THE PRESERVATION OF LIVING RED BLOOD CELLS IN VITRO.

I. Methods of Preservation.

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There is practically no mention in the literature of attempts to keep red blood cells alive for a long time *in vitro*. Yet methods for their preservation might have much practical importance, and certainly would possess theoretical interest. Kept cells could be utilized for serum reactions, or for culture media, or even under certain circumstances for transfusion. This last possibility is the more worth considering because of the recent experiments of Abel and his coworkers[1] on plasmaphaeresis. They have demonstrated that the body tolerates well the repeated withdrawal of large amounts of plasma, rapidly placing new fluid in circulation; from which it follows that only the formed elements of blood need be supplied to a healthy animal depleted by hemorrhage.

The essential peculiarities of the red blood cells must be taken into account in any attempt to preserve them. They have little of the ability to adjust themselves to changes in external conditions which is possessed by many somatic cells[2] in common with the unicellular organisms. As bits of protoplasm without a nucleus, multiplication is impossible to them, and their existence is necessarily limited, whether they are in the circulation or *in vitro*. If they are to be kept alive outside the body, it must be in what one might term a state of suspended

[1] Abel, J. J., Rowntree, L. G., and Turner, B. B., *Jour. Pharmacol. and Exper. Therap.*, 1914, v, 625.

[2] Striking instances of this ability are afforded by the *in vitro* growth of tissue cells in plasma diluted with distilled water, in plasma from other animal species, in synthetic media, etc.

animation. Experience has taught that they are best kept in the cold. But even then whether the medium be serum or physiological salt solution, they break down within a few days,—much sooner than they are supposed to do in the circulation. Evidently there is room here for improvement in method, or else current estimates of the length of life of the red cell are faulty.

Injury during Washing.

For our experiments we have used cells of the rabbit, dog, sheep, and man. It seemed best in the initial tests to free the cells of plasma and suspend them in solutions of known constitution. Meltzer[3] has shown that red cells shaken in plasma to the slight extent necessary for defibrination break down much sooner on keeping than when the blood is allowed to clot undisturbed. We have asked ourselves whether injury during washing might not be responsible for the brief survival of washed cells. That in the case of dog, rabbit, and sheep cells, especially the first mentioned, some injury may occur is evident from the hemolysis frequently observed during washing even when the cells are handled most carefully.

Experiment 1.[4] *Cells Washed and Kept in Ringer's Solution Break Down Sooner than Those Left Undisturbed in Citrated Blood.*—Dog blood was taken in Ringer's solution containing 1 per cent of sodium citrate in the proportion of 1 part of blood to 4 of the solution. Some of the mixture was at once tubed and set aside. The cells of the remainder were washed twice with ordinary Ringer's solution and distributed in Ringer's plus citrate and in Ringer's solution, respectively, to the same amount as in the original blood-citrate mixture. The tip of the pipette with which the washing was carried out was kept beneath the surface of the fluid, and in other ways the handling was careful. There was no immediate hemolysis. Tubing was done as in the case of the unwashed blood. The preparations were kept in the ice box.

Two days later hemolysis was well marked in the preparations of washed blood. The cells of the original citrated blood were still intact.

Experiment 2. Cells Washed and Kept in a Plasma-Locke's-Citrate Mixture Hemolyze Sooner than Those Left Unwashed in the Same Mixture.—Rabbit blood

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[3] Meltzer, S. J., *Rep. Johns Hopkins Hosp.*, 1900, ix, 135.

[4] The experiments are all specimen instances selected from a number giving the same results.

was taken in Locke's[5]-citrate,—1 part of blood to 4 of the solution, which latter contained 1 per cent of citrate,—and some of the mixture was tubed and set aside. The cells of the remainder were washed twice with Locke's fluid and to some of the cells the original supernatant fluid, a mixture of plasma and Locke's-citrate, was restored, in the original proportion, and tubing done as usual.

After five days in the ice box there was well marked hemolysis of the washed red cells but none of those left undisturbed.

Experiment 3. Red Cells That Break Down Rapidly when Shaken in Locke's Solution Remain Intact in Plasma-Locke's-Citrate. The Plasma is the Protective Agent.—Some blood was taken from two normal sheep, two rabbits, two dogs, and two men into Ringer-citrate, in the usual 1:4 proportion. Half of each mixture was washed twice with Ringer's solution and the original volume restored with it. There was no hemolysis. The washed and unwashed specimens of blood were now placed in shaking tubes of uniform size. The same considerable air space was left in each tube and duplicate preparations were made. All were now shaken in a machine for 15 minutes, the tubes centrifugalized, and the amount of hemolysis read.

