is released the patch of redness presently becomes defined; this redness disappears in about ten minutes after the circulation is released. Thus the reddening of the cooled forearm is not a direct response of the vessels to cold, for it can persist long after the skin has become warm again. It is due, so it is suggested, to the release of vasodilator substance or substances in the skin as a result of cellular damage; this substance is held *in situ* in the one arm, and its effects begin to decline only when, the circulation being restored, the substance begins to be removed (Lewis,* 1930).

Supercooling.—The skin, as will be described later in more detail, can be cooled below its freezing-point without freezing. It occasionally happens that such supercooling is followed by the formation of an acute wheal and flare on the skin (Lewis and Love, 1926). Such a reaction, were it a usual phenomenon, would in itself form conclusive evidence that the normal skin is directly injured by cold; for the reaction typifies the response to injury. But it is not a usual, it is an exceptional, phenomenon, depending on conditions that are still undefined. For that reason no further conclusion can be drawn from this evidence than that in subjects who display this conspicuous reaction cold can directly injure the skin.

Sensitivity to Cold.—A similar limitation applies to evidence derived from those who show extraordinary sensitivity to cold. In these a piece of ice laid on the skin for a minute is followed by conspicuous whealing. These subjects are rare; some of them suffer simultaneously from paroxysmal haemoglobinuria. The sensitivity of these skins to cold can be reproduced in other subjects by infiltrating the skin of the latter with serum from the original patient (Harris, Lewis, and Vaughan, 1929). The cases are remarkable in that whealing may be produced by temperatures as high as 18° C. (64° F.) or even, as in one case recently seen, as high as 25° C. (77° F.). I hesitate to use these two exceptional instances in evidence of the injurious effects of cold upon skins in general, in that they are not sufficiently understood and may be irrelevant; nevertheless, it is necessary here briefly to describe them, for it is also clearly possible that one or both may be no more than exaggerations of normal reactions.

Prolonged Cooling.—In recent observations I have found that if a normal hand or foot is immersed in water at 5° C. (41° F.) for a period of two hours, at the end of this time the reddened member is quite definitely swollen. The skin is less mobile: when thrown into folds these folds are broad; fine crinkling of the skin cannot be produced by manipulating it. The fingers may be swollen enough to prevent complete or easy flexion of the phalanges—either active or passive flexion. The pulps of the fingers are tenser than usual. The backs of the hands may pit perceptibly; sometimes they pit to an extent that makes it clear that not only skin but subcutaneous tissue is involved. This swelling, which may amount to as much as 15% increase of volume, gradually subsides in a few hours. It is not the simple result of imbibition of water by the skin, though some imbibition by the horny layer undoubtedly occurs, for it happens in a hand protected by grease or by the wearing of a loose rubber glove. It can occur recognizably in hands immersed in water for periods of hours at temperatures as high as 10° or 15° C. (50° or 59° F.). This effect is attributed to an injury by cold; presumably the reaction, as is customary in injury, involves the release of H-substance, which in turn acts on the blood vessels. Here it should be stated that histamine (1 c.cm. of 1 in 30,000 base) injected into the skin of the forearm, deep to the skin, is followed by obvious subcutaneous swelling. If several such swellings are made near to each other, distinct pitting of the arm occurs with firm pressure. Thus release of a histamine-like substance would account for swelling both of skin and of subcutaneous tissue.

In the swelling of a hand to cold, as much as 50 c.cm. increase of volume may be found. It is obvious, therefore, that if several of the limbs become cold for a period of hours, as when immersed in sea water or when submitted to other comparable exposure, a very substantial amount of fluid might be lost from the circulation. Perhaps this reaction may account for or con-

tribute to a state of circulatory collapse in wounded or unwounded men exposed to severe cold.

The observations upon human skin here referred to are all such as arise soon after cooling, and are in the nature of acute reactions of the skin; the reactions illustrate the simplest forms of inflammation, characterized by heat, redness, and swelling. There are in addition a number of conditions associated with more prolonged actions of cold and with more prolonged reactions. These will be considered separately in the next lecture.

