THE PRESERVATION OF LIVING RED BLOOD CELLS IN VITRO.

II. The Transfusion of Kept Cells.

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In the foregoing paper methods are described whereby red blood cells may be kept intact for long periods *in vitro*. It has remained to determine whether cells kept according to these methods are alive in the sense that they are capable of functioning in the animal body. This can be told by transfusion of the kept cells in bulk, with appropriate control. We have performed many such experiments, using rabbits.

The cells for preservation were obtained by bleeding etherized rabbits from the carotid or aorta into a Locke's-citrate mixture or an isotonic solution of citrate (3.8 per cent) in water. In some instances, the cells were washed, with gelatin-Locke's ($\frac{1}{8}$ per cent gelatin), and placed in a preservative solution; in others they were kept, without washing, in the original mixture of citrate and blood, or in citrate and blood plus a sugar. When they were to be used the supernatant fluid was pipetted away; and sometimes the sediment was suspended directly in Locke's solution to the original blood bulk and used for injection. More often it was washed once or twice with gelatin-Locke's and then suspended in ordinary Locke's. The suspension was filtered through two thicknesses of gauze; warmed to body temperature; and introduced under slight pressure into the ear vein of a rabbit which had just been bled.

The rabbits which furnished and received the cells were selected from a number tested against one another to rule out the presence of iso-agglutinins and isolysins. In the rabbit such antibodies are weak and infrequent. None of our results suggest their action. The animals chosen as recipients were bled in large amount from the ear

and the hemoglobin loss was replaced by the transfusion of kept cells. The bleeding and injection were repeated if the amount of kept cells allowed, as in instances when the cells from two rabbits had been preserved for the injection of a single individual. In every case a large proportion of the blood was drawn and replaced. The fate of the transfused cells was followed by blood counts, hemoglobin estimations, direct microscopic observations on the appearance of the blood elements, and by daily tests of the urine for hemoglobin (guaiac and spectroscopic tests) and bile (Gmelin and Bouvais tests). In addition, the percentage of reticulated red cells in circulation was noted in wet preparations stained with cresyl blue. This was done because an increase in the reticulated cells indicates an abnormal activity on the part of the bone marrow, such as would be the result of an increased destruction of the circulating erythrocytes. The rabbit's temperature was taken twice daily and its weight frequently recorded.

Some of the results may be briefly summarized and thus a repetition of data avoided. The bleeding and replacement of blood were always well borne. They took from one-half to three-quarters of an hour, and at the conclusion the animal usually went at once to eating. There was never a suppression of urine. Bile was never found in it and blood only in one instance, in a control experiment. The rabbit was, in this case, injected with cells kept, not in a preservative, but in Locke's solution, and a copious breaking down resulted with hemoglobinemia, hemoglobinuria, and death (Experiment 6).

In the specimen protocols that follow, the amount of blood drawn and replaced is recorded in percentage of the animal's total hemoglobin content. This is not to be confused with the percentage of hemoglobin in the circulating blood as obtained with the hemoglobinometer. It gives a much more precise idea of the proportion of formed elements dealt with, than would the blood volume. As is well known, when bleeding is done, even very rapidly, the last portion of blood contains much fewer cells than the first.[1] In thirteen instances in which we have taken into citrate from 30 to 50 per cent of the total calculated volume of a rabbit's blood and have com-

[1] Boycott, A. E., in Pembrey, M. S., and Ritchie, J., Text-Book of General Pathology, New York and London, 1913, 9.

pared the hemoglobin concentration of the drawn blood with that of the animal originally, it was found to be on the average 17 points lower by the Sahli scale. In general we have noted both the volume and the hemoglobin of the drawn blood, and have calculated from these the amount of cell suspension of known hemoglobin content that should be injected to restore the animal's hemoglobin content to the normal. In some of the early experiments this was not done and replacement was made by volume. According to Boycott and Douglas[2] a rabbit possesses 5.5 cc. of blood for 100 gm. of body weight. We have used this figure with hemoglobin estimations by the Sahli scale in determining the proportion of total blood pigment, and thus of erythrocytes, drawn and replaced. Since only 93 per cent of a rabbit's total hemoglobin is in the circulating blood (Boycott

TABLE I.

Time before or after transfusion.	Red blood corpuscles.	Hemoglobin.
		per cent
Before	4,890,000	87
3 hrs. after	4,590,000	85
24 " "	4,460,000	85
3 days "	4,510,000	83
6 " "	4,450,000	81

and Douglas), our estimate must be considered as rather less than the actual proportion. When two withdrawals and injections were carried out, allowance was made in the calculations for the mixed character of the second portion of blood withdrawn.

