

DELAYED HEMOLYSIS OF HUMAN ERYTHROCYTES IN SOLUTIONS OF GLUCOSE

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When normal oxygenated blood is diluted 1 in 20 in a series of glucose solutions of different concentrations at 20°C., hemolysis does not begin until the concentration is reduced to about 2.2 gm. per 100 cc. and is complete when the concentration is further reduced to about 1.2 gm. per 100 cc. No standard series of osmotic hemolysis curves in glucose solutions has been carried out; suffice it to say that the fragility curve is a typical sigmoid curve exactly comparable to that obtained when solutions of sodium chloride are used. But apart from the ordinary osmotic hemolysis phenomenon there also occurs, under certain conditions, a sequence of events which may conveniently be described as "delayed hemolysis."

Delayed hemolysis consists of an interval after the addition of the blood to the glucose solution, during which there is no visible alteration in the system (the prolytic phase), followed by a period during which hemolysis slowly proceeds to completion (the hemolytic phase). There need be no manipulation of the system other than gentle mixing at long intervals. The prolytic phase normally lasts about 1 to 1½ hours and the hemolytic phase about 2 hours. The following procedure describes the conditions under which delayed hemolysis may best be observed, and it forms the prototype for all subsequent experiments.

Normal human venous blood (oxalated or heparinized) is fully oxygenated by exposure to pure oxygen for 20 to 30 minutes with occasional shaking. 2.00 cc. of the whole blood is added to 38.00 cc. of a solution containing 2.200 to 2.300 gm. anhydrous glucose per 100 cc. (a dilution of 1 in 20). After mixing, the tube is allowed to stand in a water bath at 20°C. with occasional gentle shaking. 4 cc. samples are removed every 15 minutes and are immediately centrifuged at 2,000 R.P.M. for 2 minutes. 2.00 cc. of the supernatant is taken for determination of the liberated hemoglobin, and the percentage hemolysis is determined by a method already described (1).

The blood is used within 30 minutes of its being withdrawn. The glucose solution is made up immediately before use. No precautions need be taken to ensure sterility but few experiments have been carried on for more than 4 hours. Graph A in Fig. 1 shows a typical result obtained under these conditions.

Delayed hemolysis depends on a number of physical factors, most of which can be controlled and their effects examined. The experiments described here were designed to investigate the matter further and discover if it could add anything to our knowledge of hemolytic processes.

The Glucose Concentration.—In selecting a concentration of 2.200 to 2.300 gm. of glucose per 100 cc. one is dealing with a concentration which just fails to produce osmotic hemolysis under the given conditions, or which produces a minimum degree of hemolysis depending on the particular sample of blood.

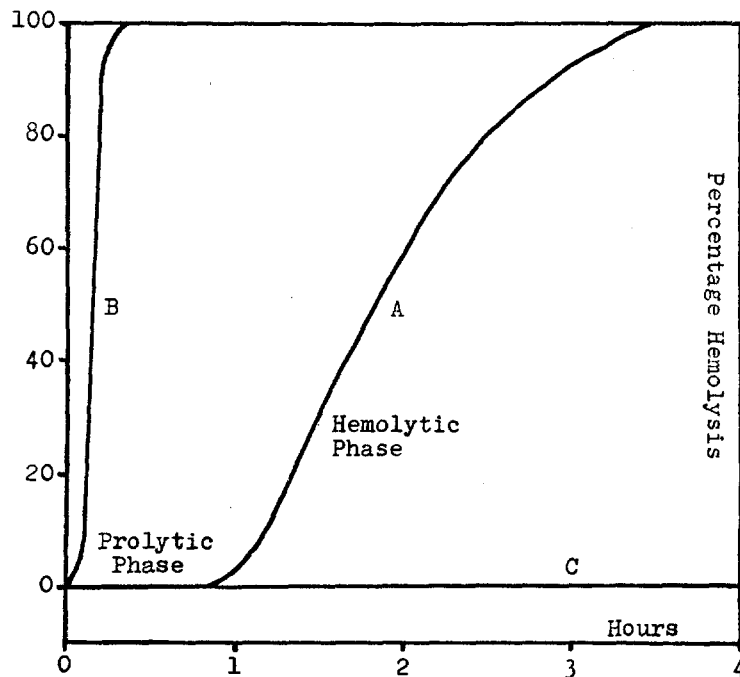


FIG. 1. Graphs of delayed hemolysis; A at 20°C., B at 36°C., C at 8°C. Glucose concentration = 2.200 gm./100 cc. Dilution = 1 in 20.