The washed human bloods shaken in Ringer's solution showed each a trace of hemolysis, the two sheep bloods considerably more, an amount which may be indicated by +, the rabbit bloods +++, and the dog bloods +++ and ++++, respectively. There was no hemolysis in any of the tubes containing plasma-Ringer's-citrate. The duplicate preparations confirmed these results.

As a corollary to this experiment, tests were made to see whether Ringer's-citrate without plasma had a protective action. Washed cells shaken in it went to pieces with the same rapidity as in ordinary Ringer's solution.

It is evident that the handling of red cells in salt solution, even to the small extent necessary to wash them, may be very injurious. Much of the injury is immediate and mechanical in character. Plasma has a notable influence to prevent it.

Protection.

From these observations it is plain that if washed red cells are to be properly preserved they must be protected during washing. Plasma obviously cannot be used for this purpose. Some simple agent is needed. And this was found in gelatin. $\frac{1}{8}$ to $\frac{1}{4}$ per cent of gelatin in Locke's solution protects cells absolutely against injury during washing, and even during prolonged shaking.

[5] The Locke's solution referred to here and elsewhere is Locke's modification of Ringer's fluid, but without any sugar: 9.2 gm. sodium chloride, 0.05 gm. sodium bicarbonate, 0.1 gm. potassium chloride, 0.1 gm. calcium chloride in 1,000 cc. of water.

Experiment 4. The Protective Influence of Gelatin.—Dog blood was taken in Locke's-citrate as usual (1 part to 4), distributed in equal quantity in eight tubes, and centrifugalized at high speed. From all except one of the tubes the supernatant fluid was now pipetted off as completely as possible, by means of a capillary pipette. The original volume was restored to six tubes with Locke's solution containing graded amounts of gelatin, from $\frac{1}{2}$ per cent to $\frac{1}{64}$ per cent, while to the seventh tube ordinary Locke's solution was added. All were now stoppered, shaken in a machine for 15 minutes, centrifugalized, and the hemolysis was noted. Shaking was then renewed for 15 minutes, and the tubes were placed in the ice box and examined after 3 days. The results are given in Table I.

TABLE I.

Hemolysis.

Time of shaking.	Locke's solution	+ $\frac{1}{64}$ per cent gelatin.	+ $\frac{1}{32}$ per cent gelatin.	+ $\frac{1}{16}$ per cent gelatin.	+ $\frac{1}{8}$ per cent gelatin.	+ $\frac{1}{4}$ per cent gelatin.	+ $\frac{1}{2}$ per cent gelatin.	Citrated blood.
min.								
15.....	+++	+++	+++	+−	0	0	0	0
30.....	+++	+++	+++	+−	0	0	0	0

Many subsequent experiments confirm this one. The protection afforded by the addition of $\frac{1}{8}$ per cent of gelatin to Locke's solution is for practical purposes perfect, and we have employed it regularly when blood was to be washed. Cells of the sheep, dog, and rabbit thus protected last days longer than when handled in ordinary Locke's solution.

Specific Differences in Fragility.

Protection is especially needed in the case of the red cells of the dog. Ottenberg, Kaliski, and Friedman[6] in some experiments on the normal hemagglutinins of this animal found that erythrocytes washed and placed in salt solution broke down too rapidly to be used. They were obliged to employ cells suspended in their own serum. Usually we have noted an abundant hemolysis within a few hours of dog cells washed after the ordinary methods. But if gelatin-Locke's be the washing medium, they may remain intact for several days. The erythrocytes from different dogs show marked differences in their period of survival.

[6] Ottenberg, R., Kaliski, D. J., and Friedman, S. S., *Jour. Med. Research*, 1913, xxviii, 141.

It has proved interesting to compare the resistance to mechanical injury (shaking) of the cells of different species. The results obtained in Experiment 3 express a general rule. Human cells have by far the greatest resistance. In their case, as we have repeatedly found, washing with Locke's solution after the ordinary method entails no perceptible injury, the cells remaining unhemolyzed as long as when gelatin-Locke's is used. Sheep cells come next in point of endurance. But washing in ordinary Locke's solution injures them somewhat, and it affects much more considerably rabbit cells and dog cells. All of these require protection by gelatin. An absolute scale of the fragility of bloods is difficult to prepare because marked variations are observed with the cells of different individuals and with the length of time that shaking is carried on. Dog blood at first breaks down far more than rabbit blood, but as shaking is continued the latter shows the greater destruction. In order properly to illustrate the findings, curves should be constructed like those that Smith and Brown[7] have used to record the percentages of erythrocytes breaking down in salt solutions of graduated hypotonicity.

Resistance to Shaking versus Resistance to Hypotonic Solutions.

Has the resistance of erythrocytes to mechanical injury any relation to their behavior in hypotonic salt solution?

TABLE II.

