ml. of suspension. For practical purposes, the concentration of lysin which produces complete lysis in 300 minutes may be taken as the asymptotic concentration. The position of these two asymptotes and that for the standard curve determines $R_{s}$ and $R_{t}$. A series of systems containing 0.8 ml. of lysin, 0.4 ml. of accelerator, 0.4 ml. of inhibitor, and 0.4 ml. of cell suspension is then set up, and the asymptote for

<table>
<thead>
<tr>
<th>Saponin 1 in</th>
<th>Accelerator and inhibitor</th>
<th>$R_{s}$</th>
<th>$R_{t}$</th>
<th>$R_{al}$</th>
<th>$R_{calc}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>10,000</td>
<td>Indol, 1 mM/l, 0.4 ml.</td>
<td>0.70</td>
<td>1.70</td>
<td>1.92</td>
<td>1.19</td>
</tr>
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<td></td>
<td>Plasma 1/100, 0.4 ml.</td>
<td></td>
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<tr>
<td>15,000</td>
<td></td>
<td>0.73</td>
<td>1.67</td>
<td>1.06</td>
<td>1.22</td>
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<tr>
<td>20,000</td>
<td></td>
<td>0.70</td>
<td>1.60</td>
<td>1.00</td>
<td>1.12</td>
</tr>
<tr>
<td>25,000</td>
<td>Indol, 1 mM/l, 0.2 ml.</td>
<td>0.76</td>
<td>1.27</td>
<td>0.91</td>
<td>0.96</td>
</tr>
<tr>
<td></td>
<td>Plasma 1/1000, 0.2 ml.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30,000</td>
<td></td>
<td>0.78</td>
<td>1.27</td>
<td>0.95</td>
<td>0.99</td>
</tr>
<tr>
<td>35,000</td>
<td>Indol, 1 mM/l, 0.2 ml.</td>
<td>0.74</td>
<td>1.14</td>
<td>0.84</td>
<td>0.85</td>
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<tr>
<td></td>
<td>Plasma 1/100, 0.05 ml.</td>
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the time-dilution curve is found. This gives $R_{ai}$. Table II shows the results for three experiments of this kind.

When we take these results together with others (accelerators, Ponder, 1941; inhibitors, Ponder, 1943; and mixed lysins, Ponder, 1933) I think that it can be said that the behavior of mixtures of accelerators, inhibitors, and lysins is fairly well established, provided that the systems are relatively simple. The net effects can usually be obtained by appropriate calculations in terms of $R$-values, although sometimes enhancement or partial neutralization of effects occurs. When dealing with hemolysins in vivo, however, we are dealing with a mixture of unknown lysins, and in the case of any one inhibitory or acceleratory substance the question must always arise as to whether its effect on an in vitro system containing lysins such as saponin, a bile salt, or a soap, is any guide to its effect on the in vivo system of unknown composition. At present there is no way of answering this question except by direct experi-

<table>
<thead>
<tr>
<th>Lysin</th>
<th>Accelerator and inhibitor</th>
<th>$R_a$</th>
<th>$R_i$</th>
<th>$R_{ai}$</th>
<th>$R_{anel}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saponin</td>
<td>Indol, 1 mm/l, 0.4 ml.</td>
<td>0.75</td>
<td>3.0</td>
<td>2.14</td>
<td>2.25</td>
</tr>
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<td></td>
<td>Plasma 1/100, 0.4 ml.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Taurocholate</td>
<td>Same</td>
<td>0.33</td>
<td>1.5</td>
<td>0.50</td>
<td>0.50</td>
</tr>
<tr>
<td>Oleate</td>
<td>Same</td>
<td>0.30</td>
<td>2.5</td>
<td>0.75</td>
<td>0.70</td>
</tr>
</tbody>
</table>

TABLE II

II. The Nature of the Intravascular Lysins

Principally because of Rous and Robertson's observation (1917) that fragmentation plays such an important part in red cell destruction in vivo, evidence for the existence of intravascular lysins in normal blood has been less energetically sought than it might have been. Rous says "At the present time (1923) the thesis that hemolysis is concerned in normal blood destruction must be looked upon as unproven. . . . Only two methods have so far been discovered whereby worn out cells leave the circulation, namely, phagocytosis and fragmentation. . . . Both processes may quite possibly be of little importance as compared with some other unrecognized as yet. One is privileged to believe as one wishes about the matter, but scarcely to draw conclusions." Even today, it must be admitted that the evidence for the destruction of red cells
by lytic processes is not very impressive as long as we confine ourselves to normal material; if evidence obtained from pathological material is admitted, on the other hand, there never has been much doubt about the matter (see Rous's review). The weight of the evidence will appear greater, moreover, if we bear in mind that hemolytic processes may exist in vivo although the normal method of red cell destruction is fragmentation and phagocytosis. Suppose that the effective concentration of intravascular lysis is \( c_1 \) and that in this concentration lysis would take 50 days. If there is a simultaneous process of fragmentation by means of which red cell destruction is effected in 30 days, fragmentation will appear as the method of normal destruction, although the slower process of lysis is operating all the time. If the concentration \( c_1 \) were to double, however, the new concentration \( c_2 \) might be able to produce lysis in 25 days, and the destruction of the cells by the hemolytic process would occur before the destruction by fragmentation. Fragmentation and phagocytosis as the usual methods of red cell destruction are thus by no means incompatible with the continual operation of in vivo lysins, the effects of which may become prominent if their concentration increases.

The three normal in vivo hemolytic processes for which there is substantial evidence involve (1) the action of the soaps in chyle after a meal rich in fat, (2) the action of the spleen in modifying red cells so as to aid in their destruction, and (3) the hemolytic effect of substances derived from tissues such as lung, liver, and spleen.

1. Freeman and Johnson (1940), and Loewy, Freeman, Marchello, and Johnson (1943), have shown that the soap content of the chyle rises to 3 to 6 mg. per ml. during rapid fat absorption, and that this quantity of soap is sufficient to bring about a certain amount of hemolysis when it enters the bloodstream. During fasting, soaps are found in amounts too small to cause experimentally demonstrable hemolysis, but when the individual is eating an ordinary diet their continual action probably provides a normal mechanism for red cell destruction. Again it should be emphasized that the important thing from the standpoint of this discussion is the existence of the in vivo hemolytic system, even although the concentration of lysin in it is not sufficient to hemolyze a single cell before the cell's life is terminated by other processes (fragmentation, phagocytosis, etc.). If the hemolytic system exists normally, there are always the possibilities (a) that the concentration of lysin will increase and that intravascular lysis will become an important mode of cell destruction, and (b) that in the presence of an accelerator, or because of the reduction in the concentration of an inhibitor, the lysin will become more effective than it was previously. Whenever one can show that an increase in the concentration of a normally occurring blood constituent results in intravascular hemolysis, I think that one can assume that a normal in vivo hemolytic system exists; Johnson and his collaborators have done this for the soaps, and by the same sort of
reasoning there is satisfactory evidence for the existence of a hemolytic system containing the bile salts.