To be quite sure that there shall be no trace of hemolysis (as will be necessary in certain experiments) a concentration of 2.400 or even 2.500 gm. per 100 cc. should be used.

As the concentration of glucose is increased, the prolytic phase is slightly prolonged, and the rate of hemolysis, once it starts, is slower. With an initial concentration of 3.0 gm. per 100 cc. or more, the onset of the hemolytic phase is delayed for more than 4 hours, and in isosmotic solution (5.5 gm. glucose per 100 cc.) no hemolysis is observed even after 12 hours at 20°C.

Lowering the glucose concentration below 2.0 gm. per 100 cc. always results in an initial hemolysis the degree of which depends on the concentration, but

even then, delayed hemolysis still occurs but the length of both phases is much reduced (see Fig. 2).

The Shape of the Delayed Hemolysis Curve.—The delayed hemolysis curve differs from the osmotic hemolysis curve in sodium chloride (or glucose) solutions. It is not a regular sigmoid curve. At the end of the prolytic phase, hemolysis begins quite sharply—it is much easier to guess where hemolysis “begins” in the case of delayed hemolysis than in the case of osmotic hemolysis.

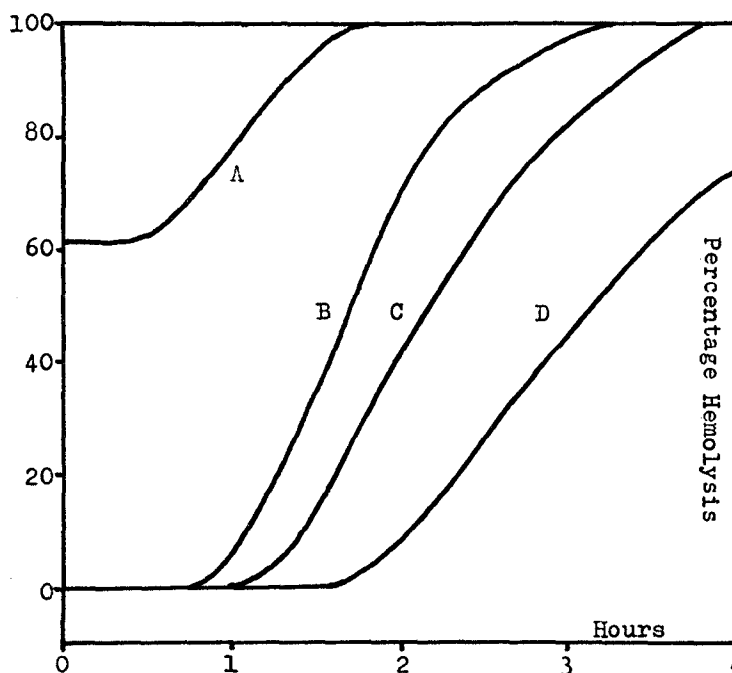


Fig. 2. Delayed hemolysis curves in varying concentrations of glucose; A = 1.50, B = 2.10, C = 2.20, D = 2.60 gm. per 100 cc. Dilution = 1 in 20; temperature = 20°C.

Once the 10 per cent hemolysis point is reached, the curve is practically a straight line until about 60 per cent of the cells have lysed. Thereafter, the rate of hemolysis gradually falls off, but one very noticeable feature is that the curve does not linger in the region of 99 to 100 per cent hemolysis, but reaches 100 per cent hemolysis quite abruptly.