	Sheep C.	Sheep M. A.	Dog B.	Dog Dal.
Minimum resistance in salt solution=	0.76 per cent.	0.70 per cent.	0.58 per cent.	0.50 per cent.
Hemolysis on shaking.	Tr.	++	++++	++++

Experiment 5. The Resistance of Erythrocytes to Hypotonic Salt Solution, and to Shaking Vary Independently.—Blood was taken from two dogs often bled previously and from two sheep immunized against a bacterium and also bled often. 15 cc. of each blood were allowed to flow into an equal amount of a solution containing 4 per cent of sodium citrate, 0.6 per cent of sodium chloride, and the other salts of Locke's solution in the usual amount.

[7] Smith, T., and Brown, H. R., *Jour. Med. Research*, 1906, xv, 425.

Each specimen was divided into two equal parts; these were washed twice with Locke's fluid, and one was made up with it as a 10 per cent suspension for shaking, while the other, in thick suspension, was used for tests of resistance to salt solution. Shaking was carried on for 10 minutes and the tubes were centrifugalized and read. Two drops of the thick suspension were added to a number of tubes containing 3 cc. of hypotonic sodium chloride solution. The tubes of hypotonic salt solution were so prepared that each differed from the next by 0.02 per cent of sodium chloride. Readings were taken from these after 3 hours and again after 12 hours.

The results are shown in Table II. The percentages of salt solution recorded are those giving the faintest trace of hemolysis.

In this experiment the cells of the sheep were far less resistant to hypotonic salt solution than those of the dogs, but to shaking they exhibited much the greater resistance. The same phenomenon was evident in the case of individuals of one species (Sheep C. and M. A.). This inverse relationship between the two resistances does not always hold. Human erythrocytes, as we have found, are very resistant to shaking and quite resistant to hypotonic solutions. Hamburger[8] has pointed out that resistance to hypotonic salt solution is the result of many factors, and Rywosch[9] and Rous[10] respectively have proved that it is independent of resistance to chemical hemolysins and to a specific serum hemolysin. The fact that it is also independent of resistance to mechanical injury shows that the term fragility, so often used in connection with it, is a misnomer. Resistance to hypotonic solution is in no real sense an index to the fragility of red cells. A clinical investigation of this fragility as determined by shaking experiments might be not without importance.

Protection versus Preservation.

The injury sustained by cells washed and kept in salt solution is one cause for their rapid breaking down, but it is not the only one.

Experiment 6. Locke's Solution Is Injurious to Cells Kept in It after Washing.— Dog blood was taken as usual in citrate and twice washed, part with Locke's

[8] Hamburger, H. J., Osmotischer Druck und Ionenlehre in ihrer Bedeutung für die Physiologie und die Pathologie des Blutes, Berlin, 1912.

[9] Rywosch, D., *Arch. f. Physiol.*, 1906–07, cxvi, 229.

[10] Rous, P., *Jour. Exper. Med.*, 1909, xi, 763.

solution and part with Locke's solution containing ½ per cent of gelatin. The fluid was taken off, as far as possible, by means of a capillary pipette, and the cells were made to 12½ per cent suspension with ordinary Locke's. 1 cc. of each suspension was now added to 3 cc. of the original plasma-Locke's-citrate and to ordinary Locke's solution, respectively. Examination of the tubes after they had been 4 days in the ice box showed that there was no hemolysis of the cells washed in gelatin-Locke's and kept in plasma-Locke's-citrate, and only the faintest trace in the case of those washed in Locke's. Cells kept in Locke's after washing in gelatin-Locke's showed a + hemolysis, and those washed in plain Locke's a ++ hemolysis.

Here the protective action of gelatin is evident. But it is also plain that, whether red cells are protected during washing or not, they break down sooner in Locke's solution than in a mixture of Locke's with plasma and sodium citrate.

Preservation.

The search for a preservative was now begun. The form of the experiments was simple. Blood was taken in Locke's solution containing 1 per cent of sodium citrate, and its cells were twice washed with Locke's containing ⅛ per cent of gelatin. All possible fluid was then pipetted off, the cells made up in suspension with Locke's, and portions added to the various preservative fluids. These had, for the most part, Locke's solution, without sugar, as a base. We are aware that other solutions, Tyrode's for example, might have proved better; but Locke's fluid has the advantage of simplicity, and some preliminary observations suggested that cells last longer in it than in 0.95 per cent sodium chloride or in ordinary Ringer's fluid. The substances tested for preservative action were all of high purity. Small vials with sterile corks were used as containers, and only enough cells were placed in each to cover the bottom thinly. This was done because it was found that cells allowed to sediment in a thick layer exhibit very slowly the action of the supernatant fluid. So many vials were used in each experiment that it was not practicable to test the sterility of each preparation by means of culture, and instead reliance was placed on duplicate preparations, on repetition of the work, and on the graded character of the tests which ensured graded results except when some technical error was present. The preparations were kept in the dark under aseptic conditions at a temperature of 1° to 3°C. At first the

appearance of hemolysis was taken as the limit of the period of survival of the cells; but as the work progressed and more precise evidence was needed, it was obtained by transfusing the kept cells in bulk. The transfusion experiments are dealt with in the second part of this paper.