REFERENCES

- Grant, R. T. (1930). *Heart*, 15, 281.
 — and Bland, E. F. (1931). *Ibid.*, 15, 385.
 — and Pearson, R. S. B. (1938). *Clin. Sci.*, 3, 119.
 Harris, K. E., Lewis, T., and Vaughan, J. M. (1929). *Heart*, 14, 305.
 Lewis, T. (1930). *Ibid.*, 15, 177.
 — (1931). *Ibid.*, 15, 351.
 — (1933). *Clin. Sci.*, 1, 175.
 — and Love, W. S. (1926). *Heart*, 13, 27.
 — and Pickering, G. W. (1936). *Clin. Sci.*, 2, 149.
 Pickering, G. W. (1932). *Heart*, 16, 115.

FAILURE OF IN VITRO TESTS AS A GUIDE TO THE VALUE OF STORED BLOOD

BY

P. L. MOLLISON, M.B., M.R.C.P.

AND

I. MAUREEN YOUNG, B.Sc.

(S.W. London Blood Supply Depot)

Many different solutions are still in use for the preservation of blood during storage. This state of affairs seems to be due mainly to the different criteria that are accepted for the comparison of one solution with another. It will be generally admitted, first, that the value of stored blood, contrasted with that of stored plasma or serum, lies chiefly in its content of erythrocytes, and, secondly, that the real worth of these stored erythrocytes must lie in their ability to survive in the recipient's blood stream after transfusion. Nevertheless, most workers have accepted changes occurring in the erythrocytes *in vitro* as an indication of the value of a given method of preservation—in some cases, at least, assuming that such changes are correlated with *in vivo* survival. The changes most commonly measured are alterations in the osmotic fragility of the erythrocytes and the rate at which spontaneous haemolysis occurs during storage.

Criterion of Positive Value of Preservatives

The power to inhibit haemolysis during storage is certainly a desirable property in any preservative solution, since there are limits to the amount of haemolysed blood that may be safely injected into the blood stream. This, however, is a negative property, correlated with safety but not necessarily with value. The only procedure which seems to us to provide a criterion of the positive value of a given preservative solution is to follow the fate of the transfused erythrocytes in the recipient's blood stream. The *in vivo* survival may be measured by the differential agglutination method originally described by Ashby (1919). In brief, this consists in the transfusion of cells of Group O (IV) to a recipient of Group A (II). After transfusion the recipient's blood contains two types of cells, O and A. The A cells can be "removed" with anti-A serum and the free (O) cells counted. Actually a small fraction of the A cells are not agglutinated, and constitute a "blank" value to be deducted from the total of free cells. By counting the number of donor cells surviving at different intervals of

* Correcting the interpretation of an earlier observation (Lewis and Love, 1926).

time after transfusion, a sufficiently accurate picture may be formed of the survival of the donor cells in the recipient's blood stream.

Using this method, we have tested eighteen different preservatives in a series of over 100 transfusions of stored blood of various ages. In each case the commonly accepted *in vitro* tests were also applied to the blood immediately before transfusion. Data are presented here which emphasize that these *in vitro* qualities are unsound guides to the actual survival of the same erythrocytes when transfused.

In Table I is set out the constitution of three different preservative solutions. In Table II observations on blood stored for varying lengths of time with these solutions are recorded. The *in vitro* properties of the erythrocytes of a given sample and the corresponding *in vivo* survival of the erythrocytes from the same sample are tabulated.

TABLE I.—Constitution of the Three Preservatives

(I) Citrate - Glucose (Rous - Turner):					c.cm.
3.8%* sodium citrate	..	..	..	..	400
5.4% glucose	..	..	..	..	1,000
Blood..	..	..	..	..	600
(II) Citrate - Glucose:†					c.cm.
3% sodium citrate	..	..	..	..	100
30% glucose	..	..	..	..	10
Blood..	..	..	..	..	430
(III) Citrate - Sucrose (Wilbrandt, 1940):					c.cm.
10% sodium citrate..	..	..	..	..	63
10.3% sucrose	..	..	..	..	375
Blood..	..	..	..	..	500

TABLE II.—Findings with the Three Preservatives described in Table I: (1) *In vitro* Tests. (2) *In vivo* Survival of Cells from the Same Sample. All the Recipients were Women suffering from a Secondary Anaemia