Blood Samples.—In all the following work, blood has been obtained from normal healthy human beings, and, given the same conditions, the delayed hemolysis graphs are almost identical. The sex of the donor is immaterial. No experiments have yet been carried out with blood from pathological cases.

In one or two experiments (not described in the text) the cells have been washed five times with physiological saline, and then suspended in a volume of saline equal to that of the original plasma. This in no way alters the delayed hemolysis curve.

Most of these experiments have been carried out with heparinized blood but the same curve is obtained with oxalated blood using the usual mixture of potassium and ammonium oxalates in minimal amount. Although standard series for comparison have not been carried out, there is no evidence that the nature of the anticoagulant makes any difference.

Temperature.—This is one of the most interesting factors concerned in delayed hemolysis. It has been well established that when a fragility curve is determined in sodium chloride solution, the curve is dependent, *inter alia*, on the temperature. At constant concentration of salt, the lower the temperature the greater the degree of hemolysis, and *vice versa*.

Using a solution of glucose containing 2.20 gm. per 100 cc. under the standard conditions already described, the typical graph of delayed hemolysis is obtained at 20°C. If the same solution of glucose be equilibrated at 36°C. before the addition of the blood, the prolytic phase is reduced to about 5 minutes and the hemolytic phase to about 10 minutes (Graph B, Fig. 1). At 8°C., it is found that no hemolysis occurs even after 4 hours (Graph C, Fig. 1).

It has already been shown that in hemolysis in sodium chloride solution, the effect of changes of temperature can be correlated with the resulting changes of osmotic pressure (2), and that if the concentration of the sodium chloride solutions be so adjusted that the hemolyzing systems have the same osmotic pressure at different temperatures, then temperature changes have no effect on the degree of hemolysis or on the fragility curve. Because of the very great differences in the delayed hemolysis curves at 8, 20, and 36°C., it was not anticipated that changing from constant concentration to constant osmotic pressure would bring the curves into alignment, and so it turned out. At constant osmotic pressure, the delayed hemolysis curves at these three temperatures are as widely different as they are at constant concentration. In view of this finding, it is necessary that the temperature variation should not exceed 0.5°C.

Using a glucose concentration of 5.50 gm. per 100 cc., and working at 37°C., delayed hemolysis is again observed—the curve being very similar to that obtained by using a glucose concentration of 2.40 gm. per 100 cc. at 20°C.

Dilution of the Blood.—A dilution of 1 in 20 was originally chosen largely as a matter of convenience. The ratio of blood to hemolyzing system affects the degree of osmotic hemolysis because of the difference in osmotic pressure between the blood (approximately 6.5 atmospheres) and the hypotonic solutions of sodium chloride and of glucose which will produce some degree of hemolysis (approximately 2.8 atmospheres). The greater the ratio of the volume of blood to the volume of hemolyzing solution, the less will be the degree of osmotic hemolysis.

If delayed hemolysis curves are carried out at different dilutions (1 in 10, 1 in 20, 1 in 40) it is found that the length of the prolytic phase is the same for all dilutions, but the rate of hemolysis during the hemolytic phase is most rapid in the 1 in 10 dilution and slowest in the 1 in 40 dilution. This is the reverse of what might be anticipated from the figures given in Table I.

Oxygenation.—Oxygenation has a considerable effect on the osmotic fragility of red cells suspended in glucose solution; as in sodium chloride solution, oxygenation decreases the degree of hemolysis. The typical curve of delayed hemolysis can be obtained with venous as well as with oxygenated blood, but when venous blood is employed, a higher concentration of glucose should be used. In all experiments described here, oxygenated blood has been used.