The action of sodium citrate was tested out because of the results with plasma-Locke's-citrate. But though a 1 per cent solution of it in Locke's fluid proved better for dog and rabbit corpuscles than plain Locke's solution, it had not sufficient preservative action for practical purposes.

The first step toward preservation of the cells seemed to be to obtain conditions that would not be deleterious to them. In salt solutions such as Ringer's and Locke's the conditions are far from natural. Red cells placed in them undergo a change of form, becoming spherical (Hamburger).[11] Weidenreich[12] attributes this to the absence of colloids. So tests were begun with Locke's solution to which colloids of various sorts had been added. Gelatin was first employed. It has no preservative action, but, on the contrary, in the amount of $1\frac{1}{2}$ per cent, which restores the normal shape to the cell (Weidenreich), it causes a gradual hemolysis and browning of the blood pigment. Washed agar, soluble starch, plain starch, and dextrin were also tried, but only the last proved useful, and that only for dog blood. The fluid pressed from serum coagulated by heat, and serum water made up to isotonicity with the salts of Locke's gave poor results.

A number of observers[13] have shown that the red cells are almost totally impermeable to sugars in contrast with other crystalloid substances. Solutions of sugar, then, not inconceivably might act like colloids in their effect on the shape of the cells. Sugars dissolved in Locke's fluid were accordingly tried and in them red cells were found to be preserved intact for a remarkable length of time. Dextrose and saccharose had the most marked action, but levulose, maltose, and lactose were not far behind. The cells still remained spherical, however.

[11] Hamburger, H. J., Osmotischer Druck und Ionenlehre in ihrer Bedeutung für die Physiologie und die Pathologie des Blutes, Berlin, 1912.

[12] Weidenreich, F., *Folia Haematol.*, 1905, ii, 95.

[13] Hedin, Hamburger, Kozawa.

The best results were obtained with mixtures of Locke's solution and isotonic solutions of the sugars in water.[14] Isotonic mixtures are better than hypertonic, though slight variations in tonicity are well borne. The small amount of sugar present in Locke's solution, when it is made up after the usual formula, is far below that which exerts a preservative influence. In isotonic sugar solutions alone erythrocytes keep fairly well, but they sediment in a firm layer which can hardly be suspended without hemolysis. The sugar solution and Locke's solution to be mixed together must be autoclaved separately, because in mixtures of them the sugar caramelizes during the autoclaving.

Optimum Preservative Fluids.

For the cells of each species a special preservative mixture is required. In our experience the optimum preservative for the washed cells of the sheep is one made by adding to Locke's solution sufficient saccharose in isotonic, watery solution to give an ultimate concentration of 2.8 per cent of saccharose. Cells twice washed in gelatin-Locke's remain unhemolyzed in this sugar medium for at least 3 or 4 weeks; and even after 2 months hemolysis is exceedingly slight. As is well known, the cells begin to break down within 3 or 4 days when washed and kept in Locke's solution in the ordinary way. For their proper preservation, it is essential that they be washed in an abundance of fluid, since a trace of plasma soon leads to hemolysis. The properly washed and kept cells retain their color; they take up and give off oxygen readily; and though they sediment into a rather firm layer they are easily made into a uniform suspension which passes readily through a filter paper. The cells in such a suspension are discrete, not stuck together. They will withstand repeated washings, but after washing they break down somewhat sooner in Locke's solution than do fresh cells. Cells kept for 3 weeks and for a month have been used for the Wassermann reaction and compared with cells freshly obtained from the same sheep. They had the same hemolytic titer and gave identical results in the reaction.[15]

[14] Saccharose 10.3 per cent, dextrose 5.4 per cent.

[15] For these tests we are indebted to Dr. Russell L. Cecil.

Dextrose is a fairly good preservative for washed sheep cells and human cells. But the latter are best kept in a saccharose-Locke's mixture containing about 5 per cent of the sugar (4.9 per cent). We have thus preserved them for 4 weeks without any hemolysis in the supernatant fluid or on washing. They are easily suspended, and like sheep cells take up and give off oxygen readily. Cells of both sorts begin to break down in about 10 days in a plasma-Locke's-citrate mixture.

Washed rabbit cells remain unhemolyzed longest in plasma-Locke's-citrate, slightly over 2 weeks as a rule. In a mixture of Locke's with glucose and saccharose, containing 3 and 6 per cent of the sugars, respectively, they stay intact from 9 to 12 days.

