HEMOLYSIS CONSIDERED AS A PROGRESSIVE REACTION IN A HETEROGENEOUS SYSTEM

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(Received for publication, July 31, 1951)

This paper is concerned with two experiments on hemolysis, designed to throw light on two points which have been the subject of considerable debate. The first experiment shows, at least in the case of certain lysins, that the cells are heterogeneous with respect to their resistance to the lysin. The second shows that the lysin initiates a process in the cell which precedes hemolysis and which cannot be stopped by diluting the system. It is proposed to interpret the results of these experiments, so far as is possible, without making special assumptions which would apply to types of reaction (simple irreversible and simple reversible) different from the *progressive* reaction which the hemolytic process turns out to be.

When red cells are suspended in a solution of a hemolysin, they survive for very different lengths of time. Some are hemolyzed almost instantly but others may survive for hours. Two possible causes of this variation have been differently emphasized by different writers on hemolysis. Arrhenius and his pupils, whose work was summarized by Arrhenius himself in 1915, supposed that the hemolysis of a cell is the almost instantaneous result of its interaction with a single molecule of the lysin. The cells were regarded as identical, and the variation among their times of survival was ascribed entirely to the randomness of their encounters with molecules of the lysin. In a population of cells, the chance of hemolysis in a given time would be the same for each cell. The number hemolyzed per unit time would thus be proportional to the number present and would decrease as the hemolysis of the population progressed. Hence a graph showing the percentage of the population hemolyzed during any time as a function of the time would be a curve with a continuously decreasing slope and would be everywhere concave to the time axis.

The later discovery that the actual graphs were not of this character but were on the contrary sigmoid caused this hypothesis to be abandoned. Now sigmoid curves are typical of the continuous distribution of characteristics in a heterogeneous population. This fact gave reason for several authors (Brooks, 1919; Ponder, 1923) to emphasize, as a likely cause of variation in the time of survival of cells, the differences in resistance to hemolysis which might be expected to exist in a population.

On the other hand, other investigators (Yule, 1910; Rahn, 1945; Alberty and Baldwin, 1951) have pointed out that sigmoid curves of percentage cell destruction *vs.* time are to be expected even with a homogeneous population if it is supposed that not one but several molecules of the lysin must interact with a cell before lysis can occur. On this assumption, the initial rate of lysis is small because the chance is slight that a cell will encounter several molecules of the lysin within a short time. The rate is small again as the process nears completion either by the destruction of the whole cell population or by the exhaustion of the lysin. Between these early and late periods of slow lysis, the rate passes through a maximum, and thus the graph of percentage lysis *vs.* time has a sigmoid shape.

Alberty and Baldwin have applied these considerations not only to the graphs of percentage lysis *vs.* time but also to "static lysis curves." To obtain an experimental point on such a curve, the cells are left in the solution containing the lysin until lysis has practically ceased, when the percentage lysis is measured. By preparing a number of systems with equal volumes of solution and equal quantities of cells but with different initial concentrations of the lysin, it is possible to obtain a graph of percentage lysis *vs.* initial lysin concentration. It is found that over a range of low lysin concentrations the percentage lysis remains nearly zero and at higher concentrations it approaches 100 asymptotically; *i.e.*, the static lysis curve is also sigmoid. To explain this fact Alberty and Baldwin suppose, when hemolysis comes to a stop short of lysis of the whole cell population, that it is because the lysin has been all used up in an irreversible reaction with the cells. How many lysin molecules will have combined with any one cell is a matter of chance encounters. For a random cell there is a calculable probability that this number will have attained some value assumed necessary for lysis. If the initial lysin concentration was somewhere within the low range for which the average number of reacting molecules per cell is well below the critical number required for lysis, the chance of lysis is small and very few cells will have been hemolyzed. On the other hand, if the initial concentration was in the high range for which the average number of reacting molecules is several times the critical number, few cells will have escaped lysis. In both these ranges, the percentage lysis is insensitive to small changes in the initial concentration and the static lysis curve has only a small slope. At an intermediate range of initial lysin concentrations, for which the average reaction is near the critical value, the percentage lysis is very sensitive to changes in concentration and the curve is therefore steep. Thus the static lysis curve predicted on these assumptions, even for a quite homogeneous cell population, has the general sigmoid shape found experimentally.