Time of Storage (Days)	1. In Vitro Tests			Recipient's Age (Years)	2. Survival In Vivo			
	Spontaneous Haemolysis (% Total)	Mechanical Fragility (% Free Hb after Shaking)	Osmotic Fragility (M.C.F.) (% NaCl)		Percentage Survival at Various Intervals of Time			
					4-8 hours (%)	24 hours (%)	1 week (%)	1 month (%)
<i>Blood Stored in Preservative I</i>								
16	0.2	25	0.64	22	99	101	87	70
18	0.4	15	0.74	30	—	96	89	58
21	0.1	29	0.67	28	105	99	94	69
24	0.6	32	0.76	35	85	66	76	52
27	0.3	34	0.72	29	111	84	73	44
28	1.2	34	0.80	38	—	88	65	42
34	0.9	50	0.78	31	84	54	0	0
<i>Blood Stored in Preservative II</i>								
12	0.1	18	0.47	45	112	93	100	71
18	0.2	22	0.51	38	122	88	67	46
19	0.7	32	0.62	30	89	76	73	36
23	2.2	34	0.64	22	—	62	39	13
29	3.0	40	0.68	30	84	39	32	0
<i>Blood Stored in Preservative III</i>								
7	0.3	20	0.27	33	90	93	83	66
9	0.1	28	0.28	73	82	91	75	49
12	0.5	37	0.29	55	80	76	54	0
14	0.6	38	0.37	28	92	32	—	—
16	0.7	40	0.37	—	88	29	—	—

Explanation of Methods and of the Terms used in Table II

On the day of transfusion the stored blood to be tested was carefully removed from the refrigerator. The bulk of the supernatant plasma was siphoned off and the remaining cells from two or more bottles of identical age were pooled.

* DeGowin, Harris, and Plass (1940) have pointed out that Rous and Turner used a different salt of sodium citrate from that in present use. The solution employed by us contains a higher percentage of sodium citrate than the original mixture.

† This is a minor modification of the preservative solution recommended in the Medical Research Council's War Memorandum No. 1.

A sample of this concentrated cell suspension was kept for the performance of *in vitro* tests, and the rest was used for transfusion. Three *in vitro* properties were measured:

1. The Degree of Spontaneous Haemolysis—i.e., the amount of free haemoglobin in the plasma expressed as a percentage of the total haemoglobin content of the sample.

2. The Increase in Haemolysis following a Standard Amount of Shaking in the Presence of Glass Beads.—The figures in Table II express the percentage of haemoglobin (Haldane) found in the plasma after shaking a sample of standard concentration. Previous workers have pointed out that the fragility of erythrocytes to shaking increases during storage, but the relationship of this increasing "mechanical fragility" to subsequent *in vivo* survival of stored erythrocytes has not previously been studied. It is interesting to note that Rous and Turner pointed out in 1916 that there is no constant relation between the mechanical and osmotic fragilities of erythrocytes.

3. Fragility to Hypotonic Saline.—In the table only the mean corpuscular fragility (M.C.F.) is recorded—i.e., the percentage NaCl in which 50% of the cells lyse (Dacie and Vaughan, 1938).

After the preliminary withdrawal of a sample of blood from the recipient the transfusion was begun, and was usually completed in two hours or less. A second venous sample was obtained five to ten minutes after the end of the transfusion. Further samples were taken four to eight hours after the transfusion, at twenty-four hours, and thereafter at suitable intervals.

The number of donor cells found in the first sample after transfusion was taken as 100% survival. Subsequent counts were expressed as percentages of this figure. To take this figure as 100% is, of course, not strictly accurate. If the

transfusion causes an increase in blood volume, and this is subsequently readjusted (i.e., after the collection of the first sample), the number of donor cells will appear to increase. Conversely, if destruction of erythrocytes has already occurred by the time that this sample is taken, to reckon as 100% the figure found makes survival seem better than it really is. From Table II it will be seen that a sample obtained four to eight hours after the transfusion often shows a relative increase of surviving cells compared with the number found immediately after transfusion and that destruction is dominant with the older bloods only. It may

be assumed from this initial relative increase that considerable destruction does not occur during the first few hours after transfusion, except with the older bloods, and the influence of this factor is therefore small.