2. The evidence for the spleen being an organ in which red cells are destroyed or prepared for destruction has been reviewed by Rous (1923), by Krumbhaar (1926), and more recently by Dameshek (1941, 1942). The frequent observation that red cell resistance to certain forms of lysis is increased in vitro after splenectomy was shown by Gordon and Kleinberg (1937) to correspond to an increased resistance to destruction in vivo in the guinea pig, and the increased resistance to hypotonic saline is due to the cell being able to attain a greater volume before it hemolyzes (Gordon, Kleinberg, and Ponder, 1937). The removal of the spleen is accordingly followed by an alteration in the structure of the red cell, and the increase in resistance to lysis by taurocholate (although not to lysis by saponin) points to the same conclusion. After splenectomy in man the alteration in structure is accompanied by a change in shape, the red cell population containing an increased number of flat cells ("platycytes," some of which may even be "target cells," Singer, Miller, and Dameshek, 1941). The reason for these shape changes is still obscure, but according to the view which prevails at the moment, the spleen produces substances which tend to make red cells less flat and more spheroidal, the continued action of which may eventually produce spheres and hemolysis. Removal of the spleen with its spherocyte-producing substances thus results in a less spheroidal, or flatter, population. The possible nature of these substances has been dis-

1 An extreme statement of this point of view would be that the red cell is originally produced as a very flat body ("platycyte") with an $a/b$ ratio of, say, 6 to 1, that as a result of the continued action of substances produced by the spleen and perhaps by other tissues it "tends to spherocytosis" by becoming less and less eccentric as it grows older, and it finally becomes a spherocyte and hemolyzes. The whole process would have to take weeks, starting in the marrow where the youngest red cells, although not yet in circulation, would nevertheless be exposed to the action of circulating lysins, and the mean diameter and thickness of the cell as ordinarily measured would correspond to the diameter and thickness of the cell of mean age, thinner cells being younger and thicker cells older. So far as the kinetics of intravascular hemolysis are concerned, there is no objection to this hypothesis, which would correspond very well to the transformation of $S$ to $S'$ described in section IV, provided that the cell shape were a function of $S$ or $S'$. The objection to it is that lysins do not usually convert red cells into spheres with a series of biconcave forms of varying eccentricity as intermediates. At least, when studied in vitro, the intermediates are crenated or thorn-}

apple forms (Ponder, 1935, 1942), and these are not often seen in the circulation. It is possible that a lysin might produce regular forms of diminishing eccentricity instead of crenated forms if it were to act slowly enough, but until such a phenomenon is shown to exist the chain of evidence is incomplete. (Some evidence for it may be found in a paper by Tigertt, Duncan, and Hight (1940), but the red cell diameters were measured in dried films.) One might put forward a hypothesis to the effect that the red cell is
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cussed by Singer (1940, 1941), Singer, Miller, and Dameshek (1941), Ham and
Castle (1940), and Dameshek and Schwartz (1938). Bergenhem and Fahraeus
(1936) have shown that serum contains an enzyme which splits off a substance
from the serum lipoids after some hours in vitro; this substance can be adsorbed
on the red cell, and produces a change from the biconcave to the spherical form.
The enzymatic process proceeds most rapidly at 42°C. and at pH = 7.2, and
is inactivated at 56°C. Bergenhem and Fahraeus have identified the substance
as lysolecithin, small amounts of which produce spherocytosis and larger
amounts of which produce hemolysis (cf. the action of lecithin, Ponder, 1935,
1942). It is possible that the spherocyte-producing substance of the spleen is
actually lysolecithin. 3

3. Maegraith, Findlay, and Martin (1943a, b) have recently described a
lytic agent, which they think is an erythrocytase, in a variety of human and
animal tissues. Its presence can be demonstrated by adding small pieces of
washed tissue to a washed red cell suspension and incubating for 24 hours at
initially a very flat body, that the continued action of lysins from the spleen and else-
where makes it less and less eccentric, but that fragmentation normally occurs before
the stage of crenation and sphere formation is reached. This would account for the
absence of crenated and spherical cells from normal blood, and only when there was
an increase in the activity of in vivo hemolytic activity might we expect to see circu-
lating spherical forms and the forms (spheroidal or crenated) preceding them. This
would be an interesting working hypothesis, but I think that it should be borne in
mind that the spleen may also control the shape of the red cells as produced at their
source.

A different mechanism by means of which decreased eccentricity may be produced
is that described by Ham and Castle (1940). Under conditions of stasis, such as
probably occur in the spleen, red cells swell as a result of changes in permeability which
follow on the accumulation of metabolic products, and in swelling they tend to become
first less eccentric and later spherical. This change in form is well recognized (Castle
and Daland, 1937; Ponder, 1937), and is due to quite a different mechanism from that
responsible for the disk-sphere transformation produced by lysins like lysolecithin.
While it might be effective in producing red cell destruction, especially if the a/b
ratio for the cell is less than normal, it is not necessary to think of it as a mechanism
which regulates the mean shape of the red cell in the circulating blood.

2 Great caution has to be exercised in identifying substances extracted from
blood and tissue, and the fact that a lytic substance can be extracted cannot be taken
as evidence that it exists, as a lysin, in the blood or tissue in vivo. A great number of
fatty acids, soaps, and related substances can be obtained by various extraction
processes, but before extraction these are probably combined with other tissue com-
ponents in such a way as to render them non-lytic. The apparent amount and nature
of these extractable substances depends on such details as the temperature and pH at
which the extraction is carried out, and different mixtures of substances, some lytic,
some inhibitory, can be obtained by using different concentrations of alcohol; e.g.,
50 per cent, 75 per cent, and so on.
37°C. The lysin is described as being species specific, destroyed by heating to 80°C. for 5 minutes, and inhibited by plasma in dilutions as great as 1 in 1000. It seems to bear a relation to the tissue lysins originally described by Metchnikoff, of which an excellent account was given by Well in 1907. Most of Well's work was done with kidney tissue, freed from blood by perfusion, but he also used a variety of tumor tissue, some of which was necrotic. He found that normal tissue, if chopped up in 10 times its weight of saline and stirred for some hours, yields substances in saline extract which are hemolytic for the washed red cells of the same animal (2 hours at 37°C., followed by 18 hours in the ice box; Well did not ascertain whether the lysin hemolyzed the cells of other species as well as the cells of the same species). Liver and kidney extracts were found to contain a variable amount of lysin, which is activated by the addition of red cell stromata, inhibited by serum, and which can be shown to become bound to the surfaces of the red cells of the system. Well points out, however, that the situation is more complex than appears at first sight, for some of the lytic substances which appear in extracts of incompletely washed tissues are derived from the residual blood. Extracts of non-necrotic tumors act like extracts of normal tissues, but necrotic tissue contains entirely different lytic substances in addition. These are diffusible, and are presumably products of necrosis; Well suggests that the frequent anemia which accompanies malignancy in its later stages may be due in part to the hemolytic action of these products of necrosis.

I have had no difficulty in confirming Maegraith, Findlay, and Martin's results in so far as the lytic effect of pieces of tissue is concerned. Most of my material has been either rat lung or human tissue removed in the operating room. All procedures, including the removal of the tissue, must be carried out aseptically. The tissue is cut into pieces about 4 mm. by 2 mm., and these are washed several times in saline. Several of the pieces are transferred to each of a series of test tubes, to which 2 ml. of a suspension of washed red cells are added; the cells may be those of the same species or of another species, and buffers, inhibitory agents, etc., can also be added as the experiment requires. The tubes are kept in a water bath at 37°C. with occasional shaking, and the results are read after 18 to 24 hours. Lysis rarely begins before 12 hours have elapsed, but proceeds fairly rapidly once it starts. The existence

3 These experiments must be carried out with the strictest aseptic precautions, and the hemolytic systems must be shown to give no growth on blood agar plates at the time the results are read. I have found this degree of sterility very difficult to attain, for animal tissues often contain organisms which grow under the almost ideal conditions of incubation for 24 hours at 37°C. in the presence of autolyzing tissue, and hemolytic contaminants can be derived even from the air, at least in Mineola. One large gram-positive "hay bacillus" is strongly lytic on culture under the conditions of these experiments.
of such a long “latent period” in itself suggests that the lytic material is derived from precursors in the tissue.

The lysin (or lysins) does not diffuse through either collodion or cellophane, and is destroyed by heating to 60°C. for 5 minutes. It has a pH optimum at about 7.1; at pH = 6.5 and 8.1 (phosphate buffers) some lytic activity can be observed, but at pH 4.5, 5.8, and 8.5 the lysin is apparently inactive. These observations are in agreement with those of Macraith, Findlay, and Martin. The point upon which I cannot agree with them is that of the species specificity. I have obtained lysis of human cells with pieces of rat lung and lysis of rat cells with pieces of human thyroid, and, in general, have not found that the lytic material affects the cells of the same species only.  