The inference to be drawn is that neither the kinetic nor the static lysis curves give any clear evidence that the cells are heterogeneous in respect to their resistance to hemolysis. This inference concerning the ambiguous meaning

of the static lysis curves is not valid, however, unless the lysin actually is, as it was assumed to be in the argument, all used up when hemolysis ends with some of the cells still intact. The first experiment to be described here was designed to see whether or not this is true.

1. Demonstration of Heterogeneity of Red Cell Resistances

A population of red cells is added to a series of lysin concentrations. Hemolysis is allowed to proceed for a long time, at the end of which its velocity has become negligibly small. The intact red cells are removed by centrifuging; the supernatant fluids are removed from each system, and the percentage hemolysis in each concentration is found. A second population of red cells is now added to each supernatant fluid. Lysis is again allowed to proceed for a long time, at the end of which the percentage hemolysis of the cells of the second population is measured.

Method.—Four ml. of each of a series of concentrations of a lysin in NaCl-buffer are placed in each of a series of tubes. For saponin at pH 7.0, a suitable series is 40, 30, 25, 20, 15, 10, 6, 4, and 3 γ/ml.; for digitonin at pH 7.0, a suitable series is 10, 7, 5, 4, 3, 2.5, 2, 1.5, and 1 γ/ml.

A concentrated red cell suspension is prepared by suspending the thrice washed cells of 10 ml. of heparinized human blood in 20 ml. of saline, and a dilute suspension is made from this by diluting it fivefold. One ml. of this dilute suspension is added to each of the tubes containing the lysin, and the systems are allowed to stand, with occasional mixing by inversion, at 25°C. for 5 hours. At the end of this time, the intact cells of each system are thrown down by centrifuging, and the supernatant fluids are transferred to a series of clean tubes. Some of each supernatant fluid is used for a photometric determination of P_1, the percentage hemolysis present in the system at the end of the 5 hours. Two ml. of each supernatant fluid are transferred to another series of clean tubes, and 0.08 ml. of the concentrated red cell suspension is added to each. The resulting hemolytic systems are allowed to stand, with occasional mixing, at 25°C. for 5 hours. At the end of this time the intact cells of each system are thrown down by centrifuging, and the amount of Hb in each supernatant fluid is found photometrically. Each supernatant fluid contains the Hb from the hemolyzed cells of the first population plus the Hb from the hemolyzed cells of the second population; since the former is known, the percentage hemolysis P_2 of the cells in the second population, at the end of 5 hours, can be calculated.

The type of result obtained when these measurements are made in systems containing saponin is shown in Fig. 1, in which the sigmoid curve I shows the relation between the initial concentration c and P_1, the percentage hemolysis of the first population, while the sigmoid curve II shows the relation between c and P_2, the percentage hemolysis of the second population. In any initial concentration, the velocity of lysis becomes very small after 5 hours, as may be shown by allowing the system to stand for 10 hours and observing that P_1

increases only a few per cent in the interval between 5 and 10 hours. That this is not due to the quantity of lysin being exhausted is shown by the fact that the second population undergoes a percentage lysis P_2 equal to about half of P_1. For example, in the initial concentration 12 γ/ml., $P_1 = 55$ and $P_2 = 28$. The fact that the lysin which has almost ceased to hemolyze the first population can be so active in hemolyzing the second shows that at least a part of the second population is less resistant to the lysin than the surviving portion of the first. The difference is most reasonably ascribed to an initial heterogeneity of the cell population. Thus the lysin in a concentration of

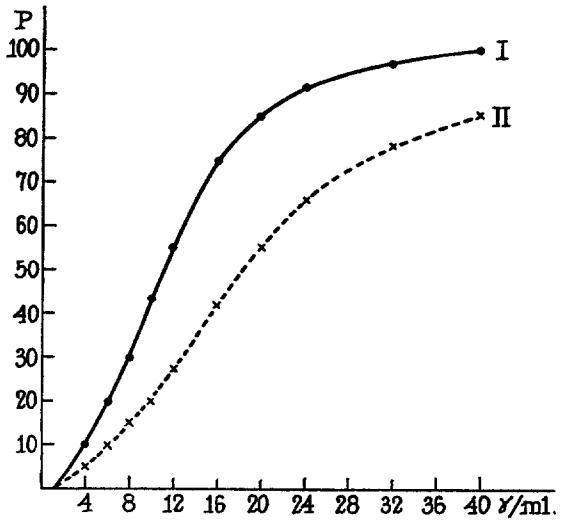

FIG. 1. Static hemolysis curves for a population of human red cells and saponin (curve I) and for a second equal population added after lysis of the first (curve II). Ordinate, percentage hemolysis; abscissa, lysin concentration. For further explanation, see text.