A further check upon the actual number of donor cells found in the recipient's circulation was made by counting the red cells and measuring the volume of the blood transfused; then, by taking a figure for the recipient's blood volume based on the known height and weight, the expected concentration of donor cells in the recipient's blood stream could be calculated. In this way it could be demonstrated that approximately the expected number of donor cells appeared in the recipient's blood stream.

Results

The findings set out in Table II are representative of the larger number of samples that have been studied. In order to facilitate comparison the *in vitro* qualities of four samples stored with each of these preservatives have been averaged and set against the average *in vivo* survival of the same samples (arbitrarily taken at one month after transfusion) (see Table III). The ages of the samples stored with

TABLE III

Preservatives	In Vitro Tests			Survival In Vivo
	Spontaneous Haemolysis (% Total)	Mechanical Fragility (% Total)	Osmotic Fragility (M.C.F.) (% NaCl)	Percentage Survival at One Month
I. Citrate-Glucose (Rous-Turner)...	0.5	38	0.74	54
II. Citrate-Glucose (usual)...	1.5	43	0.61	24
III. Citrate-Sucrose (Wilbrandt)...	0.6	51	0.34	0

In the above table average figures for *in vitro* findings are compared with *in vivo* survival. For this comparison 4 examples of storage with Preservative I (at 18, 21, 24, and 28 days) have been contrasted with 4 examples with Preservative II (at 18, 19, 23, and 29 days). In the case of Preservative III, with which survival is so much inferior, 3 younger samples (12, 14, and 16 days) were chosen for comparison.

Preservatives I and II are comparable, but in the case of Preservative III younger samples have been chosen because of the much poorer survival. Since the cases are representative of a larger series that has been studied, the following conclusions seem justified:

The survival of transfused cells is better after a given period of storage with Preservative I than with Preservative II and is much less good with Preservative III.

The osmotic fragility of stored erythrocytes is no guide to their subsequent *in vivo* survival. It might appear from this small series that there is even an inverse correlation between osmotic fragility and survival; findings with other preservatives, however, show that there is no constant relation.

A small amount of haemolysis in a given sample does not necessarily imply good *in vivo* survival—e.g., Preservative III.

Fragility to mechanical trauma is only a very approximate guide to survival.

Other Preservatives

Other anticoagulant solutions studied in this way include heparin, sodium citrate with and without glucose in different concentrations and dilutions, "I.H.T.,"* citrate-dextrin, and combinations of sucrose, glucose, and sodium citrate; defibrinated blood was also tested. The findings with these other systems support the contention that fragility to hypotonic saline is no guide, and other commonly measured *in vitro* properties are at best a poor guide, to subsequent survival.

* "I.H.T." is the name given to an anticoagulant solution containing 0.5% sodium citrate, 0.7% NaCl, and traces of other salts; this solution has been much used in Russia (Bagdassarov, 1937).

On the basis of *in vivo* survival tests the preservative solutions used fall into two main groups: (1) systems containing no glucose; (2) systems containing glucose.

1. The first group includes heparinized blood, defibrinated blood, blood stored with "I.H.T.," with sodium citrate, and with citrate-sucrose and citrate-dextrin mixtures. After the transfusion of blood previously stored with any of these anticoagulant solutions for less than seven days the erythrocytes are found to survive extremely well—i.e., virtually as well as those of fresh blood. (After a fresh-blood transfusion it is found that the donor cells are slowly and steadily eliminated over a period of approximately 100 days—i.e., about one-third are eliminated during each month after transfusion (Ashby, 1919; Martinet, 1938).) Subsequently deterioration is rapid, and after two weeks' storage the majority of the transfused cells are usually eliminated from the recipient's circulation within twenty-four hours of the transfusion. To particularize, blood stored with a large relative volume of 3% sodium citrate falls below the average for this group, whereas blood stored with dextrin survives a little better than the average.