Dog cells are most difficult to keep. They are very frail, and hemolyze rapidly in their own plasma. In a saccharose-dextrin-Locke's fluid containing 1.6 to 2.7 per cent of the sugar and 2 per cent of dextrin, they show no breaking down for from 5 to 12 days, when they have been thoroughly washed with gelatin-Locke's before being placed in this medium. Preservation for so long a time must be regarded as notable for elements which under ordinary circumstances, washed and kept in Locke's or sodium chloride solution, begin to hemolyze at once. 2 per cent of dextrin added to the Locke's-sugar mixture aids the preservation of dog cells; but it cannot be regarded as uninjurious, since in amounts of 10 per cent it causes a gradual browning of the blood pigment to methemoglobin. This alteration is not evident with 2 per cent dextrin.

We are inclined to attribute the hemolysis that eventually occurs in the optimum preservatives to autolysis within the substance of the cells. That it is not due to the action of the preservatives themselves is well shown by comparing the results with cells allowed to settle in these fluids and cells agitated each day. In such an experiment gelatin must be present to prevent mechanical injury. If the fluid used as a preservative is harmful the cells brought in daily contact with it by agitation break down much sooner than those left to sediment. This is what happens in Locke's solution, for example. But with fluids such as the saccharose-Locke's that is optimum for washed sheep cells, the sedimented and agitated cells show the same gradual breaking down.

Concomitant Factors.

There are many factors besides the character of the preservative which conceivably may affect the period of survival of the cells. Sedimented cells lose their oxygen within a few days; they are exposed to a possible digestive action of the leukocytic pellicle, and to possible injury of the living erythrocytes by contact with dead cells scattered in the mass. Cells that are stirred remain bright and are not exposed to the dangers mentioned. But in experiments specifically directed to these points, we have found that red cells deprived of oxygen last no longer than those well supplied with it, that the leukocytic pellicle of normal blood does not cause hemolysis of the erythrocytes, and that contact injury of living cells by the dead can be disregarded. *A priori* one might suppose that cells would keep best in a very small quantity of preservative fluid, since in this there would be less loss of their diffusible substances. But tests in which the cells were stirred daily and allowed to settle through long and short columns of fluid, have failed to show that this has any importance. It is our practice to place the washed cells in five or six times their bulk of preservative fluid and allow them to remain in sediment until wanted.

Preservatives Are Not Protectives.

If the preservative solutions protect the cells against mechanical injury, gelatin can be dispensed with and a single solution used throughout.

Experiment 7. Dextrose and Dextrin Are Not Protectives.—Dog blood was caught in Locke's-citrate as usual and the mixture distributed in equal amount in 5 centrifuge tubes. The cells of 4 were washed twice with ordinary Locke's solution, Locke's containing 5 per cent of dextrin (Merck), 3 per cent of dextrose, and 1½ per cent of gelatin, respectively, and were suspended to the original blood-citrate bulk in these fluids. With the 5th tube the form of washing was twice gone through, using the original fluid. All were now corked, shaken for 15 minutes, and centrifugalized. There was no hemolysis in the tubes containing gelatin-Locke's and plasma-Locke's-citrate, but in the others it was abundant and of about the same amount in all.

Plasma possesses both protective and preservative qualities, but it is, of course, no simple fluid.

The Preservation of Leukocytes.

Some tests were made to determine whether leukocytes require the same protective and preservative media as the red cells. An aleuronat exudate of the dog containing many large mononuclear cells was washed, half in gelatin-Locke's, half in ordinary Locke's solution, and portions were distributed in various fluids for preservation. As a control, the red cells of the same dog were similarly treated. After 1 week in the cold, all were washed, this time in ordinary salt solution, and the ability of the mononuclear cells to take up rat erythrocytes was tested, with fresh dog serum as complement. Only those cells which had been placed for keeping in the original citrated plasma now showed phagocytosis, and this was independent of whether gelatin had been present in the original wash fluid. Mononuclears kept in Locke's solution, and in the sugar-Locke's, and the sugar-Locke's-dextrin mixtures most favorable to the red cells, failed entirely to ingest the rat corpuscles.

The Preservation of Unwashed Red Cells.

The results with red cells gave some ground for the hope that the erythrocytes of blood received directly into a medium preservative for the washed cells and thus kept, would remain unhemolyzed longer than under the usual conditions. Experiments along this line were undertaken. It was necessary, of course, to use some anticoagulant, and for this purpose sodium citrate was employed. Considerable quantities of blood were taken, distributed with the preservative mixtures in large test-tubes, and kept in the cold. To estimate hemolysis not only was the color of the supernatant fluid noted but the cells were stirred up in $\frac{1}{8}$ per cent gelatin-Locke's and centrifugalized. This frequently brought to light a marked hemolysis of which there had been no trace when the cells lay in sediment. Our freezing point determinations showed that a watery solution of sodium citrate containing 3.8 per cent of the salt is isotonic with 0.95 per cent sodium chloride. Tests were first carried out for an optimum blood-citrate mixture.