12 γ/ml. hemolyzes cells of a resistance less than a certain value (in this case 55 per cent of the cells belong to the less resistant group), while it does not hemolyze cells of a resistance greater than this value (in this case, 45 per cent of the cells belong to the more resistant group). Similarly, any other concentration of lysin divides the cells of the population into a less resistant group and a more resistant group, and we can get as many such divisions as we have values of c between the concentration which produces just commencing hemolysis and the concentration which produces complete hemolysis.

A different alternative should perhaps be considered. It might be imagined that the cell population is initially homogeneous and that the greater resistance of the surviving portion is an immunity acquired by exposure to the lysin. To

suppose this, however, is to suppose that the operation of pure chance on cells initially alike can hemolyze some of them while it renders others immune to hemolysis. This seems to be ascribing a great deal to chance.

Considering that individual red cells of populations have been shown to be unlike, rather than identical, with respect to every attribute which has been measured (dimensions, density, fragility in hypotonic media, content of Na and K (Kruszinski, 1950), etc.), and that bimodal percentage hemolysis curves have been found, both with saponin and with hypotonic saline (Handovsky, 1911; Ponder, 1949) under conditions in which two red cell populations are mixed, it seems entirely reasonable to conclude that the form of the static lysis curve is determined by the initial heterogeneity of the red cell population.

The fact that the velocity of lysis becomes negligible while there are both unhemolyzed cells and active lysin present in the system strongly suggests that we are dealing with a reaction which does not go to completion but instead attains an equilibrium between the active lysin and that which is in some way altered by interaction with the cells. The attainment of a state of equilibrium rather than a completed reaction would indicate that the alteration of the lysin, whatever the process involved, is reversible. If the alteration is attachment to the cells, then there must also be the possibility of release.

The supposition that the attachment of lysin molecules to the cells is reversible has been employed before (Ponder, 1948, pp. 195 *ff.*) to explain the way in which the asymptotic concentration of lysin varies with the cell concentration.

When the experiment just described is carried out with digitonin as the lysin, the results are quite different from those obtained with saponin. With digitonin the partial lysis of the first population is not followed by a partial lysis of the second population; the cells of the second population, indeed, are not hemolyzed at all unless the system contains much more lysin than is necessary to completely hemolyze the cells of the first population within 5 hours. This suggests that the reaction of digitonin with the cells, in contrast with what appears to be true for saponin, goes to completion; when hemolysis stops with intact cells present in the system, it is because the lysin has been exhausted, as is shown by the fact that there is no lysis of the second population. This is what would be expected if the alteration of the lysin is irreversible. Systems containing sodium taurocholate at pH 6.5 are similar in the results of this experiment to systems containing digitonin. The difference between the results found with digitonin and with saponin supports other observations to the effect that these two lysins produce hemolysis by quite different means (Ruyssen and Loos, 1946, film penetration studies; Wolpers, 1949, electron microscope studies). As between the largely reversible case, found with saponin, and the largely irreversible case, found with digitonin, there may be intermediate cases; this is indicated by the separation of curves I and II, in experiments similar to the foregoing, varying considerably even in systems containing saponin and human red cells.

2. *Observations on the Type of the Hemolytic Reaction*

Although, in the experiment described with saponin, the alteration of the lysin appeared to be reversible, the alteration of the cell is certainly not so, since it involves the hemolysis of the cell. A second experiment, now to be described, gives some evidence as to the type of the hemolytic reaction.

In this experiment a hemolysin is allowed to react with red cells until partia hemolysis has occurred. The system is then diluted with saline to such an extent that the concentration of lysin becomes less than that which would be asymptotic for this percentage hemolysis, and the progress of hemolysis, if any, is followed in the dilute system. When the velocity of hemolysis has become very small, the original concentration of lysin in the system is re-established, and the rate at which lysis occurs is again observed.