2. In general, blood stored with glucose survives extremely well after transfusion—i.e., virtually as well as fresh blood—when it has been stored for less than fourteen days. (This is a slightly more conservative estimate than one previously made with a similar type of anticoagulant solution (Mollison and Young, 1940).) There is a progressive deterioration during the third week of storage, but blood stored with glucose for three weeks is usually not completely eliminated from the recipient's circulation for more than a month after transfusion. These remarks apply to citrate-glucose mixtures in which the volume of preservative solution is small compared with the volume of blood (e.g., Preservative II). Survival was not found to be improved by increasing the concentration of glucose in the same relative volume of preservative solution. By adding a relatively greater volume of 5.4% glucose, however, survival is improved, and when the proportions of Preservative I are reached a clear-cut superiority is demonstrable. Thus after three weeks' storage with Preservative I survival is still almost as good as if the erythrocytes were fresh. It might appear from this that dilution is one of the factors responsible for the superiority of the Rous-Turner preservative solution. However, in the absence of large amounts of glucose, dilution was found to have an unfavourable effect upon survival, and it therefore seems that the problem requires further investigation.

The addition of sucrose to various citrate-glucose preservative solutions has a favourable effect upon the osmotic fragility of the stored erythrocytes but does not significantly influence their subsequent *in vivo* survival.

Practical Application of these Results

Owing to the low protein content of plasma obtained from blood stored with Preservative I it is not expected that this type of preservative will come into general use. In any case, the solution has to be modified if it is to be used directly for transfusion, because of the large amount of citrate it contains. DeGowin and Hardin (1940) have reported very satisfactory results from such a modification. Storage of blood with Preservative II gives adequate preservation for two weeks and has the advantage of providing plasma with a protein content of approximately 5 grammes %. This preservative solution is virtually the same as that at present in use at the Medical Research Council's Blood Supply Depots, and, as has been mentioned above, it does not seem to be improved by increasing the concentration of glucose.

It may be well to call attention to a few instances from the literature in which fallacious conclusions have been

drawn from *in vitro* tests, and to emphasize that some of the solutions that have been recommended are not in fact the best for preservation. Thus Rous and Turner (1916) concluded that sucrose was practically as good a preservative as glucose for human erythrocytes because of its power to inhibit haemolysis during storage. As we have shown, sucrose is an inferior preservative, judged by its failure to improve *in vivo* survival. Maizels and Whittaker (1939) suggested the use of dextrin because it, too, inhibits the haemolysis of blood during storage. *In vivo* survival tests show that the use of dextrin during storage has only a very slight effect on the survival of the subsequently transfused erythrocytes.

In the selection of the optimal citrate-glucose preservative solution, osmotic fragility tests are, as we have shown, positively misleading. Nevertheless, such tests have been adopted as the main criterion of value by many workers (Bushby, Marriott, Kekwick, and Whitby, 1940; Denstedt, Osborne, Roche, and Stansfield, 1941; Dubash, Clegg, and Vaughan, 1940); the preservative solutions recommended by these authors therefore contain only small amounts of glucose.

It is clear that no new preservative solution should be recommended until its effect on the subsequent *in vivo* survival of the erythrocytes has been determined.

Fate of "Fragile" Cells after Transfusion

A corollary of the view that the osmotic fragility of stored erythrocytes is an indication of their ability to survive *in vivo* must be that cells with an increased osmotic fragility soon rupture when introduced into the recipient's blood stream. Such a view has been expressed by Denstedt *et al.* (1941), for example. If this were true there would be two possible findings immediately after the transfusion of blood with a raised osmotic fragility. Either the recipient's blood stream would contain numerous fragile erythrocytes, in which case the osmotic fragility curve of the recipient would be found to be altered, or, if the donor cells had already ruptured, there would be free haemoglobin in the recipient's plasma and *in vivo* survival tests would show far less than the expected number of donor erythrocytes.

In practice neither of these possibilities is observed. On the contrary, first, transfusion of blood with an increased osmotic fragility does not affect the fragility curve of the recipient; secondly, following the transfusion of such blood, haemoglobinaemia is not observed; and, further, the expected number of donor cells is found, at least temporarily, in the recipient's blood stream. From this one can only conclude that the osmotic fragility of the donor cells has undergone a considerable change.

The above statement is based upon observations with preservatives containing glucose. For example, when blood that had been stored for twenty-one days in Preservative I was transfused, the curve of osmotic fragility of erythrocytes taken from the recipient, both before and after the transfusion, was estimated. Although the M.C.F. of the donor erythrocytes had been found to be very high (i.e., 0.78% NaCl) immediately before being used for transfusion, the osmotic fragility curve of samples taken from the recipient before and after the transfusion showed no difference. The transfusion occupied approximately forty-five minutes, and the sample was obtained about five minutes after the transfusion had been completed. Both before and after transfusion, in estimating the osmotic fragility, it was found that haemolysis began in 0.46% saline and that 50% of the cells lysed in 0.39% saline.