Bacterial Infection.—Although most unlikely that delayed hemolysis is due to, or is associated with, the growth of bacterial contaminants, the standard experiment

TABLE 1
*Percentage Hemolysis of Oxygenated Blood in Glucose and in Sodium Chloride Solutions at 20°C.
at Various Dilutions*

Time of hemolysis = 10 minutes.

| Solute | Concentration. <i>gm. per 100 cc.</i> | Dilution | | |
|----------------------|--|----------------------------|----------------------------|----------------------------|
| | | 1 in 10 <i>per cent</i> | 1 in 20 <i>per cent</i> | 1 in 40 <i>per cent</i> |
| Glucose..... | 1.700 | 19 | 31 | 48 |
| Sodium chloride..... | 0.366 | 25 | 52 | 70 |

was repeated with all precautions to ensure sterility. Delayed hemolysis occurred as usual. Nor does the addition of 0.3 per cent *p*-cresol affect the delayed hemolysis curve.

pH Changes.—The changes in pH which occur during delayed hemolysis are slight. Immediately after diluting the oxygenated blood 1 in 20 with the glucose solution, the pH lies between 7.9 and 8.1 at 20°C. By the end of the prolytic phase, it has risen to about 8.3, and during the hemolytic phase, it gradually falls until it reaches its original level once hemolysis is complete. The actual pH values vary slightly from one blood sample to another, but the changes are always in the same direction and of the same order.

It was early discovered that this system is very sensitive to changes in the initial pH. Addition of small amounts of various substances (in order to determine their effect on delayed hemolysis) produced changes which, in most cases, corresponded to a change in pH. Thus addition of sodium dihydrogen phosphate to a final concentration of 0.1 per cent lowered the pH of the system by about 1.2 units, prolonged the prolytic phase slightly, and slowed down the hemolytic phase. Addition of disodium hydrogen phosphate to a final concentration of 0.1 per cent increased the pH of the solution by about 0.7 unit,

decreased the length of the prolytic phase slightly, and markedly accelerated the hemolytic phase.

In general, addition of any substance which alters the pH of the system, alters the delayed hemolysis curve.

Effect of Added Solute.—If delayed hemolysis be allowed to proceed until the degree of hemolysis has reached 40 to 50 per cent, and enough solid sodium chloride is then added to double the osmotic pressure of the mixture, the hemolytic phase is abruptly stopped. Although the final concentration of sodium chloride itself is not sufficient to prevent osmotic hemolysis, the combined osmotic pressures of the sodium chloride and the glucose are enough. If the experiment be repeated adding solid glucose in place of solid sodium chloride, the hemolytic phase is again stopped (see Fig. 3). This finding was somewhat unexpected but it agrees with the observation that delayed hemolysis occurs only within a restricted range of glucose concentrations. Although osmotic and delayed hemolysis are quite different phenomena, they are not entirely independent. The position is clarified when the accompanying changes in cell shape are examined.

When erythrocytes are suspended in isosmotic glucose solution, they retain their characteristic shape for many hours. Suspended in a solution containing 2.20 gm. glucose per 100 cc., they immediately assume the spherical shape of impending lysis. No change visible under the microscope occurs during the succeeding prolytic phase. When the hemolytic phase has begun, ghost cells begin to make their appearance and can be detected without difficulty. Stomatolysis follows, so that the percentage of ghost cells never reaches a very high figure until near the end of this phase. After hemolysis is complete, all ghost cells soon vanish. Agglutination does not occur.

If, during the prolytic phase or early in the hemolytic phase, the concentration of glucose be gradually increased by addition of the solid sugar, a curious transformation occurs. In the course of a few minutes, many of the cells assume what may be described as the shape of a "monoconcave disc;" *i.e.*, a sphere with a very obvious indentation. Further increase in the glucose concentration results in the formation of the normal biconcave discs. This reversion to the normal shape must be the result of increased osmotic forces, and since the assumption of the spherical shape is a necessary preliminary to osmotic and other types of hemolysis, it explains why the addition of solid glucose (or sodium chloride) suddenly stops the progress of the hemolytic phase.

Certain of the following experiments involve the addition of small quantities of various substances to the glucose solution to which the blood is later added. It is necessary that these substances be colorless and water-soluble; that they be neutral or almost so; and that they do not react with N/10 sodium hydroxide to give colored products or otherwise interfere with the determination of the percentage hemolysis.