Method.—The experimental procedure will be described as it is carried out at 25°C. with a Model 6A Coleman Junior spectrophotometer set at a wave length of 6500 A.

The washed red cells of 4 ml. of human blood are suspended in 20 ml. of NaCl-buffer at a selected pH. Two calibration curves are constructed, the first giving the optical densities corresponding to values of percentage hemolysis P of 10, 20, . . . 100 per cent in a system consisting of 1.6 ml. of saline and 0.4 ml. of this suspension, and the second giving the optical densities corresponding to the values of P in a system containing 19.6 ml. of saline and 0.4 ml. of the suspension; *i.e.*, to the values of P in the first system diluted tenfold. A large cuvette (6-300, 25 × 105 mm.) is used to contain these dilute systems.

To a small cuvette (6-311, 10 × 75 mm., one of a matched pair, the other cuvette being used to determine that the zero of the photometer does not shift during long experiments) is added 1.6 ml. of saponin in a concentration c_0; 30 γ/ml. is a convenient concentration, and is the one used in the experiment shown in Fig. 2. At zero time, 0.4 ml. of the suspension is added. Lysis begins in a minute or so, and is allowed to proceed until there is 40 per cent hemolysis in the system; this is known from the value of the optical density corresponding to $P = 40$ on the calibration curve. The entire hemolytic system of 2 ml. is very quickly transferred to a large cuvette containing 18 ml. of saline; this is placed in the light path, and the progress of the lysis, referred to the calibration curve for the dilute system, is followed. When the velocity of lysis has become very small, 0.18 ml. of a concentration of saponin equal to 100 c_0 (3 mg./ml. in the experiment shown in Fig. 2) is added to the dilute system. This raises the lysin concentration to c_0, its original value. The progress of lysis, still referred to the calibration curve for the dilute system, is followed until its completion.

The type of result obtained when these measurements are made in a system containing saponin is shown in Fig. 2. The curve marked B is that for the system containing 1.6 ml. of lysin in the concentration $c_0 = 30$ γ/ml. and 0.4 ml. of the cell suspension. The course of the reaction, which would otherwise proceed along the curve marked B, is interrupted at the point marked 1,

i.e. after 3.75 minutes and when $P = 44$ per cent, by diluting the system tenfold with saline. The lysin concentration in this diluted system is less than the asymptotic value for 44 per cent hemolysis of the initial cell population. Nevertheless the lytic process continues along the part of the curve marked with crosses, and P increases from 44 to 71 during the next 6 minutes. When $t = 10$ minutes, *i.e.* 6 minutes after the dilution of the system, the velocity of lysis has become very small, and at this point (marked 2) the original lysin concentration c_0 is reestablished by the addition of lysin dissolved in a very small

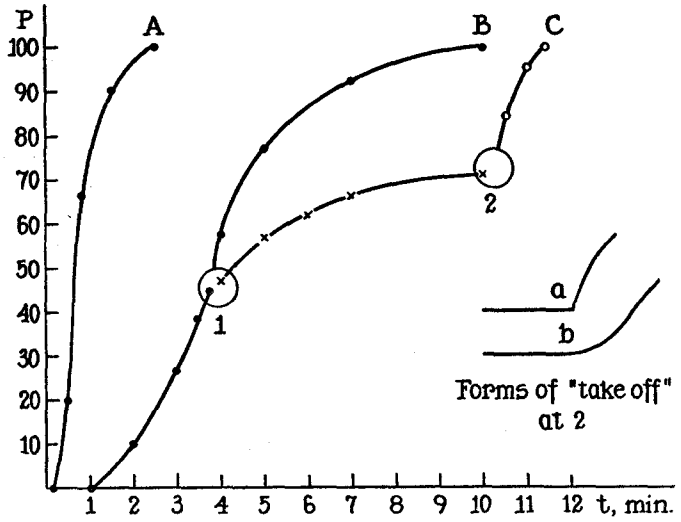

FIG. 2. Percentage hemolysis curve C for a system diluted at point 1, and brought to its original concentration with respect to lysin at point 2. Curves A and B, reference curves, for the use of which the text should be consulted. Ordinate, percentage hemolysis; abscissa, time in minutes. Inset shows two forms of "take-off" at point 2; for further explanation, see text.

amount of saline. Hemolysis now takes place rapidly, as is shown by the measurements indicated by circles on the part of the curve marked C.