The absence of species specificity puts the matter in a different light, for if we are dealing with a non-specific lysin we have to consider the possibility that it is lysolecithin or a substance related to it. If pieces of rat lung, etc., which prove to be lytic after 12 to 24 hours are incubated at 37°C. in saline for 12 hours, quite large amounts of lysolecithin (or, more properly, the substance identified as lysolecithin) can be extracted from them by the process described by Singer (1940) for the extraction of lysolecithin from serum. The amount is about the same as that contained in normal human plasma, and it is sufficient to produce lysis of a suspension of washed red cells within the second 12 hours of the 24 hour experiment. Since lysolecithin is produced by an enzymatic process with a pH optimum of about 7.2, both the heat sensitivity of the tissue lysin, the effect of pH on it, and, indeed, all of its properties which I have observed, would be explainable on the assumption that it is lysolecithin or a related substance. If so, lysolecithin is probably a normal intravascular lysin produced by the tissues in a wide-spread manner, and not in the spleen only. Its lytic activity would be dependent on its concentration in the plasma, together with the concentration and effectiveness of its plasma inhibitors.

An apparent specificity may be due to the cells of one species being less resistant to a lysin than the cells of another; e.g., lysolecithin, in sufficient dilution, would appear to be specifically hemolytic for dog cells if its effects were observed on suspensions of the red cells of man and of the dog. It is possible, of course, that both species-specific and non-species-specific lysins are present at the same time, and that sometimes the former are so active that the effects of the latter pass unnoticed by comparison. I have never observed this state of affairs. The question as to the species specificity of the tissue lysins played quite an important part in the early history of the subject (see Weil, 1907), and it was ultimately decided that the lysins then under discussion, at any rate, were non-specific.

The hemoglobin of the hemolytic system may be converted to a brownish derivative both by the tissue lysins and by the extracted substances.

A real difficulty, however, lies in the smallness of the quantity of lysolecithin which can be extracted from plasma by Singer’s method. If one dissolves the lytic
III. The Nature of the Plasma Inhibitors

The nature of the inhibitory substances contained in plasma and serum has been under investigation ever since the inhibitory effects were first described. Both the plasma proteins and the plasma lipoids are inhibitory, and the first problem is to find how much of the inhibition is due to the former and how much to the latter. This is a point on which observers have held different views, some attributing most of the inhibition to the proteins, and others believing that the lipoids are principally responsible. The second question is whether there are diffusible inhibitors in addition, for a powerful inhibitor of digitonin hemolysis, the quantity of which is stated to be dependent on the intake of thiamine, has been recently described (Farley, 1939, 1942; Horwitz and Farley, 1940).

1. Inhibition Produced by Plasma Proteins and Lipoids.—There are two ways in which we may identify the inhibitory substances and determine their inhibitory powers. The first is to fractionate the plasma and to measure the inhibition produced by the various fractions; the second is to start with pure substances, such as serum albumin, fibrinogen, cholesterol, etc., in known concentration, and to compare the inhibitory effect of each with that of plasma as a whole.

All the inhibitory substances of plasma seem to be non-diffusible, and I have been unable to obtain evidence for the existence of the diffusible inhibitors described by Farley (see below).

A partial separation of the constituents of plasma can be effected by adding dilute acetic acid to plasma diluted 1 in 10 with saline, until the pH is about material extracted from 2 ml. of normal human plasma, after incubation at 37°C. for 24 hours, in 2 ml. of saline, one obtains a solution 0.2 ml. of which usually hemolyzes 0.1 ml. of standard red cell suspension (the thrice washed cells of 1 ml. of human blood finally suspended in 20 ml. of saline) in 24 hours in vitro at 37°C. Now Ponder, Hyman, and White (1941) and Ponder and Hyman (1943) have shown that the activity of a lysin in a system containing whole blood is very much less than its activity in an in vitro system such as this, even when allowances are made for the maintenance of the lysin at a constant level, as it is maintained in vivo. This relatively small activity in the system containing whole blood is due to the greater concentration of red cells, to some of the lysin being used up in reacting with the vessel walls, and to the action of the plasma inhibitors. The inhibitory power of normal human plasma for lysolecithin is such that plasma diluted 1 in 100 gives an R-value of between 1.5 and 2.0, and so is of the same order as the inhibitory power for saponin or digitonin hemolysis. The extracted lysolecithin of plasma, a feeble lysin even in vitro, would therefore be so much feebler in vivo as to raise doubts as to its ability to produce an appreciable degree of intravascular lysis. Alternatively, Singer’s method may extract only a small fraction of the lysolecithin present in plasma, or may extract inhibitors along with it.
5.0, heating for 15 minutes in boiling water, filtering through paper, and neutralizing to pH 7.0 with 0.1 n NaOH. The removal of protein is very complete, and the inhibitory power of the filtrate for saponin and digitonin hemolysis is only from one-tenth to one-twentieth that of the original plasma. A variable and surprisingly large amount of lipoid, however, is carried down with the flocculated protein. Not realizing this in 1923, I thought that the result of this separation was evidence that the greater part of the inhibition is due to the plasma proteins, and it was not until recently that I found the parallelism between the inhibitory effect and protein content to be very incomplete (Ponder, 1943). By means of the same separation procedure, however, it can be shown that only about one-quarter to one-third of the total inhibition of saponin (or digitonin) hemolysis is due to plasma cholesterol. In a typical experiment, the inhibitory power of the plasma for saponin hemolysis is about 16 times that of the protein-free filtrate, and the amount of cholesterol in the diluted plasma is about 3 times that left in the filtrate. From these ratios, it appears that the plasma protein and the inhibitory substances other than cholesterol which are carried down with it are responsible for about 77 per cent of the total inhibition, while the cholesterol accounts for the remaining 23 per cent. In different experiments, the figure varies from 20 to 35 per cent.

Through the kindness of Dr. E. J. Cohn, Dr. H. B. Vickery, and Dr. Hans Neurath, I have obtained a number of plasma protein fractions in a state of known purity, and have measured their inhibitory power for different lysins. In each case the protein, dissolved in a phosphate buffer saline at pH 7.1, was introduced into a hemolytic system containing 0.8 ml. of lysin, 0.4 ml. of protein in known concentration, 0.4 ml. of saline, and 0.4 ml. of red cell suspension, and R-values were found in the usual way. The principal results are shown in Table III, which gives the R-values together with the concentrations of protein for which each was obtained.

It will be clear from Table III that none of the protein fractions has an inhibitory power of the same order as that of plasma itself. The most inhibitory preparation is the mixed globulin Fraction II + III, and this has only 10 to 15 per cent of the inhibitory power of whole plasma. The horse serum albumin and pseudoglobulin, the beef globulin, and the fibrinogen preparations exhibit their inhibitory effect, if any, in 1 per cent solution, and so their inhibitory power is less than 1 per cent of that of whole plasma. Since the sum of the inhibitory effects of the protein fractions does not amount to more than 25 per cent of the total inhibition, and since the cholesterol content ac-

It is quite likely that purification of the protein fractions alters their physical state in such a way that part of the inhibitory power is lost. Both Tsai and Lee and I have noticed that merely drying the plasma reduces its inhibitory power by 20 to 30 per cent, and Tsai and Lee attribute this to denaturation. Since some of the inhibitory effects appear to take place at the red cell surfaces rather than in the bulk
counts for only about 25 per cent, some 50 per cent of the inhibition has so far remained unaccounted for. This is also the conclusion reached by Tsai and Lee (1941).

The position has recently been greatly clarified by the investigations of Lee and Tsai (1942 a, b; and see also Tsai and Lee, 1941). They have approached the problem by studying the effect of cholesterol and lecithin sols on hemolysis by different lysins (saponin, digitonin, bile acids and salts, oleate, etc.), and have found that lecithin, although lytic per se, enhances the inhibitory effect of cholesterol 3 to 10 times in saponin systems. This enhancement depends on the value of the lecithin/cholesterol ratio as well as on the concentration of saponin, and seems to be due to an interaction between the lecithin, the cholesterol, and the saponin. A similar reinforcement is observed in systems containing digitonin or the bile salts. In addition to this, Lee and Tsai find that lecithin per se is inhibitory in saponin, digitonin, and crude bile acid systems, although not in systems containing the bile salts or the oleates. Their evidence is sufficient to lead to the conclusion that the 50 per cent of the system it would not be surprising to find that small changes in physical characteristics would produce appreciable changes in inhibitory power. The process of purification, moreover, eliminates the enhancing effects of one component on another, and it is very doubtful if the sum of the inhibitions of the purified components of plasma, taken separately, would equal the inhibitory power of plasma itself. On the other hand, all the inhibitory substances acting together would probably produce a greater inhibition than that of whole plasma, in which some of the effect of inhibitors is offset by the opposed effects of accelerators.