Such additions will necessarily increase the osmotic pressure of the hemolyzing system and their concentrations must not be such that they will interfere with the process by virtue of the increased osmotic pressure which they will certainly produce. If 4 mg. of sodium chloride be dissolved in the 38.0 cc. of glucose solution used in the experiment (a final concentration of 0.01 per cent (weight per volume) when the blood has been added) and if the glucose solution have a concentration of 2.40 gm. per 100 cc., then the osmotic pressure of the hemolyzing system will be increased from 3.20 atmospheres at 20°C. (glucose only) to 3.28 atmospheres (glucose plus sodium chloride). The addition of this quantity of sodium chloride in no way affects the curve of delayed hemolysis but the addition of sodium chloride to a final concentration of 0.05 per cent slows down the rate of the hemolytic phase appreciably.

With these various provisos it is obvious that the range of substances, the activity of which can be investigated, is severely limited. Potassium cyanide, for example, even in a concentration as low as 0.01 per cent shortens the prolytic phase and accelerates the hemolytic phase. This is due to its effect on the pH of the system.

Sodium fluoride in concentrations up to 0.05 per cent has no effect on delayed hemolysis, compared with a control containing an osmotically equivalent amount of sodium chloride. Sodium iodoacetate in concentrations up to 0.1 per cent has no effect on delayed hemolysis. Above these concentrations, the osmotic effect of these two compounds becomes apparent and inhibits the phenomenon.

Cupric ion in concentrations up to 10^{-4} M, and mercuric ion in concentrations up to 10^{-5} M, have no effect; but the latter, in a concentration of 10^{-4} M prolongs the prolytic phase and slows down the hemolytic phase. Saturation with carbon monoxide (in place of oxygenation) does not alter the delayed hemolysis curve; nor does the presence of urethane in a concentration of 0.25 per cent.

One of the most interesting actions is that of phlorhizin. Addition of phlorhizin to a final concentration of 0.05 per cent (*ca.* 0.001 M) completely inhibits delayed hemolysis for several hours. Phlorhizin is intrinsically hemolytic, for its addition to a suspension of cells in sodium chloride solution increases the degree of hemolysis markedly. It is also slightly acidic and its addition to the glucose solution at this concentration lowers the pH (once the blood has been added) by about 0.3 unit. It has been stated above that addition of substances causing a lowering of the pH prolongs the prolytic phase and slows down the hemolytic phase, but the effect of phlorhizin is far beyond what would be expected were an alteration in pH its only effect (see Fig. 3). It is beyond doubt that the action of this substance is a genuine inhibition of delayed hemolysis.

Potassium Loss.—It was first shown by Davson and Danielli (3) that potassium escapes from cells suspended in hypotonic solutions prior to any

escape of hemoglobin. Such a leakage of potassium also occurs during the prolytic phase of delayed hemolysis.

Potassium has been determined in the supernatant fluid by the cobaltinitrite method using the modification of Abul-Fald (4). Where phlorhizin had been used as inhibitor, it is necessary to ash the specimen prior to precipitation for phlorhizin interferes with precipitation by cobaltinitrite. The potassium con-