Experiment 1. Transfusion with Washed Cells Kept in a Dextrose-Locke's Solution and Washed before Use.—An animal weighing 1,700 gm. was bled 41 cc., and 41 cc. of a suspension of kept blood cells at once injected. By calculation some 30 per cent of the total hemoglobin was thus taken and replaced. The kept blood had been taken eight days previously, twice washed, and placed in Locke's solution to which 2½ per cent of glucose and ⅛ per cent of gelatin had been added. It was washed twice just previous to injection and suspended in Locke's solution. This handling brought about no hemolysis (Table I).

[2] Boycott, A. E., and Douglas, C. G., *Jour. Path. and Bacteriol.*, 1909, xiii, 256.

The animal was killed on the sixth day because of an infected ear. The reticulated red cells were not followed in this case. Fresh preparations of the blood failed to show shadows.

Experiment 2. Transfusion with Cells Kept in a Blood-Citrate-Saccharose Mixture and Washed before Use.—For this experiment the erythrocytes of two rabbits were used after they had been kept separately in mixtures of the citrated blood with saccharose for 11 and 12 days, respectively. Three parts of the blood were mixed for keeping with 2 of isotonic citrate and 5 of isotonic saccharose solution. The cells were washed once just before injection and suspended in Locke's solution. Following the injection there was no hemoglobin in the supernatant or wash fluid. The rabbit that acted as recipient was twice bled (40 and 6 cc.) and twice injected. 31 per cent of the animal's hemoglobin was thus replaced (Table II).

TABLE II.

Time before or after transfusion.	Red blood corpuscles.	Hemoglobin.	Reticulated cells.	Weight.	Urine.	Temperature.
		per cent		*gm.*	*cc.*	*°F.*
1 day before	6,500,000	91	25 in 500	2,025	65	102
Just before	6,100,000	90	19 " "		38?	102
3 hrs. after	6,500,000	90				
1 day "	6,640,000	87	24 " "		150	102.6
2 days "	6,540,000	86	14 " "		80	101.9
3 " "	6,540,000	83	12 " "	2,050	150	101.9
4 " "	6,840,000	91	13 " "		180	101.6
5 " "	6,420,000	85	11 " "		353	101.9
7 " "	6,730,000	89	9 " "		110	101.8
9 " "	6,640,000	90	11 " "	1,875	80	101.9

Fresh preparations of the blood made daily appeared normal throughout. On the third day after the transfusion a slight polychromatophilia was noted.

Experiment 3. Transfusion with Cells Kept in a Blood-Citrate Mixture and Washed before Use.—The blood of two rabbits was taken into citrate as usual (3 parts of blood to 2 parts of citrate) and kept for 11 days. The cells were washed once just prior to injection and suspended in Locke's solution. There was a slight hemolysis in the supernatant and wash fluids. The amount was calculated by comparing the color of these fluids with a laked preparation of the ultimate suspension, and it was found to equal about 0.2 per cent of this suspension. The recipient rabbit was bled 60 cc., or 36 per cent of the total hemoglobin content, and an equivalent amount was injected (Table III).

Throughout in fresh preparations the blood appeared normal. On the second day after transfusion occasional polychromatophilia was noted.

TABLE III.

Time before or after transfusion.	Red blood corpuscles.	Hemoglobin.	Reticulated cells.	Weight.	Urine.	Temperature.
		per cent		*gm.*	*cc.*	°*F.*
3 days before	6,320,000	87	12 in 500	2,250		101.8
1 day "	6,480,000	85	14 " "		70	101.8
Just "	6,010,000	80	18 " "		35	102.0
5 hrs. after	6,460,000	85				
1 day "	6,380,000	86	9 " "		60	101.4
2 days "	6,820,000	90	16 " "		35	102.2
3 " "	6,820,000	86	11 " "		64	102.2
4 " "	6,440,000	86	6 " "		51	101.9
5 " "	6,560,000	87	8 " "	2,375	140	101.5
7 " "	6,850,000	86	10 " "		250	101.2
9 " "	7,390,000	93	5 " "		172	101.4
11 " "	6,720,000	88	6 " "	2,300	170	102.0

Experiment 4. Transfusion with Cells Kept in a Blood-Citrate-Saccharose Mixture and Suspended Directly in Locke's Solution.—The blood of two rabbits was taken and kept as in Experiment 14 for 13 and 15 days, respectively. The supernatant fluid was pipetted off just prior to injection and the cells suspended in Locke's solution without washing. Two bleedings (of 40 and 50 cc.) were done and two injections. About 64 per cent of the total hemoglobin was thus withdrawn and more than replaced, as the blood examination showed (Table IV).

On the 2nd day after operation there were slight anisocytosis and polychromatophilia.

TABLE IV.