### TABLE III

<table>
<thead>
<tr>
<th>Substance</th>
<th>Concentration</th>
<th>Saponin</th>
<th>Digitonin</th>
<th>Taurocholate</th>
<th>Oleate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human serum albumin*</td>
<td>0.25</td>
<td>0.95</td>
<td>0.92</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Human serum globulin fraction II + III†</td>
<td>0.02</td>
<td>1.11</td>
<td>1.14</td>
<td>1.12</td>
<td>1.20</td>
</tr>
<tr>
<td>Horse serum albumin§</td>
<td>1.00</td>
<td>0.95</td>
<td>1.35</td>
<td>1.80</td>
<td>1.45</td>
</tr>
<tr>
<td>Horse serum pseudoglobulin¶</td>
<td>1.00</td>
<td>1.00</td>
<td>1.45</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Human fibrinogen</td>
<td>1.00</td>
<td>1.10</td>
<td>1.17</td>
<td>1.14</td>
<td>1.20</td>
</tr>
<tr>
<td>Beef serum albumin¶</td>
<td>1.00</td>
<td>1.35</td>
<td>1.00</td>
<td>1.75</td>
<td>1.35</td>
</tr>
</tbody>
</table>

* Contained 1 in 1500 merthiolate, which gives R = 0.92 with digitonin. 97 per cent pure by electrophoresis, the balance being globulins.
† Also contained merthiolate. A mixture of gamma and beta globulins.
§ Electrophoretically homogeneous.
¶ Electrophoretically homogeneous.
¶ Contained 4 per cent globulin impurities.
inhibitory effect of plasma, which has so far remained unaccounted for, may represent the inhibitory effect of the plasma lecithin together with the enhancement which this substance confers on the cholesterol inhibition. The sum of the inhibitions produced by protein, cholesterol, and lecithin, acting singly, together with a generous allowance for enhancing effects, is thus enough to account for the whole inhibitory effect of plasma.° The complete elucidation of the enhancing effects will probably turn out to be very difficult, and will have to take account of the fact that some of the lipoids and proteins of plasma are actually chemically combined (Bloor, 1943, p. 185).

2. “X-Substance,” “OBT Principle,” etc.—Farley (1939, 1942) and Horwitz

°In connection with these inhibitory effects, Lee and Tsai have raised the old question as to whether the inhibition takes place in the bulk of the system, i.e., between inhibitor and lysin, or at the red cell surface. There are two distinct mechanisms by means of which inhibition can be produced. In the first, a reaction occurs between the lysin and the inhibitor, so that a quantity Δ of the lysin is rendered inert, the original concentration of lysin c₁ falling to a lower concentration c₂, and Δ being equal to c₁ - c₂. In this case the added cells act merely as an indicator of the amount of free lysin. In the second, the inhibitor acts in the neighborhood of the cell surfaces, producing some kind of over-all effect which increases the resistance of the cells to the lysin R times, where \( R = c₁/c₂ \). These two mechanisms may be referred to as the \( \Delta \)-mechanism and the \( R \)-mechanism respectively, and which of the two is operative in any given case requires to be decided on experimental grounds.

In the case of the inhibition of hemolysis (saponin, digitonin, crude bile acid, and natural hemolysin) by cholesterol sols, Lee and Tsai have shown that the inhibition increases with time and temperature when lysin and cholesterol react in the absence of red cells. This is good evidence for a \( \Delta \)-mechanism. When cholesterol is added to lecithin, oleates, or the bile salts, and when lecithin is added to saponin, digitonin, crude bile acid, the bile salts, and the oleates, the inhibition observed when cells are added subsequently does not depend on the conditions of either time or temperature under which the cells and the inhibitor have stood together. Lee and Tsai propose that in these cases the inhibitors react with the lysins in a physical rather than in a chemical manner; there remains, however, the possibility that the inhibition is primarily due to an \( R \)-mechanism.

Both mechanisms are probably coexistent even in the simpler forms of inhibition. 80 per cent of the inhibition in arginine-saponin systems and 90 per cent of that in sucrose-saponin systems, is the result of an effect on the cells themselves, but the remainder may be the result of a reaction of the inhibitors with the lysin (Ponder, 1926). Yeager and I (1928) concluded that the inhibitory effect of the sugars is a double one both in saponin-sugar-cell systems and in taurocholate-sugar-cell systems, an \( R \)-mechanism being the more prominent in the case of the former, and a \( \Delta \)-mechanism in the case of the latter. I have recently emphasized the effects of the plasma inhibitors at cell surfaces, or the \( R \)-mechanism (Ponder, 1943) partly because in the past I have treated this form of inhibition almost exclusively as a \( \Delta \)-mechanism, or interaction between lysin and inhibitor. It seems likely, however, that both mechanisms are involved.
and Farley (1940) have described an inhibitor of digitonin hemolysis which they call “X-substance,” or “OBT principle.” This inhibitor is stated to be present in plasma in quantities which depend on the intake of thiamine or on the thiamine level, and Farley has proposed that the inhibitory effect of plasma on digitonin hemolysis be used as a method for thiamine assay. The inhibitory power of rabbit plasma is reported to be enormously increased by the daily administration of 100 mg. of thiamine subcutaneously for 7 days, plasma initially inhibitory in dilutions of about 1 in 2000 becoming detectably inhibitory in dilutions of about 1 in 1,000,000. Farley believes that the inhibitory substance is of low molecular weight, and related to, if not identical with, another inhibitory substance which he has obtained in crystalline form from tissues (“vitatropin”).

I have been unable to confirm these results, either as regards the presence of a diffusible inhibitor or as regards the alleged effect of thiamine on the inhibitory power of serum on digitonin hemolysis. No measurable quantity of inhibitory substance diffuses from serum or plasma through either collodion or cellophane, and I have not obtained any significant increase in the inhibitory power of serum or plasma on digitonin or saponin hemolysis either in man or in the rabbit. In one experiment I gave each of 5 rabbits 100 mg. of thiamine chloride subcutaneously daily for a week. The inhibitory values for the sera, measured both by Farley’s technique (1942) and by the method described in part 3 of this section, were substantially unchanged. In another experiment 6 persons received 50 mg. of subcutaneous thiamine daily for a week, and in 2 persons the injections were continued for 2 weeks. No changes in the inhibitory power of the sera were observed, other than the small differences which may occur from day to day in the untreated animal.

3. Variations in the Inhibitory Power of Plasma.—Very few investigations have been made on the variations in the inhibitory power of plasma. When investigating them, one may use saponin, digitonin, one of the bile salts, or any other lysin as the lysin to be inhibited, and the results found with one lysin need not necessarily be the same as those found with another. At this stage of our knowledge, however, we can suppose that a general idea of the variations in the power of inhibiting intravascular lysins will be provided by measurements of the power of inhibiting hemolysins such as saponin and digitonin in vitro. These lysins, being very stable, are more suitable for quantitative work than are the bile salts or the soaps.

A solution of digitonin (or saponin) is prepared, and decreasing amounts,

9 Dissolve 100 mg. of Merck’s digitonin in 1000 ml. of saline. There seems to be some doubt about the solubility of this substance, which takes a considerable time to go into solution. The exact concentration is not important, but one wants it to be such that 1 ml., made up to 1.6 ml. with saline, gives complete lysis of 0.4 ml. of standard cell suspension in about 15 seconds. If lysis is more rapid, the stock solution can be diluted appropriately. It keeps well in the refrigerator.
1.2 ml., 1.1 ml., ... 0.5 ml., are added to a series of tubes. The volume of each is made up to 1.6 ml. with saline, and the tubes are placed in a water bath at 37°C. To each is added 0.4 ml. of a red cell suspension (the thrice washed cells of 1 ml. of human blood suspended in 20 ml. of saline) and the time for complete hemolysis is determined. The end point with digitonin and human cells is very sharp. Plotting the quantity of lysin used against the time required for complete lysis gives a time-concentration curve. This curve is not always the same for suspensions made from day to day from the blood of the same individual, the small variations observed apparently reflecting small variations in the resistance of the cells to digitonin, such as are also found in the resistance of cells to saponin hemolysis (see section IV).