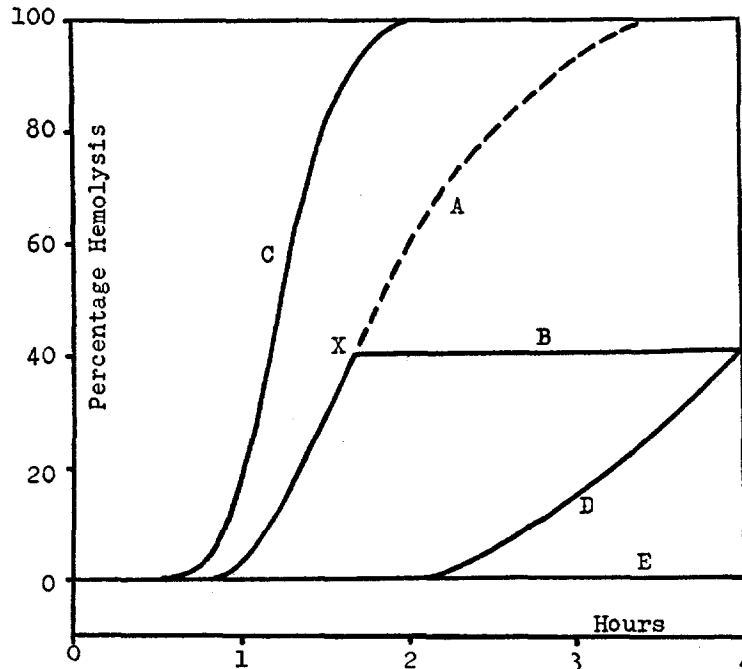


FIG. 3. A, Continuation of the normal delayed hemolysis graph; B, with addition of solid sodium chloride or glucose at the point X; C, with 0.1 per cent disodium hydrogen phosphate; D, with 0.1 per cent sodium dihydrogen phosphate; E, with 0.05 per cent phlorhizin. Glucose concentration = 2.20 gm. per 100 cc.; dilution = 1 in 20; temperature = 20°C.

tents of the plasma and cells of the individual blood samples have not been analyzed; these values for normal blood are very constant and can be taken as 20 and 400 mg. per 100 cc. respectively without invalidating any conclusions that may be drawn from the following type of experiment. Assuming also, a packed cell volume of 40 per cent for normal blood, it may be calculated that, in the standard experiment, 1 cc. of the supernatant will contain approximately:—

- 6 μ g. potassium if no potassium escapes from the cells, and,
- 86 μ g. potassium if all the potassium diffuses out of the cells.

Analyses have been confined to the escape of potassium during the prolytic phase; no determinations have been made on any sample which showed hemolysis. Typical results are shown graphically in Fig. 4. The rate of escape of potassium decreases when the temperature is lowered, but the more interesting observation is that it is not affected by phlorhizin, and that 90 per cent of the cell potassium can escape without the occurrence of hemolysis.

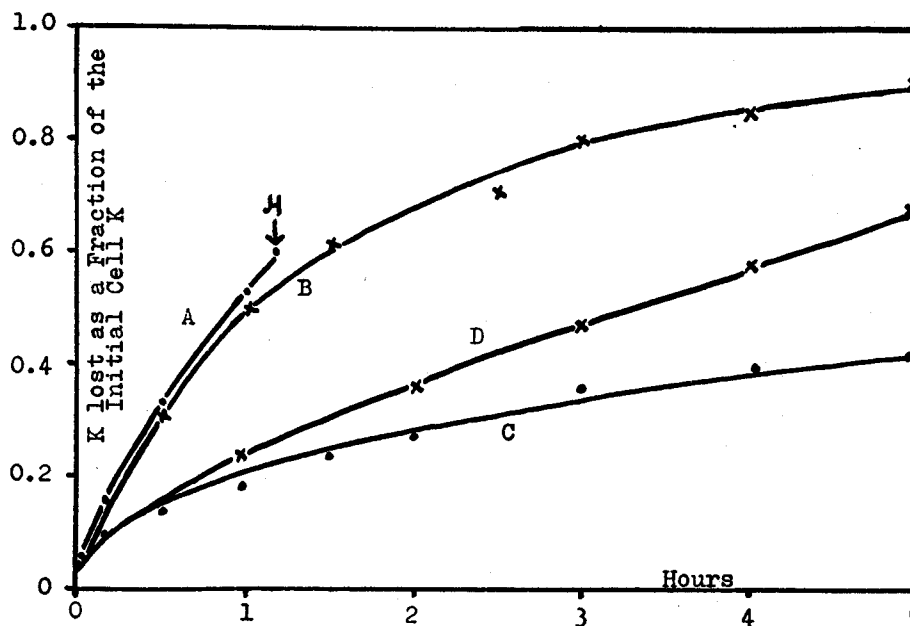


FIG. 4. Escape of potassium from erythrocytes during the prolytic phase of delayed hemolysis; A, at 20°C.; H = hemolysis. B, at 20°C. in presence of 0.1 per cent phlorhizin. C, at 8°C. D, at 20°C., in 5.50 per cent glucose.