Time before or after transfusion.	Red blood corpuscles.	Hemoglobin.	Reticulated cells.	Weight.	Urine.	Temperature.
		per cent		*gm.*	*cc.*	°*F.*
1 day before	4,860,000	68	16 in 500	1,800		103.4
Just "	4,790,000	65	20 " "		150	103.2
3 hrs. after	5,670,000	81				
1 day "	5,220,000	76	16 " "		130	102.6
2 days "	5,280,000	73	21 " "		10?	103.0
4 " "	5,360,000	75	19 " "	1,825	170	102.4

Many experiments similar to these were done and with the same general results. Rabbit red blood cells kept for two weeks *in vitro* under suitable conditions can be used with good results to replace the blood lost in a hemorrhage. It is unnecessary to wash the cells which

may be simply suspended in Locke's solution after the preservative mixture is pipetted off. The preservative mixture which we have found best,—blood plus sodium citrate plus an isotonic saccharose solution—cannot be injected with the cells because of its content in citrate, but the small portion of it that remains with the cells after pipetting is not harmful. In blood-citrate mixtures without sugar the cells show some slight hemolysis (Experiment 2) after 2 weeks; and in several instances a slight drop in the cell count and hemoglobin percentage following transfusion, together with a rise in the number of reticulated red cells, has indicated that the kept cells were disappearing from the circulation and that the bone marrow was active in repairing the loss.

TABLE V.

	Time.	Red blood corpuscles.	Hemoglobin.	Reticulated cells.
			per cent	
Rabbit A.				
35 cc. taken of the total 78 cc. of blood.	Before operation.	5,500,000	106	2 in 500
	Day after "	2,400,000	48	53 " "
	19 days after "	5,000,000	77	27 " "
Rabbit B.				
45 cc. taken of the total 139 cc. of blood.	Before operation.	6,100,000	112	1 " "
	Day after "	3,600,000	65	15 " "
	18 days after "	5,870,000	110	14 " "
Rabbit C.				
42 cc. taken of the total 94 cc. of blood.	Before operation.	6,800,000	125	2 " "
	Day after "	3,000,000	54	13 " "
	25 days after "	6,000,000	112	35 " "

In striking contrast to these results are some that were obtained in control experiments.

Experiment 5. Effects of Bleeding Alone.—Two rabbits were bled as usual, but received no injection afterwards. Both died within a few minutes. The calculated blood volume of the animals was 88 and 60 cc., and the bleedings were for 50 and 28 cc., respectively.

Experiment 6. Effects of Bleeding Followed by Injection of Locke's Solution.—Three rabbits were bled and an equivalent amount of Locke's solution was injected intravenously. There was an immediate great drop in hemoglobin percentage and number of red cells. Regeneration was still incomplete after many days (Table V).

Experiment 7. Bleeding Followed by Transfusion of Cells Washed and Kept in Locke's Solution.—A rabbit with a calculated blood volume of 87 cc. was twice bled (44 and 28 cc.) and an equivalent amount of kept cells was introduced. Thus about 56 per cent of the total hemoglobin was replaced. The kept cells had been washed and preserved in Locke's solution for 11 days and they were again washed just previous to injection. At this time some hemolysis was noted. The animal died in less than 24 hours after the injection and on autopsy there were hemoglobinuria, hemoglobinemia, spodogenous spleen, and other findings typical of the breaking down of blood in large quantities.

These control rabbits all fared badly. Evidently the aid rendered by a transfusion of cells kept in a proper preservative is a real one. As Experiments 4 and 5 show, these cells function normally even after they have been kept *in vitro* for 2 weeks. We have performed a number of transfusions with cells kept longer. They remain unhemolyzed for as long as 4 weeks, but by the end of the 3rd week have largely lost their ability to be useful when reintroduced into the body, as shown by the fact that within a few days they disappear from the circulation. This disappearance is unaccompanied by any signs of hemolysis or, indeed, of other derangement. The animal eats well and may gain weight. The anatomical findings in such cases have interest as bearing on methods of blood destruction. Discussion of them will be reserved for another paper. From among the many experiments one will be given here to illustrate the facility with which the body disposes of blood elements no longer useful in the circulation.

Experiment 8. Transfusion with Cells Preserved Too Long in Vitro.—A rabbit weighing 1,925 gm. was bled 54 cc. and transfused with kept cells. 44 per cent (actual) of the total hemoglobin was thus taken and an amount equal to only about 37½ per cent put back. By calculation this should have caused a fall in hemoglobin percentage as determined with the Sahli instrument to 75 per cent after the transfusion, and that indeed was the figure obtained. The cells had been kept for 23 days in a mixture of 3 parts of blood, 2 parts of isotonic citrate solution, and 5 parts of isotonic saccharose solution. For injection they were washed once in gelatin-Locke's solution and suspended in ordinary Locke's. Neither the supernatant nor the wash fluids showed the slightest trace of hemolysis (Table VI).