The sera are obtained by drawing blood from the finger into Wright's tubes and centrifuging. Each serum is diluted 1 to 500 with saline (20 mm. to 10 ml.). To each of a series of tubes are added 1.2 ml., 1.1 ml., ... 0.5 ml. of digitonin, 0.4 ml. of the diluted serum, and enough saline to bring the volumes up to 1.6 ml. The tubes are allowed to stand for a measured time (usually 1 hour) at room temperature (25°C.), and the time for complete lysis of 0.4 ml.

As in the case of saponin, the asymptotic concentration of digitonin required for complete hemolysis increases with increase in temperature. Thus at 37°C. complete lysis is brought about by 0.08 ml. of diluted stock solution of digitonin; at 30°C., 0.09 ml. is required, at 35°C., 0.10 ml., and at 40°C., 0.11 ml. The sharpness of the end point becomes gradually less as the temperature rises.

The inhibition of digitonin hemolysis by serum and plasma increases with the length of time during which the lysin and the inhibitor are allowed to stand together before the completion of the hemolytic system by the addition of the cells. This seems to be the result of a reaction between cholesterol and other components of the plasma and the lysin. The time course of this reaction has been studied as a function of temperature by Lee and Tsai (1942b) for systems containing a variety of lysins. They have found that the inhibitory action of cholesterol for saponin and digitonin hemolysis is increased by an increase in temperature, whereas that for bile salt and soap hemolysis is not; the inhibitory effect of lecithin is not affected by temperature either. The time course of the reaction between inhibitor and lysin is roughly exponential, being rapid at first and complete only after some hours. In the case of the inhibition produced by plasma, the temperature effect is small and the inhibition actually decreases with rise of temperature (Ponder, 1943). The time effects are also less conspicuous than in the case of inhibition by cholesterol sols, a plasma-digitonin system which gives an R-value of 1.37 when the plasma and the digitonin are allowed to stand together for 3 minutes before the cells are added giving an R-value of 1.43 when the reaction is allowed to go on for 30 minutes, and an R-value of 1.51 when it continues for 3 hours. It should not be concluded from this, however, that the inhibition is entirely due to a reaction between the inhibitor and the lysin in the bulk phase of the system (see footnote 8).
of cell suspension at 37°C. is then found. By comparing these times with those on the standard curve, a series of R-values are obtained in the usual way, and these, or their average, measure the inhibitory power of the serum.

Proceeding in this way, we can make a number of observations.

(a) The inhibitory power of the serum of normal individuals varies from day to day, e.g. the following R-values were found for one person on 12 consecutive days: 1.39, 1.38, 1.35, 1.37, 1.29, 1.24, 1.36, 1.39, 1.44, 1.30, and 1.22. There seems to be a cyclic character to the variations, the values decreasing for a few days and then increasing again. The variation in the R-value for the inhibitory plasma from 1.20 to 1.44 would have the same effect in the intact animal as a ±10 per cent variation in the lysin concentration in the bloodstream, and so would correspond to a variation of ±10 per cent in the life of the average red cell.

(b) The inhibitory power of plasma is not highly correlated either with the cholesterol content or with the plasma protein content. In a series of 25 observations, the coefficient of correlation between the R-value and the cholesterol content was found to be 0.57 ± 0.14, while that between the R-value
and the plasma protein content was 0.55 ± 0.14. This result is to be expected in view of Lee and Tsai’s demonstration that the inhibitory effects are very complicated.

(c) Nevertheless, sharp changes in the cholesterol content or in the plasma protein content are usually associated with changes, in the same direction, in the $R$-value, e.g. an $R$-value of 1.33 was found on one day associated with a cholesterol content of 230 mg. per cent, and a fall in the cholesterol content to 138 mg. per cent on the following day was accompanied by a decrease in the $R$-value to 1.23 (the plasma protein content remaining constant at 7.0 gm. per cent). This associated variation would result from the cholesterol being responsible for part of the inhibitory power, but it may be contributed to by the same factors which bring about the change in the cholesterol content also bringing about a simultaneous change in the concentration of other inhibitors.

(d) Under conditions of poor nutrition and after major surgical operations, when the concentration of protein and cholesterol in the plasma is usually lowered, the inhibitory power of the plasma is very often reduced. I have already suggested that this reduction in inhibitory power, which would operate in the direction of favoring red cell destruction by intravascular lysins, may contribute to those postoperative anemias which are out of all proportion to the amount of blood lost (Seaman and Ponder, 1943).

IV. The Steady State and Hemolysis in Vivo

Suppose that the membrane of the least resistant red cell in a hemolytic system contains an amount of a component $S$ and that the lysin acts irreversibly on $S$ to convert it into another substance $S'$,

$$ S + x \rightarrow S' $$

So long as a certain amount of $S$ remains in the membrane, the semipermeability remains complete, but when a certain fraction of $S$ is converted into $S'$ the semipermeability is lost.\(^{14}\) Suppose that this loss occurs when $S'$ has the

\(^{12}\) I have not been able to obtain as high a correlation between the cholesterol content of plasma and its inhibitory power as some investigators report. Tsai and Lee (1941) give a correlation coefficient of 0.922 for the inhibitory effect and the cholesterol content, and one of 0.267 for the inhibitory effect and the protein content. Yi and Meng (1941) also conclude that cholesterol is almost wholly responsible for the inhibitory effects. My experience agrees more closely with Lee and Tsai's later view (1942) that the total inhibitory effect of plasma is more complex than that produced by cholesterol alone.

\(^{14}\) For simplicity's sake, one can think of the lysin as digitonin, and as combining with the cholesterol $S$ in the cell membrane to form cholesterol digitonide $S'$, but the situation is not as simple as this, and, except as an illustration, the idea is probably
particular value \( S'_0 \); the loss of semipermeability is the result of the breakdown of the membrane at its weakest or least resistant "key spots" (Ponder, 1941), and is followed by an all-or-none hemolysis. The transformation of \( S \) to \( S' \) corresponds to the utilization of a quantity of lysin \( x_0 \), and the resistance of cell will be properly defined by this quantity \( x_0 \). Another cell of greater resistance \( x_1 \) will not become semipermeable until a greater quantity of lysin \( x_1 \) is used up in forming compound \( S' \) in greater amount \( S'_1 \); a cell of still greater resistance will not become semipermeable until the still greater quantity \( x_2 \) of lysin has formed the still greater quantity of compound \( S'_2 \), and so on. If there are \( N \) cells of varying resistance in the system, the distribution of the resistances, each measured in terms of the quantity of \( x \) necessary for the breakdown of semipermeability, will be described by some form of frequency distribution; i.e., by

\[
N = F(x)
\]

In an \textit{in vivo} hemolytic system a concentration of lysin \( c \) is maintained at a constant level, the lysin used up in reacting with \( S \) to form \( S' \) being constantly replaced by fresh lysin, and so the rate of utilization is constant and proportional to \( c \), or

\[
\frac{dx}{dt} = kc
\]

and

\[
t = \frac{x}{kc}, \quad \text{or} \quad x = kc \cdot t
\]

Let the line AB in Fig. 1 represent the abscissa of the frequency distribution of the cells which are being added to the circulation from the hematopoietic tissues. The distribution itself must be imagined in a plane at right angles to the paper, a total number \( P \) being distributed into \( P_0 \) cells of least resistance \( x_0 \), \( P_1 \) cells of resistance \( x_1 \), ..., \( P_m \) cells of mean resistance \( x_m \), ..., up to \( P_g \) cells of greatest resistance \( x_g \). Alternatively we can think of the cells as being distributed according to the amount of the component \( S \) which each contains; thus the cells of resistance \( x_0 \) contain an amount of the component \( S_0 \) and hemolyze when this is all transformed into \( S' \). And so on for the groups \( x_1 \) ..., \( x_m \) ..., \( x_g \).