Experiment 8. The Optimum Blood-Citrate Mixture for Rabbit Cells.—The blood of two normal rabbits, X and Y, was taken in portions of 3 cc. into sodium

TABLE III.

Hemolysis.

Mixture.	Rabbit X. 26 days.		Rabbit Y. 34 days.		Rabbit X. 34 days.		Rabbit Y. 47 days.	
1 ½ cc. blood +	Supernatant fluid.	Wash fluid.	Supernatant fluid.	Wash fluid.	Supernatant fluid.	Wash fluid.	Supernatant fluid.	Wash fluid.
1/20 cc. 10% sodium citrate.....	++	+++++	Clotted.		++		Clotted.	
3/16 " 10" " "	++−	++++	++−	++−	++−		++−	++++
3/16 "7.6" " "	++−	++++	+	++−	++−		+−	++
3/8 "7.6" " "	++−	+++	Tr.	++−	++−		Tr.	++
3/8 "3.8" " "	++−	++	+	Tr.	++−		+−	+
1 "3.8" " "	+	+	Ft. Tr.	"	+		0	Tr.
1½ "3.8" " "	Tr.	+−	0	Ftest. Tr.	+		0	"

citrate solutions of various concentration and amount. Each mixture was divided into two equal portions and tubed. After many days one tube of each sort was tested for hemolysis, and still later (after 47 days, all told, in the case of Y) the second was examined. The color of the supernatant fluid was recorded and also the amount of hemolysis when the cells were suspended in 6 cc. of $\frac{1}{8}$ per cent gelatin-Locke's and centrifugalized.

The results are shown in Table III.

As this experiment shows, the preservation of the erythrocytes of citrated rabbit blood is much influenced by the amount and concentration of the citrate. We have repeatedly found that the best results are obtained, not with the smallest amount of citrate that will prevent clotting, but with equal parts of blood and isotonic citrate solution. The same amount of a somewhat hypotonic solution (2.5 per cent citrate) also gives good results. With human blood, on the other hand, the proportion and concentration of the citrate seem to have little influence. With sheep and dog blood no quantitative tests have been made.

As Experiment 8 shows, when rabbit blood is mixed with the right amount of citrate its cells remain intact for a long time. The addition of sugars further increases the preservation and the real length of life of the cells to a slight extent, as our transfusion experiments have shown. Sheep cells keep no longer in the presence of sugar. But for human blood cells, it has a remarkable preservative effect.

Experiment 9. The Preservative Action of Sugars on the Cells of Citrated Human Blood.—20 cc. of two human bloods were taken, that from X in an equal bulk of $2\frac{1}{2}$ per cent citrate in water, that from Y in a citrate-salt solution containing 2 per cent of citrate, 0.3 per cent of sodium chloride, and the other salts of Locke's in the same relative proportion. To equal amounts of the blood-citrate mixtures, Locke's solution and isotonic saccharose and dextrose solutions, respectively, were added. One tube of each of these preparations was examined after 13 days and another after 20 days. The sedimented corpuscles were tested for concealed hemolysis by suspending them in $\frac{1}{8}$ per cent gelatin-Locke's and immediately centrifugalizing. The results are given in Table IV.

Experiment 10. The Preservative Action of Sugars on the Cells of Citrated Human Blood.—Three human bloods were taken in various amounts of citrate, as in the case of Experiment 8 with rabbit bloods. To one of the mixtures which contained 3 parts of blood and 2 parts of isotonic (3.8 per cent) citrate solution, 5 parts of isotonic dextrose solution were added. After 4 weeks all the preparations were examined as usual, the cells being suspended in gelatin-Locke's and centrifugal-

TABLE IV.

Hemolysis.

Mixture	Blood X. 13 days.		Blood Y. 13 days.		Blood X. 20 days.		Blood Y. 20 days.	
3 parts citrated blood +	Supernatant fluid.	Wash fluid.	Supernatant fluid.	Wash fluid.	Supernatant fluid.	Wash fluid.	Supernatant fluid.	Wash fluid.
0	+−	+−	Tr.	+−			Tr.	+
1 part Locke's.	Tr.	+−	+−	+−	Tr.	++−	+	+
3 " "	+−	+−	++−	++−	++−	++	++−	++
7 " "	++−	++	+++	+++	++	++++	++	+++
1 " saccharose.	0	Tr.	0	+−	Ft. Tr.	+−	Ftest. Tr.	+
3 " "	0	Ft. Tr.	0	+−	0	Tr.	0	+−
5 " " / 2 " Locke's.	Ft. Tr.	" "	0	+−	0	Tr.	0	+−
1 " dextrose.	0	" "	0	Ftest. Tr.	Ft. Tr.	0	Tr.	Tr.
3 " "	0	0	0	0	" "	0	Ft. Tr.	0
5 " " / 2 " Locke's.	0	0	0	0	" "	0	+	+−

ized. There was no hemolysis of those kept with dextrose, but those of the citrate mixtures all showed a marked breaking down, independent apparently of the amount of citrate present.