Using a glucose concentration of 5.50 gm. per 100 cc. at 20°C., the potassium escapes more slowly, but at the end of 5 hours, about 70 per cent of it has been lost without the occurrence of hemolysis (Fig. 4, Graph D).

Phosphate Estimations.—Estimations of the total acid-soluble and inorganic phosphate in the cells during the prolytic phase by the method of Fiske and SubbaRow (5) gave results which added little information. 5.00 cc. aliquots of the system set up as in the prototype experiment were removed at intervals up to the onset of hemolysis, centrifuged rapidly, the cells washed once with 10 per cent sucrose, lysed with water, and precipitated with 10 per cent trichloroacetic acid. The total acid-soluble phosphate of the cells remained constant. The inorganic phosphate gradually rose from 2.7 mg. P per 100 cc. cells (after 1 minute) to 3.9 mg. P per 100 cc. cells (after 75 minutes). This change is not much beyond the limits of accuracy of the method with the amounts which have been employed, and is in the direction to be expected.

DISCUSSION

There are so many points of difference between osmotic and delayed hemolysis that they must be regarded as separate and distinct phenomena. The prolytic phase in osmotic hemolysis lasts only a few seconds with human blood; in delayed hemolysis in glucose solution it lasts 60 to 80 minutes under the conditions of the experiment. An increase in temperature at constant concentration reduces the degree of osmotic hemolysis, but greatly accelerates the process of delayed hemolysis, and *vice versa*. Decreasing the ratio of blood to hemolyzing system decreases the degree of osmotic hemolysis, but accelerates the hemolytic phase of delayed hemolysis, and *vice versa*. Addition of phlo-rhizin increases the degree of osmotic hemolysis, but acts as an inhibitor of delayed hemolysis. But delayed hemolysis is not entirely independent of osmotic pressure, for it occurs only within a limited range of concentration.

Now it may fairly be reasoned that the development of the hemolytic phase of delayed hemolysis is not a spontaneous reaction, and all experience suggests that it is a result of processes going on during the prolytic phase. Something is apparently happening during the prolytic phase which culminates in a state which permits of a fairly sudden onset of hemolysis, and the conditions are such that the cells have received the minimum of manipulation and the only chemical which has been added is glucose in solution.

The possibility of enzyme activity being involved must first be considered. No hemolytic agent, in the widest sense of the word, is present in lytic concentration at the outset and if one is developed during the prolytic phase it is presumably, but not necessarily, developed as a result of enzyme action; *e.g.*, the production or activation of a lysolecithin. The prolytic phase would then be an interval during which enzyme activity was increasing until it reached a critical point. The considerable effect of alteration in temperature would support a theory of enzyme action. Glycolysis is an obvious possibility and the experiments described above do not completely exclude glycolysis. Fluoride and iodoacetate do not interfere with delayed hemolysis in the maximum concentrations which can safely be used without interfering with the process by increasing the osmotic pressure of the system to a level at which hemolysis will not occur. There seems to be considerable doubt about the concentration of fluoride required to inhibit glycolysis: Danowski (6) finds that concentrations of 0.05 and 0.025 per cent retard glycolysis effectively; Bueding and Goldfarb (7) have shown that not even 2 per cent sodium fluoride will completely inhibit glycolysis. But a cursory review of the literature of the last 10 years shows that a concentration of 0.05 to 0.10 per cent fluoride is generally used to inhibit glycolysis; and the corresponding concentrations of iodoacetate are in the region of 0.01 to 0.05 per cent.

The highest concentrations of fluoride and iodoacetate which can be used in these experiments without introducing an osmotic pressure effect, are 0.05 and 0.1 per cent respectively. Neither of these compounds has any effect on

delayed hemolysis at these concentrations, which makes it unlikely that glycolytic mechanisms are involved.