The *in vivo* survival test showed that the recipient's blood stream contained approximately 560,000 donor cells per c.mm. (the expected number) immediately after the trans-

fusion, and thus the only possible conclusion is that the donor cells must have undergone a change in osmotic fragility.

This reversal of changes in osmotic fragility may also be studied *in vitro*. For instance, in the case referred to above a sample of the donor cells was placed in fresh compatible plasma for one hour and its osmotic fragility was then re-estimated. It was found that the M.C.F. of the cells had practically reverted to normal (actually 0.45% NaCl). This effect was found to be constant with this particular preservative. It should be pointed out that although the majority of the cells in a given sample succeed in re-acquiring an approximately normal resistance to hypotonic saline, a few may rupture. The most likely explanation of this reversal in osmotic fragility is that glucose is washed out of the cells when they are placed in a solution of relatively high tonicity at room temperature.

Reversal of osmotic fragility was not found to occur with blood stored in certain other preservatives. For instance, when erythrocytes that had been stored with Preservative III were placed in fresh plasma their mean corpuscular fragility did not appear to be affected. This is probably because alterations in the base content of the erythrocytes have occurred and because base is not so mobile as glucose. Nevertheless, the fact that the M.C.F. of stored erythrocytes can be altered in certain circumstances by allowing them to stand in fresh plasma is a further reason for regarding the osmotic fragility, as usually measured, as being an artificial criterion of value.

Summary

The *in vivo* survival of the erythrocytes of stored blood following transfusion cannot be predicted from a knowledge of the results of the commonly accepted *in vitro* tests made upon the sample. The results of work based upon such *in vitro* tests may therefore be completely fallacious.

Many different preservative solutions have been tested for their effect on the *in vivo* survival of the subsequently transfused erythrocytes. Some details of results obtained with three of these solutions are given and results with the rest are summarized.

We should like to thank Dr. J. F. Loutit, director of this depot, for facilitating these investigations. We also wish to thank Dr. M. Maizels for helpful criticism.

REFERENCES

- Ashby, W. (1919). *J. exp. Med.*, **29**, 267.
- Bagdassarov, A. (1937). *Sang*, **11**, 466.
- Bushby, S. R. M., Marriott, H. L., Kekwick, A., and Whitby, L. E. H. (1940). *Lancet*, **2**, 414.
- Dacie, J. V., and Vaughan, J. M. (1938). *J. Path. Bact.*, **46**, 341.
- DeGowin, E. L., and Hardin, R. C. (1940). *British Medical Journal*, **2**, 1.
- Harris, J. E., and Plass, E. D. (1940). *J. Amer. med. Ass.*, **114**, 850.
- Denstedt, O. F., Osborne, D. E., Roche, M. N., and Stansfield, H. (1941). *Canad. med. Ass. J.*, **44**, 448.
- Dubash, J., Clegg, O., and Vaughan, J. M. (1940). *British Medical Journal*, **2**, 482.
- Maizels, M., and Whittaker, N. (1939). *Lancet*, **2**, 1219.
- Martinet, R. (1938). *Sang*, **12**, 15.
- Mollison, P. L., and Young, I. M. (1940). *Quart. J. exp. Physiol.*, **30**, 313.
- Rous, P., and Turner, J. R. (1916). *J. exp. Med.*, **23**, 219.
- Wilbrandt, W. (1940). *Schweiz. med. Wschr.*, **70**, 731.

The American Film Centre, 45, Rockefeller Plaza, New York City, has established a section on films in health education and medicine under the charge of Dr. Adolf Nichtenhauser. The new section will be a clearing-house and information centre on the use and production of technical films of this character. From it the centre will evaluate existing health films in collaboration with health experts and publish lists of recommended films. Attention will also be paid to the technical medical film, especially with regard to its use in medical schools. The American Film Centre is an educational organization established three years ago and supported by the Rockefeller Foundation, which has now made a three-year grant towards the Section on Health and Medical Films.