If digitonin is used as the lysin, a similar result is obtained, the significant point being that the hemolytic action continues after the dilution of the lysin in the system at the point marked 1. If hemolysis were the immediate effect of the capture of lysin by a cell, a dilution of the system to this extent would be expected to result in an almost complete cessation of lysis, since the lysin concentration has been brought below the asymptotic concentration. The graph of percentage hemolysis *vs.* time would then become a practically horizontal line. What the experiment shows is that hemolysis is slowed down but by no means stopped by the dilution.

To explain this it seems necessary to suppose that the interaction between the lysin and the cell, although it may be reversible in respect to the alteration of the lysin is, in its effect on the cell, so far from being reversible that it is not even adequately described as irreversible. The characteristic of the process taking place in the cell is that, once it is well enough under way, it cannot be stopped by diluting the lysin in the system. A reaction of this kind can be called a *progressive* reaction. In the case of the experiment illustrated in Fig. 2, when one tries to stop the progressive reaction at $P = 44$, the reaction proceeds until $P = 71$.

Reactions of this kind have been known at least since Osterhout's descriptions (1922) of the effects of injury on *Laminaria;* if the effects are small, they are reversible, but if they exceed certain measurable values, they become progressive and continue until the cell is dead. Speaking generally, progressive reactions may be looked for in systems which have structure, and in which local reactions lead to disproportionate effects. Their properties cannot be described in terms of the kinetics of reactions in homogeneous systems, and it is to be expected that these properties will depend largely on the structure of the system in which the reaction occurs, as well as on the type and concentration of the noxious agent. The least allowable heterogeneity would be that in which there are two phases, the solution in bulk and another phase localized in or around the cell. The properties of the progressive reactions which take place in systems containing red cells and lysin will have to be determined by experiments beyond the scope of this paper, which is limited to a demonstration that heterogeneity exists in the cell population and that the hemolytic process in the cell is a progressive reaction.

The increase of hemolysis after the original lysin concentration is restored, as shown on part C of the curve, appears at first sight surprisingly rapid. It must be remembered, however, that the initial concentration of cells is not restored along with the initial concentration of lysin. The slope of curve C should be compared, therefore, not with that of curve B but rather with that of curve A, which is the graph of percentage hemolysis *vs.* time for the higher concentration of lysin and the lower concentration of cells. The purpose of determining the points on curve C was to find how abrupt is the "take-off" after the initial concentration is restored. If the capture of lysin by the cells is irreversible, so that the cells retained all the lysin they accumulated before the system was diluted, it might be expected that curve C would rise sharply from point 2, as in the inset *a*. If it is reversible there would be a delay in the resumption of lysis while the cells were accumulating the lysin they lost after the dilution of the system, and curve C would be sigmoid like that in inset *b*. The measurements are not conclusive on this point, because they hardly warrant drawing the curve in the region just beyond the point 2. It is steep enough a little beyond this point that it could be drawn in the form of *a*. On the other hand, curve A has only a slight inflection, and such an inflection on curve C could easily

have escaped observation in the half minute between the last reading on the dilute system and the first reading on the reconcentrated system.

This investigation was begun as a result of discussions which took place at the Conference on Immunochemistry, held at Shelter Island in June, 1950, under the auspices of the National Academy of Sciences. We take this opportunity to thank Dr. R. A. Alberty and Dr. R. L. Baldwin for sending us their paper on immune body and complement hemolysis prior to its publication. During the preparation of our paper, we have had the benefit of conversations with Dr. W. J. V. Osterhout on the general subject of progressive reactions, and we are indebted to him for many specific suggestions.

SUMMARY

It is demonstrated, without the use of special assumptions, that red cells are heterogeneous with respect to their resistance to at least certain lysins, that the reaction between the cell components and the lysin is virtually irreversible in some cases but reversible, although to different extents, in others, and that the lysin initiates a process in the cell which is not adequately described by the terms reversible and irreversible, but rather by the term *progressive*. Progressive reactions, *i.e.* reactions which cannot be stopped once they are well under way, may be looked for in systems which have structure, and in which local reactions occurring at strategic points lead to disproportionate results.

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