It is also unlikely that a lysolecithin is involved. Were it so, there would be no apparent reason why delayed hemolysis can suddenly be stopped by the addition of solid sodium chloride or glucose. The resulting increase in osmotic pressure and the reversion to the biconcave disc form would not be likely to deter an enzyme such as lysolecithin from pursuing its activity. This argument does not necessarily apply in all cases; for example, an enzyme may assist in the transfer of glucose across the cell membrane, and a high extracellular concentration of solute would offset its action in increasing the intracellular concentration of that solute.

There are curious similarities between the actions of fluoride, iodoacetate, and phlorhizin in delayed hemolysis and in certain other phenomena. Wilbrandt (8) has shown that phlorhizin in concentrations of the order of 0.03 to 0.5 per cent inhibits the entrance of glucose into the red cell. Fluoride and iodoacetate do not. Wilbrandt's results do not lead to the conclusion that an enzyme is concerned in the transportation of glucose.

Furthermore, specific absorption of glucose depends, in part, on phosphorylation and it has been shown by other workers (9-11) that the first stage in phosphorylation, *viz.* the formation of glucose-1-phosphate, is inhibited by phlorhizin in concentrations of the order of $\text{m}/100$ (0.4 per cent), but not by fluoride or iodoacetate. These coincidences are interesting and it is not beyond the bounds of possibility that the permeability of the erythrocyte membrane to glucose has phosphorylation as a limiting factor. The process merits further investigation.

The loss of potassium during the prolytic phase of delayed hemolysis was expected, for although the erythrocyte maintains a considerable difference in concentration of potassium between the two sides of its membrane *in vivo*, it has been repeatedly shown that the cell membrane is permeable to potassium both *in vivo* and *in vitro*. Under the conditions described in the prototype experiment, about 60 per cent of the cell potassium has escaped into the extracellular fluid before hemolysis begins.

Now the escape of cell potassium cannot be the factor controlling hemolysis, for in the presence of 0.1 per cent phlorhizin (but under otherwise identical conditions) hemolysis is prevented but the escape of potassium continues. And it continues at the same rate as in the absence of phlorhizin (*i.e.* about 60 per cent in the 1st hour) until 90 per cent, or possibly more, of the potassium has been released without the occurrence of hemolysis. Under these conditions, phlorhizin apparently has the power of inhibiting lysis without interfering with the escape of potassium.

According to the colloid-osmotic hemolysis hypothesis, the development of a cation permeability results in swelling of the cell, and finally, hemolysis. The hemolysis is produced by a movement of water across the membrane

due to the difference in the colloidal osmotic pressure inside and outside the cell. The point at which the development of cation permeability produces lysis, has never been defined. Delayed hemolysis in glucose solution may be a special case of colloid-osmotic hemolysis in which glucose is slowly exchanged for potassium, but there is no evidence for this. The addition of a non-penetrating non-electrolyte to the extracellular fluid increases the osmotic pressure of this fluid, and since the solute cannot penetrate the cell, its effect is virtually that of a colloid in the sense that it can exert its osmotic pressure on only one side of the membrane. Sucrose, for example, can inhibit or greatly retard hemolysis when present in very low concentration; glucose may act similarly to a limited extent.

SUMMARY

There has been described a type of hemolysis which occurs under certain defined conditions when erythrocytes are suspended in glucose solution. It consists of a prolytic phase lasting about an hour, followed by a hemolytic phase lasting about 2 hours. The physical factors controlling this delayed hemolysis have been investigated. The system is especially sensitive to changes of pH and of temperature.

This type of hemolysis is inhibited by increased osmotic pressure and by phlorhizin, but not, as far as can be ascertained, by fluoride or iodoacetate.

It is possible, but not yet proved, that delayed hemolysis in glucose solution is dependent on enzymic activity. Phosphorylation may be the limiting factor.

During the prolytic phase the cells are easily permeable to potassium. It is concluded that the development of cation permeability is not a direct cause of hemolysis.

I would like to thank my former colleague, Dr. C. P. Stewart of the Clinical Laboratory, Royal Infirmary, Edinburgh, for the hospitality of his Department during the summer of 1950 when part of this work was carried out.

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