At \( t = 0, x = 0 \), but as \( t \) increases greater and greater quantities of lysin \( x \) combine with the cell component. The increase of \( x \) with \( t \) is represented along the line BC. Consider the group of resistance \( x_0 \), starting when \( x = 0 \) at \( t = 0 \). As \( x \) increases with \( t \), the component \( S_0 \) becomes transformed into \( S' \), misleading. Many lytic substances have no affinity for cholesterol of the same sort as digitonin has; \textit{i.e.}, do not form known compounds with it, and Tsai and Lee have remarked upon the fact that cholesterol appears to be inhibitory for lysins in general, largely irrespective of their chemical nature.
and the amount of \( S_0 \) which remains is always \((x_0 - x)\) until the quantity \( x_0 \) is combined at the time \( t_0 \). Then \((x_0 - x_0) = 0\), and the cell hemolyzes. This is expressed in Fig. 1 by moving the cells of resistance \( x_0 \) along the line AD, parallel to the time axis, until they reach the line DC, on which the quantity of component transformed \((x_0\) on the axis BC) is equal to the quantity of component initially present \((x_0\) or \( S_0 \) on the axis AB). Beyond the line DC the cells cease to exist. In this case the length of the life of the cells of resistance \( x_0 \) in the blood stream will be \( t_0 \).

Applying the same reasoning to the cells of group \( x_1 \) or to those of any other group, it will be apparent that the course of events is described by moving the percentage distribution of \( P \), erected on the line AB, along the line BC. As it moves, the distribution will at first remain intact, bounded on the one side by the line AD and by BC on the other. At time \( t_0 \), however, \( x \) on BC will become equal to \( x_0 \) on OB, and the least resistant cells of the distribution, the group \( x_0 \), will hemolyze. Next the group \( x_1 \) will hemolyze when \( t = t_1 \) and \( x = x_1 \) on BC, and so on for all the other groups \( x = x_m \). Thus as the moving distribution passes over the line DC, that part of it to the left or outside DC will be hemolyzed (e.g., when the distribution has reached the position EF), and that part of it to the right or inside DC will be intact. Eventually all of the distribution will lie to the left of DC and even the most resistant cells \( x_9 \) will have hemolyzed. Throughout, the length of the life in the blood stream of any group of cells is the distance from AB to DC, measured parallel to the time-axis; i.e., the groups \( x_0, x_1, \ldots, x_m, \ldots, x_9 \) have life's \( t_0, t_1, \ldots, t_m, \ldots, t_9 \).
Now the solid enclosed as the frequency distribution \( P \), originally at \( AB \), moves over the surface \( ABCD \) will represent the total number of cells \( N \) in the circulation at any one time. The form of the frequency distribution of \( N \) can be found at any moment by appropriate sampling, experiment, and analysis (see Ponder, 1934, p. 161). In the steady state, a number of cells \( Q \) must be destroyed to balance the number \( P \) which is added in every interval of time, so \( Q = P \), and the distribution of \( Q \) must be identical with the distribution of \( P \). It will be clear, however, that in order to maintain a given distribution of the number \( N \) in the entire solid, the distributions of \( P \) and \( Q \) cannot be the same as that of \( N \), for \( P \) must contain a greater proportion of the short lived elements and a smaller proportion of the long lived elements than \( N \) does. More specifically, if there are \( N_0 \) cells in the group \( x_0 \) in the distribution of \( N \), the number \( P_0 \) of group \( x_0 \) in the distribution of \( P \) will be proportional to \( N_0/t_0 \), \( t_0 \) being, as before, the life in the circulation of the cell of group \( x_0 \). Similarly, \( P_1 = N_1/t_1 \), ... \( P_m = N_m/t_m \), ... \( P_n = N_n/t_n \).

Several conclusions now follow from these relations between the distributions \( N \), \( P \), and \( Q \).

1. The distribution of new cells necessary for the maintenance of the population \( N \) in its steady state is more negatively skew than the distribution of the population \( N \) itself.

2. If we withdraw a sample of the population \( N \) and determine the resistance by adding a hemolysin, by reducing the tonicity of the medium, or by any other standard method, we find the resistances distributed according to the distribution \( N = \int F(x) \cdot dx \) and the percentage hemolysis curves obtained in experiment are functions of \( \int F(x) \cdot dx \). The resistance of any class of cell, however, cannot be interpreted as a measure of its age, although a relation between resistance and age is often stated or implied. The cells of least resistance \( x_0 \), for example, contain the cells of resistance \( x_0 \) which have just entered the population, the cells originally of greater resistance \( x_1 \) which entered at time \( t_1 \) previously and whose life time in the blood stream has been \( t_1 \), the cells of still greater resistance which entered at time \( t_2 \) previously and whose age is \( t_2 \), and so on. The class of cells of greatest resistance, on the other hand, contains cells of resistance \( x_0 \), and these must necessarily be recent additions to the population; it is therefore true that the group of most resistant cells observed in fragility experiments is composed of the "youngest" cells, but equally "young" cells are to be found in any other resistance group, and the group of the most resistant cells observed in the experiment is certainly not composed of the "oldest" cells. The relation between resistance and age, in fact, is apparent only at the upper extreme of the frequency curve.

3. Since the distributions \( P \) and \( Q \) are more negatively skew than the distribution \( N \), any sudden change in the rate of production or destruction will be reflected in the form of the S-shaped curve \( N = \int F(x) \cdot dx \) which is obtained
by measuring resistance by the usual methods. These S-shaped curves are
difficult to obtain in their entirety and in proper units, but the resistance of
the group at the upper extreme is defined by the position of the asymptote of
the time-dilution curve. Slight variations in the position of the asymptote,
corresponding to variations in the balance between production and destruction,
are accordingly to be expected if observations are made daily over long periods,
and sufficiently detailed observations might even reveal a relation between
variations in the position of the asymptote and the changes in the value of \( R \)
described in section III, 3. The changes in the position of the asymptote will
be familiar to anyone who has plotted time-dilution curves for suspensions of
his own red cells daily over a considerable period of time. During the last
9 years I have noticed variations amounting to about ±10 per cent in the
position of the asymptote of time-dilution curves for saponin and suspensions
of my own red cells, and in carrying out the experiments on inhibition of
digitonin hemolysis described in section III, 3, I observed variations in the
position of the asymptote of the digitonin time-dilution curve of about the
same magnitude.

Let us next consider how the total number of cells in the population \( N \) de-
ponds on the numbers in the population \( P \) and \( Q \). \( N \) is the number of cells
in the circulatory system, and there is a process producing new cells at a con-
stant rate \( P \). There is also another process removing the cells at a rate which
must be proportional to \( N \) and which may also vary explicitly with the time \( t \).
Let the rate of removal be denoted by \( Nf \), where \( f \) may be a function of \( t \);
\( f \) may be called the "fractional rate of removal." The general problem is to
find the value of \( N \) at any time \( t \), given the initial value.\(^{15}\)

The net rate of increase \( dN/dt \) is the difference between \( P \), the rate of pro-
duction, and \( Nf \), the rate of removal, so

\[
dN/dt = P - Nf
\]

(5)

Let

\[
\int f \cdot dt = F
\]

(6)

where \( F \) is a function of \( t \) which may be found by integration when the function
\( f \) is given.

The solution of the differential equation (5) is

\[
N = (k + P \int e^{-t} \cdot dt) \cdot e^{-f}
\]

(7)

where \( k \) is a constant determined by the initial conditions. The value of this
result is that it gives us the solution of the problem for any variation in the

\(^{15}\) I have to thank Dr. R. T. Cox for the general and special solutions in this sec-
tion, as well as for the development of a satisfactory method of analyzing the experi-
mental curves.
rate of fractional removal, provided that the two integrals \( \int f \, dt \) and \( \int e^{rt} \, dt \) can be evaluated.