Other experiments confirm these results. It is safe to say that the red cells of normal human beings can be kept intact for nearly or quite 4 weeks, when 3 parts of the blood are taken directly in a mixture of 2 parts of isotonic sodium citrate and 5 parts of isotonic dextrose solution. With citrate alone, in any quantity, hemolysis is well marked in less than 2 weeks.

Locke's Solution Is Injurious.

The action of Locke's solution to cause hemolysis, which is so plain in the case of washed cells kept in it, is no less evident when this solution is added to citrated blood. Experiment 9 illustrates this fact for human blood.

Experiment 11. The Injurious Effect of Locke's Solution on the Cells of Citrated Rabbit Blood.—The blood of two rabbits, X and Y, was taken in an isotonic citrate solution, and to portions of the mixtures, Locke's solution and an isotonic watery solution of saccharose, respectively, were added. The tubes were examined for hemolysis after many days had elapsed (Table V).

TABLE V.

Hemolysis.

Mixture.	Rabbit X. 26 days.		Rabbit Y. 34 days.		Rabbit X. 34 days.		Rabbit Y. 47 days.	
3 cc. blood + 2 cc. citrate +	Supernatant fluid.	Wash fluid.	Supernatant fluid.	Wash fluid.	Supernatant fluid.	Wash fluid.	Supernatant fluid.	Wash fluid.
0	+	+	Ft. Tr.	Tr.	+		0	Tr.
3⅓ cc. Locke's.	++++	+−	+++	"	++++		+++	+
3⅓ cc. saccharose.	+	Tr.	0	0	+		Ft. Tr.	0

The greater the amount of Locke's solution mixed with the citrated blood the greater is the destruction of the corpuscles (Experiment 9). But as in the case of washed cells, the injurious action of the Locke's fluid can be completely prevented by means of a sugar.

Experiment 12. The Effect of Saccharose To Prevent the Injurious Action of Locke's Solution on Rabbit Corpuscles.—Rabbit blood was taken into isotonic citrate and to portions of the mixture Locke's solution, isotonic saccharose solution, and combinations of the two were added. After many days the tubes were examined for hemolysis in the usual way (Table VI).

TABLE VI.

Hemolysis.

Mixture.	Percentage in ultimate mixture.				Rabbit X. 26 days.		Rabbit Y. 34 days.	
3 cc. blood + 2 cc. citrate +	Blood.	Citrate.	Sugar.	Locke's.	Supernatant fluid.	Wash fluid.	Supernatant fluid.	Wash fluid.
0	60	40	−	−	+	+	Ft. Tr.	Tr.
3⅓ cc. saccharose.	36	24	40	−	+	Tr.	0	0
3⅓ cc. Locke's.	36	24	−	40	++++	+−	+++	Tr.
8⅓ cc. Locke's. 9⅓ cc. saccharose.	13	9	41	37	+	Ft. Tr.	0	0
17 cc. Locke's. 19 cc. saccharose.	7	5	46	42	Tr.	0	0	Tr.

A similar action of dextrose is indicated in Experiment 9 with citrated human blood.

SUMMARY.

The erythrocytes of some species are much damaged when handled in salt solutions, as in washing with the centrifuge after the ordinary method. The injury is mechanical in character. It may express itself in hemolysis only after the cells have been kept for some days. It is greatest in the case of dog corpuscles, and well marked with sheep and rabbit cells. The fragility of the red cells, as indicated by washing or shaking them in salt solution is different, not only for different species, but for different individuals. It varies independently of the resistance to hypotonic solutions.

The protection of fragile erythrocytes during washing is essential if they are to be preserved *in vitro* for any considerable time. The addition of a little gelatin ($\frac{1}{8}$ per cent) to the wash fluid suffices for this purpose, and by its use the period of survival in salt solutions of washed rabbit, sheep, and dog cells is greatly prolonged. Plasma, like gelatin, has marked protective properties.