There was at no time bile or blood in the urine. On the 2nd and 3rd days after the transfusion, there was marked polychromatophilia and some anisocytosis. Otherwise the animal seemed normal. The blood examinations on the 2nd day indicated that nearly all the transfused cells had disappeared from the

TABLE VI.

Time before or after transfusion.	Red blood corpuscles.	Hemoglobin.	Reticulated cells.	Weight.	Urine.	Temperature.
		per cent		*gm.*	*cc.*	*°F.*
4 days before	5,960,000	78	9 in 500			101.6
3 " "	5,850,000	81	12 " "	1,925	20?	101.8
1 day "					280	
Just "	5,810,000	80	8 " "			102.7
3 hrs. after	5,490,000	75				
1 day "	4,380,000	58	52 " "		100	102.2
2 days "	3,890,000	51	45 " "		120	101.8
3 " "	3,900,000	59	44 " "	1,790	20	101.4

circulation. The rabbit was killed on the 3rd day. The organs appeared normal. The spleen weighed only 0.7 gm. It gave a well marked iron reaction, and showed many phagocyted red cells.

A transfusion such as the above with intact cells kept too long *in vitro* is not helpful, or only indirectly so as supplying the constituents for new red cells; but in our experience it is not harmful.

The Preservation of Human Red Cells.

Human red cells can be preserved *in vitro* much longer than rabbit cells; and there seems little doubt that they could be profitably used for transfusion. Recently Weil[3] has reported upon a number of transfusions with whole citrated human bloods kept in the cold for several days. He employed 1 part of 10 per cent citrate to 10 parts of blood. But Lewisohn[4] has shown that citrate in this amount is dangerous to the organism; and we have found that human blood thus kept with citrate begins to hemolyze in about a week, the hemolysis being more dangerous because it is often completely concealed amid the sedimented corpuscles. Cells kept in a blood-citrate-dextrose mixture, according to the method detailed in the first part of this work,[5] remain

[3] Weil, R., *Jour. Am. Med. Assn.*, 1915, lxiv, 425.

[4] Lewisohn, R., *Surg., Gynec. and Obst.*, 1915, xxi, 37.

[5] Three parts of blood are caught in a mixture of 2 parts of isotonic sodium citrate solution (3.8 per cent) in water, and 5 parts of isotonic dextrose solution (5.4 per cent) in water. The preparation is allowed to remain undisturbed in the ice box until wanted, when the supernatant fluid is drawn off and the sedi-

intact for 4 weeks; and it would seem preferable to use cells thus kept, suspending them for injection in a little salt solution. By this method the plasma is lost; and it is of great importance for some conditions in which transfusion is employed. But in cases of simple exsanguination, as Abel and his coworkers have shown,[6] to furnish corpuscles to the body is sufficient. One might ask whether the slight remnant of plasma, sugar, and citrate remaining with the corpuscles and injected with them would be harmful. Our experiments with rabbits, and recent work on the injection of citrate and concentrated sugar solutions[7] prove this danger to be negligible. The experiments with rabbits show that if blood cells are kept too long to function, but are still intact when restored to the circulation, they are easily disposed of by the body. Tests for iso-agglutinins and hemolysins would be necessary before the transfusion of kept human cells.

SUMMARY.

In order to determine the availability for functional uses of red cells kept *in vitro* by our methods, transfusion experiments have been carried out with rabbits by which a large part of their blood was replaced with kept rabbit cells suspended in Locke's solution. It has been found that erythrocytes preserved in mixtures of blood, sodium citrate, saccharose, and water for 14 days, and used to replace normal blood, will remain in circulation and function so well that the animal shows no disturbance, and the blood count, hemoglobin, and percentage of reticulated red cells remain unvaried. Cells kept for longer periods, though intact and apparently unchanged when transfused, soon leave the circulation. Animals in which this disappear-

ment of cells suspended in Locke's solution. The slight whitish flocculus which is sometimes present above the sediment disappears in the Locke's solution. It has been our practice to filter the suspensions of kept rabbit cells through gauze, previous to use. Needless to say, the preparation must be kept sterile. The sugar and citrate solutions should be autoclaved separately, or the mixture of them can be put through a Berkefeld filter.

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

[7] Hustin, A., *Ann. et bull. Soc. roy. d. sc. méd. de Bruxelles*, 1914, lxxii, 104. Enriquez, *Presse méd.*, 1914, xxii, 121.

ance of cells is taking place on a large scale, remain healthy save for the progressing anemia. The experiments prove that, in the exsanguinated rabbit at least, transfusions of cells kept for a long time *in vitro* may be used to replace the blood lost, and that when the cells have been kept too long but are still intact they are disposed of without harm. The indications are that kept human cells could be profitably employed in the same way.