**Special Solution 1: Constant Fractional Rate of Removal.**—If the fractional rate of removal is constant, \( f = a \), and

\[
\int f \, dt = \int a \, dt = F = at
\]

\[
\int e^{rt} \, dt = \int e^{at} \, dt = \frac{1}{a} e^{at}
\]

Substituting in equation (7),

\[
N = \left( k + \frac{P}{a} e^{at} \right) e^{-at}
\]

When \( t = 0 \), let \( N = N_0 \); then \( k = N_0 - \frac{P}{a} \), and so

\[
N = \left( N_0 - \frac{P}{a} \right) e^{-at} + \frac{P}{a}
\]

The first right-hand term approaches zero as \( t \) increases, and so \( N \) approaches a steady state with a value \( P/a \). As the term does not change sign, if the value of \( N \) is initially above the steady state, \( N_0 - \frac{P}{a} \) is positive and \( N \) never becomes less than \( P/a \). Similarly if \( N \) has an initial value below the steady-state value, it never exceeds \( P/a \). If \( N \) is initially equal to \( P/a \), it never varies.

**Special Solution 2: Uniformly Increasing or Decreasing Fractional Rate of Removal.**—Let \( f = a + bt \); then

\[
\int f \, dt = \int (a + bt) \, dt = F = at + \frac{1}{2} bt^2
\]

The integral \( \int e^{rt} \, dt \) cannot be evaluated precisely, but the integration can be performed by regarding \( e^{at+bt^2} \) as an infinite series

\[
1 + (at + \frac{1}{2} bt^2) + \frac{1}{2} (at + \frac{1}{2} bt^2)^2 + \frac{1}{6} (at + \frac{1}{2} bt^2)^3 + \ldots
\]

Expanding by the binomial theorem and collecting terms in each power of \( t \), we get

\[
e^{at+bt^2} = 1 + at + \frac{1}{2} (b + a^2)t^2 + \frac{1}{2} \left( ab + \frac{a^3}{3} \right) t^3 + \ldots
\]

Hence

\[
\int e^{rt} \, dt = t + \frac{1}{2} at^2 + \frac{1}{2} (b + a^2)t^3 + \frac{1}{8} \left( ab + \frac{a^3}{3} \right) t^4 + \ldots
\]
This expression vanishes when \( t = 0 \); also, when \( t = 0 \), \( e^{-\tau} = 1 \). Referring to equation (7), we see that when \( t = 0 \), \( N = k \), so \( N_0 \) may be written for \( k \). Substituting in (7) the values of \( F \) and of \( \int e^{\tau} dt \) given by (12) and (13), we have

\[
N = \left[ N_0 + P \left( t + \frac{1}{2}a^2 + \frac{1}{2}(b + a^2)t + \left( \frac{1}{2}ab + \frac{a^3}{3} \right)t^2 + \ldots \right) \right] e^{-\omega t + \frac{\omega t^2}{2}} \tag{14}
\]

Equations (11) and (14) can be combined to give the values of \( N \) in any case in which the graph of \( f \) against \( t \) is made up of straight segments. At any junction of two segments of the graph, the final value of \( N \) on the first segment is taken as the initial value \( N_0 \) on the second segment. A few examples follow.

**Special Solution 3: Abrupt Transition between Two Constant Values of \( f \).—** Let \( f \) be constant for a long time at a value \( a' \) and then at a certain instant \( t_1 \) let it change instantaneously to a higher value \( a'' \) (see Fig. 2, A). At \( t_1 \) and for some time before we may assume that \( N \) is at its steady state value \( P/a' \). When \( f \) suddenly assumes the new value \( a'' \), equilibrium is destroyed and subsequent values of \( N \) are given by equation (11), the time \( t \) being reckoned from \( t_1 \) the instant of transition, so that \( P/a' \) replaces \( N_0 \) and \( a'' \) replaces \( a' \). The graph of \( N \) against \( t \) is shown in Fig. 2, B. The break in the slope of the graph corresponds to the change in \( dN/dt \) as \( f \) changes from \( a' \) to \( a'' \).

**Special Solution 4: Gradual Uniform Transition between Two Constant Values of \( f \).—** Let \( f \) be constant for a long time at a value \( a' \). At a time \( t_1 \) let it start to increase linearly and let it attain a new value \( a'' \) at an instant \( t_2 \) and thereafter remain constant (Fig. 2, C). This case differs from the one just treated in that here \( dN/dt \) does not change discontinuously; consequently there will be no sharp break in the slope of the graph of \( N \) against \( t \).

The value of \( N \) at \( t_1 \) and for some time previously is \( P/a' \). Beginning at \( t_1 \), equation (14) is used, with time reckoned from the instant \( t_1 \) as zero, \( P/a' \) replacing \( N_0 \), \( a' \) replacing \( a \), and \( (a'' - a')/(t_2 - t_1) \) replacing \( b \). This solution is valid until the instant \( t_2 \) at which \( f \) becomes constant at the value \( a'' \). The value found for \( N \) at \( t_2 \) now becomes \( N_0 \) in equation (11), in which the time is reckoned from the instant \( t_2 \) as zero and in which \( a'' \) replaces \( a \). The form of the graph of \( N \) against \( t \) is shown in Fig. 2, D.

**Special Solution 5: Linear Rise and Fall of \( f \).—** Let there be initially a steady state characterized by a constant value \( a' \) of \( f \). At the instant \( t_1 \) let \( f \) start to rise uniformly, attaining a value \( a'' \) at the instant \( t_2 \). Now let \( f \) start falling, and let it attain the value \( a' \) again at the instant \( t_3 \), remaining constant thereafter. Let \( t_5 - t_2 = t_2 - t_1 \), the rate of fall being equal to the rate of rise.

\[ \text{Note: For detailed treatment of this solution, refer to the original text.} \]

\[ ^{10} \text{The instant at which } f \text{ assumes its new value often corresponds to a point of inflection on the experimental curve of } N \text{ against } t, \text{ but a point of inflection may occur under some circumstances while } f \text{ is uniformly increasing.} \]
In this case the solution up to the time $t_3$ is the same as that given in Fig. 2, D. From $t_3$ to $t_4$ the variation of $N$ is given by equation (14) with time reckoned from $t_3$, with $b = \frac{-(a'' - a')}{(t_4 - t_3)}$, with $a$ replaced by $a''$, and with the value of $N$ at $t_3$ being taken as $N_0$. After $t_4$, equation (11) is again used, with time reckoned from $t_4$, so that $N_0$ is the value of $N$ obtained at $t_4$. Also $a'$ replaces $a$. Graphs corresponding to this special solution are shown in Fig. 2, E and F.

Fig. 2. Curves illustrative of special solutions 3, 4, and 5. Ordinates, $N$ and $f$; abscissa, time. Solid curves show variations in $f$; dotted curves show variations in $N$. For explanation, see text.
The Analysis of Experimental Curves.—The general method of dealing with experimental curves which show the variation of $N$ with time can best be illustrated by the analysis of a curve such as that shown in Fig. 3, which represents the changes in the red cell count observed during the first 15 days of the development of a hemolytic anemia due to the administration of phenylhydrazine. The method requires that somewhere the system passes from a changing state to a steady one, or to one so nearly steady that one can estimate a steady-state value of $N$. In Fig. 3, a steady state seems to be attained at about 15 days, for which $N = 1.05$. Since in general $dN/dt = P - Nf$ (expression 5), and since $dN/dt = 0$ in the steady state, $N = P/f$, and so $P/f = 1.05$, $f$ being the fractional rate of removal in the steady state. Now in the approach to this steady state, $f$ must have been constant for some time, or else the steady state would never have been reached; and when $f$ is constant, we can find $N$ as a function of the time $t$. We have

$$dN/dt = P - Nf,$$

or

$$dN = -f(N - P/f) \, dt$$

(15)

Since $P$ and $f$ are constant, $d(P/f) = 0$, and we may replace $dN$ by $d(N - P/f)$. So we have

$$-f \cdot dt = \frac{d(N - P/f)}{N - P/f}$$

(16)
which gives on integration

\[ f(t_i - t) = \log \left( \frac{N - P/f}{N - P/f_1} \right) \]  

where \( t_i \) is any specific value of the time \( t \) and \( (N - P/f)_1 \) is the value of \( N - P/f \) at that instant. In the specific case of Fig. 3, we have \( P/f = 1.05 \), so

\[ f(t_i - t) = 2.3 \log_{10} \left( \frac{N - 1.05}{N - 1.05} \right) \]

We now plot \( (N - 1.05) \) against \( t \) on semi-log paper, and see how far back on the curve \( f \) is constant (Fig. 3, inset a). From 5 to 10 days, one gets a good straight line, and the value of \( f \) turns out to be 0.41.