Though gelatin acts as a protective for red cells it is not preservative of them in the real sense. Cells do not last longer when it is added to the fluids in which they are kept. Locke's solution, though better probably than Ringer's solution, or a sodium chloride solution, as a medium in which to keep red cells, is ultimately harmful. The addition of innocuous colloids does not improve it. But the sugars, especially dextrose and saccharose, have a remarkable power to prevent its injurious action, and they possess, in addition, preservative qualities. Cells washed in gelatin-Locke's and placed in a mixture of Locke's solution with an isotonic, watery solution of a sugar remain intact for a long time,—nearly 2 months in the case of sheep cells. The kept cells go easily into suspension free of clumps, they pass readily through paper filters, take up and give off oxygen, and when used for the Wassermann reaction behave exactly as do fresh cells of the same individual. The best preservative solutions are approximately isotonic with the blood serum. If the cells are to be much handled gelatin should be present, for the sugars do not protect against mechanical injury.

Different preservative mixtures are required for the cells of different species. Dog cells last longest in fluids containing dextrin as well as a sugar. The mixture best for red cells is not necessarily best for leukocytes.

A simple and practical method of keeping rabbit and human erythrocytes is in citrated whole blood to which sugar solution is added. In citrated blood, as such, human red cells tend to break down rather rapidly, no matter what the proportion of citrate. Hemolysis is well marked after little more than a week. But in a mixture of 3 parts of human blood, 2 parts of isotonic citrate solution (3.8 per cent sodium citrate in water), and 5 parts of isotonic dextrose solution (5.4 per cent dextrose in water), the cells remain intact for about 4 weeks. Rabbit red cells can be kept for more than 3 weeks in citrated blood; and the addition of sugar lengthens the preservation only a little. The results differ strikingly with the amount of citrate employed. Hemolysis occurs relatively early when the smallest quantity is used that will prevent clotting. The optimum mixture has 3 parts of rabbit blood to 2 of isotonic citrate solution.

In the second part of this paper experiments are detailed which prove that cells preserved by the methods here recorded function excellently when reintroduced into the body.

DISCUSSION.

Our findings show that in experiments with cells washed in salt solutions there is a large source of possible error in injury done during washing. That it has been so long overlooked by investigators is probably due to the fact that mechanical injury does not alter hemolytic titer, and furthermore that such injury often manifests itself only after the cells have been kept for several days. The reasons for the injury in salt solution and for the protective action of gelatin are not clear. We were led to employ the latter through some observations of Weidenreich,[16] who showed that red cells placed in salt solution containing gelatin do not become spherical as in ordinary salt solution but retain their normal shape. From this he concluded that the shape of the cell is determined not only by the osmotic pressure of the surrounding fluid, but by its molecular force (*Molecularkraft*), as determined through its content in colloidal substances. But the amount of gelatin which will confer protection in salt solution is far too little appreciably to alter the molecular force, or, as we have found, to preserve the normal shape of the cell. For this latter effect, at least

[16] Weidenreich, F., *Folia Haematol.*, 1905, ii, 95.

as much as the $1\frac{1}{2}$ per cent of gelatin, recommended by Weidenreich, is necessary. In Locke's solution containing $\frac{1}{8}$ per cent of gelatin the cells are spherical. In the preservative sugar-Locke's solutions also the cells are spherical. It is interesting that cells kept for many days in such distorted condition should retain their usefulness for the body.

The ability of plasma to protect red cells against mechanical injury may explain to some extent the relatively long survival of the latter in the circulation. A clinical investigation of the mechanical fragility of red cells as determined by shaking experiments might yield results of value.

The preservative action of the sugars on red cells kept *in vitro* is largely dependent, when Locke's solution is present, on a prevention of injury from this latter. That Locke's solution should fail to be a physiological medium for red cells is scarcely surprising. Much recent work has gone to show that for the cells of different organs, different solutions are physiological. But Locke's solution is not merely lacking in some constituent needed by the red cells. It is actively injurious. This is well seen in the experiments with citrated blood, as also in the action of sugars to prevent the injury. The further preservative action of the sugars is perhaps referable to their ability to retard proteolytic digestion;[17] and the peculiarity that it is effective only in the case of certain red cells, to the fact that the erythrocytes of different species have a somewhat different permeability for sugars.[18]

Sheep cells washed and kept according to our methods are as suitable for the Wassermann reaction as fresh cells. But considerable manipulation of them is required. Formalization of the defibrinated blood, as practiced by Armand-Delille and Launoy,[19] and by Bernstein and Kaliski,[20] would seem more practical. The blood-citrate-saccharose mixture should prove useful for the preservation of rabbit and human corpuscles for culture media.

[17] Ogáta, M., *Arch. f. Hyg.*, 1885, iii, 204.

[18] Kozawa, S., *Biochem. Ztschr.*, 1914, lx, 146, 231.

[19] Armand-Delille, P., and Launoy, L., *Ann. de l'Inst. Pasteur*, 1911, xxv, 222.

[20] Bernstein, E. P., and Kaliski, D. J., *Ztschr. f. Immunitätsforsch., Orig.*, 1912, xiii, 490.