To find the value of \( f \) at times before 5 days, we first find \( P \), and since \( P/f = 1.05 \) and \( f = 0.41 \), \( P \) must be 0.43. Now using expression (5) in the form

\[ f = \frac{P - dN/dt}{N} \]

we draw tangents of slope \( dN/dt \) to the experimental curve. With values of \( N \) taken from the curve at the points where the tangents are drawn, and with the value 0.43 inserted for \( P \) in expression (18), the value of \( f \) can be found for any time \( t \). Fig. 3, inset b, shows the way in which \( f \) varies with \( t \); for the first 4 days the fractional rate of removal rises almost linearly, and then becomes virtually constant at about 4 times its initial value.

Figs. 4 and 5 show two other examples of experimental curves and their analysis by this method. The data for Fig. 4 are taken from Ponder and Abels (1936), and show the fall in \( N \) following the injection of 80 mg. per kilo per day of quinine hydrochloride into a rabbit. In this very simple case, the fractional rate of removal seems almost immediately to assume its new value (see inset of Fig. 4). The data for Fig. 5 are taken from the paper by Dziemian (1942) on the effect of the injection of phenylhydrazine on the red cells of the rabbit, and show the result of a single injection. Here the value of \( f \) first rises and then falls to a level even lower than that for the normal steady state (see inset of Fig. 5). This probably means, in reality, that the increased rate of destruction has been followed by an increased rate of production.

In the foregoing analysis, the total number of red cells in circulation is determined in terms of the rate of production \( P \) and the rate of destruction \( Q \) or \( Nf \). If the process of destruction is an \textit{in vivo} hemolytic process, some of the factors which control it can be specified further.

As expressed in equation (1), the rate of the fundamental lytic reaction by means of which the removal of cells is effected is proportional to \( c \), and in the
Fig. 4. Analysis of a curve for the fall in the red cell count following administration of quinine (data of Ponder and Abels, 1936). See text for further description.

Fig. 5. Analysis of a curve for the changes in the red cell count after administration of phenylhydrazine (data of Dziemian, 1942). See text for further description.
steady state $f$ has some special value ($a$ in expression (8)) which makes the rate of red cell destruction equal to the rate of production. If $c$ changes from one value $c_0$ to another $c_1$, then $c_0/c_1 = R$, and $f$ changes from $a$ to $a_1$, where $a_1 = a/R$. Such a change in the value of $c$ can occur in two principal ways.

1. The rate of production of lysin may increase, as after a meal of fat, when the concentration of soaps in the blood stream rises. Many other instances of an increase in the concentration of intravascular lysins are known, particularly in pathological conditions.

2. Although the rate of production of lysin remains constant, the effective concentration of lysin in the blood stream may increase because of the addition of accelerators or the removal of inhibitors. In the normal steady state the concentration of lysin is kept at a certain effective value by the mixture of plasma accelerators and inhibitors, the net effect of which is an inhibitory one. For convenience, let us use the steady-state system as the standard one. If the net inhibitory power of the plasma decreases, so that $R$ becomes less than unity with reference to the inhibitory power in the steady state, the effective concentration of lysin, which was $c_0$ originally, will increase to $c_0/R$. The net inhibitory power of the plasma may decrease, of course, either by the decrease in the quantity of the inhibitors or by an increase in the quantity of the accelerators, and in this way the effective concentration of lysin can undergo wide variations, especially in the direction of increasing. The way in which the net inhibition $R_{\text{net}}$ depends on the individual inhibitions $R_i$ and accelerations $R_a$ has been discussed in the first section of this paper, and it is probably generally true that the inhibition or acceleration produced by any individual substance is roughly proportional to its quantity (see Ponder, 1939, 1941, 1943).

The analysis as outlined above requires in addition that $P$ remains constant, and since this is a requirement which is not always fulfilled in actual experience, experimental curves should be analyzed in this way only when it is reasonable to believe that the change in $N$ is due to changes in the extent of a destructive (usually hemolytic) process. For a more complete understanding of what occurs during the development of an anemia and recovery from it we would

It is very noticeable that there are many ways in which the red cell count can be reduced to a lower level, but only a few in which it can be raised above the normal steady state. Under no circumstances can it be raised very much, and in the few cases in which increases are observed they tend to be brought about at the expense of red cell size; i.e., the quantity of reactive material, from the point of view of a hemolytic system, tends to remain constant. This suggests that there is a mechanism, not considered here, which becomes operative when the normal level is exceeded. Decreases in the concentration of intravascular lysins below the normal steady-state values, and increases in the net inhibitory power of the plasma above the normal steady-state values, either do not occur or are ineffective because the normal steady state constitutes a "ceiling state" in the physiology of the intact animal.
have to have some method of measuring variations in either $P$ or $Q$ independently. An indication, but not necessarily an exact measure, of variations in the latter would be provided by the urobilinogen excretion. This is certainly not a very satisfactory state of affairs; taken together with the great difficulty in extracting \textit{in vivo} lysins quantitatively and the uncertainty with which the occurrences in complex hemolytic systems can be approached from the study of the effects of their isolated components, it probably means that the kinetics of \textit{in vivo} hemolysis can be established in specially selected cases only.

**SUMMARY**

This paper is concerned with a variety of questions which bear on the occurrence of hemolysis \textit{in vivo}, and with the possibility of regarding the contents of the blood stream as a hemolytic system in which a steady state is maintained by the production of new red cells to replace those which are destroyed. The material which is dealt with includes the following.

1. **Mixtures of Lysins, Accelerators, and Inhibitors.**—The effects of individual accelerators and inhibitors in mixtures, like the effects of individual lysins, are roughly additive in simple systems, the acceleration or inhibition produced by the individual substances being most conveniently measured in terms of $R$-values.

2. **Normal Intravascular Lysins.**—These probably play only a small part in red cell destruction unless their concentration rises to unusual levels, or unless their effects are enhanced by accelerators, or by the reduction of the concentration of normal inhibitors. The three normal \textit{in vivo} hemolytic processes for which there is substantial evidence involve (a) the action of the bile salts and of the soaps derived from chyle, (b) the action of the spleen, and (c) the action of hemolytic substances derived from tissues. The recent observations of Maegraith, Findlay, and Martin on the presence of widely distributed tissue lysins are confirmed except for their conclusion that these lysins are species-specific. Species-specific tissue lysins, if present, are not the only lysins derivable from tissues by simple immersion in saline, for non-species-specific lytic substances can also be obtained, and seem to be similar to the “lysolecithin” which some regard as responsible for the action of the spleen on red cell fragility and shape.

3. **Plasma Inhibitors.**—About 30 per cent of the total inhibitory effect of plasma for saponin hemolysis is due to the contained cholesterol, while 25 per cent at most is due to the plasma proteins, particularly globulins. The remaining 45 per cent is probably accounted for by enhancing effects among the inhibitors; \textit{e.g.}, the enhancing effect of lecithin on the cholesterol inhibition. The mechanism of the inhibition is still incompletely understood; probably reactions between inhibitor and lysin and reactions between inhibitor and
components of the red cell surface are both involved, and it is important to observe that the inhibitory effect of plasma or of a plasma constituent may be greater in systems containing one lysin than in systems containing another. No evidence for diffusible inhibitory substances in plasma has been found, and the variations observed in the inhibitory power of human plasma seem to be related to the combined concentrations of cholesterol, protein, and probably lecithin, rather than to the cholesterol content alone. For this reason the inhibitory power tends to be low under conditions of poor nutrition.

4. The Steady State and the Kinetics of Hemolysis In Vivo.—On the assumption that the steady state is the result of a balance between a process which produces red cells and a process which destroys them, equations have been developed for the way in which cells of different resistances are affected when the rate of destruction changes. A method for analyzing experimental curves is described and illustrated. In general, this part of the paper relates the level of the red cell count in the animal to the intensity of the hemolytic processes taking place in vivo, and does not lend itself to detailed abstraction.

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