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Historical Review

THE INTRODUCTION OF CITRATE AS AN ANTICOAGULANT FOR TRANSFUSION AND OF GLUCOSE AS A RED CELL PRESERVATIVE

In 1918, Oswald Robertson published a remarkable paper, describing transfusions of stored red cells given near the front line in France in the First World War (Robertson, O.H., 1918a). Only 4 years had elapsed since the first few transfusions of citrated blood had been given to human subjects; the method of storage which was used had not previously been applied to transfusion in humans. For the first time, a bank of units of stored blood was created and shown to be of great practical value.

Before describing Robertson's background and work, something will be said about the introduction of citrate as an anticoagulant and of glucose as a red cell preservative. These two developments were of vast importance to blood transfusion: the use of citrate made it possible to separate donor and recipient in space; the use of glucose made it possible to separate donation and transfusion by a substantial period of time.

THE INTRODUCTION OF CITRATE AS AN ANTICOAGULANT

Discovery of the role of calcium in clotting

Hammarsten (1875) noted that $CaCl_2$ had a striking effect on the speed of coagulation as well as on the amount of fibrin formed. Soon afterwards, Arthus & Pagès (1890) concluded that calcium salts were absolutely necessary for clotting. They found that if blood is mixed immediately with oxalate (1 g oxalate per 1000 g blood) it remains fluid for several weeks at +3°C and that it clots if calcium salts are added. Pekelharing (1892a) speculated that the addition of oxalate to blood might be useful in transfusion but would probably be dangerous. However, in the previous year Wright (1891) had actually used oxalate successfully as an anticoagulant in transfusion in dogs. He took as much blood as could be drawn off without causing death from haemorrhage, mixed it with oxalate (probably one-tenth volume of 1–2%) to prevent clotting and reinjected it. One of two dogs treated in this way went through the procedure twice in a fortnight. A third dog was transfused with 100 cc of blood from another dog, calculated to be equivalent to one-third of its blood volume. The dogs appeared to be none the worse for their experience.

Discovery of the anticoagulant properties of citrate

Although Griesbach (1891) stated that ammonium citrate interferes with clotting, he did not understand the mechanism. Pekelharing (1892b), who found that blood (90 cc) mixed with citrate (10 cc of a 5% solution) remained fluid, realized that the effect was due to the affinity of calcium for citric acid.

Arthus (1902) was puzzled by the fact that anticoagulants such as oxalates and tartrates precipitated calcium whereas citrate did not, and he concluded that its action could not be explained. In a paper written almost immediately afterwards, Sabbatini (1902) wrote 'Je crois, au contraire, qu'il y a une explication possible, et je dirais encore surement démonstrable; la voila.' He went on to say that citrate, in a dose of about 1·7 g/l of dog's blood, prevents coagulation indefinitely because it reduces the concentration of ionized calcium to below the minimum needed for clotting. Conductivity measurements were used to demonstrate that citrate bound calcium ions, i.e. without precipitation.

The first transfusions of citrated blood

In the period 1914–15, great excitement was caused in the medical world by the announcement of the first use of citrate for transfusion in clinical practice. No one seems to have pointed out that citrate had been used entirely successfully many years earlier for transfusions in animals. Furthermore, as early as 1893, Wright (later, Sir Almroth) had realized the potential value of citrate in transfusion (Wright, 1893). After discussing the fact that citrate keeps blood liquid by decalcifying it, he put in a footnote 'I may perhaps be allowed to remark in this connection that this variety of decalcified blood (i.e. citrated) appears to be more suitable for purposes of transfusion than even the oxalated blood proposed by me in a previous communication' (Wright, 1891). Of course, as far as humans were concerned, this was an idea too far in advance of its time: there was little point in surmounting the technical problem posed by blood clotting when transfusion remained dangerous because of the risk of incompatibility due to yet-to-be-described natural antibodies.

In many animals, in contrast, alloantibodies do not pose a threat in first transfusions and the introduction of anticoagulants had a great deal to offer. In an experiment conducted by Boycott & Douglas (1909) at Guy's Hospital, in which rabbits were transfused with allogeneic blood, the citrate method was used 'because with the artery–vein method [of transfusion] it is difficult to determine exactly how much has been transfused without intermediate manipulation *in vitro*'. A total of 0·4–0·5 g of sodium citrate was found to be enough to keep any quantity up to about 100 cc of rabbit blood liquid at 37°C for many hours, provided that blood was obtained 'from the first main gush which issues from the divided carotid'. In various

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experiments, about 50–80 cc blood was transfused; the authors commented 'we had no evidence that the citrate plasma was in any respect toxic.' The amounts transfused correspond very roughly to giving 2 l of citrated blood to humans. If the results had been known to a clinician interested in human transfusion they would surely have supplied strong justification for trying citrate in man.

Another account of the use of citrated blood was provided by Todd & White (1911). Their work was concerned mainly with demonstrating the multiplicity of red cell antigens in cattle, and large amounts of blood were injected intravenously from one animal into another. Animals were bled from the jugular vein into graduated vessels containing a known volume of 4% citrate. The citrated blood from each animal was kept at body temperature and transfused as rapidly as possible into the jugular vein of the other. Eight animals were injected in this way with quantities varying from about 2 to 4 l. In one example, a bull weighing 412 kg was bled of 2·5 l and then received an i.v. injection of 2540 cc citrated blood, equivalent to 2117 cc of pure blood. The animal must then have received 423 cc of 4% citrate, i.e. 16·9 g or about 0·04 g/kg. For comparison, an adult human weighing 70 kg and receiving 450 cc blood mixed with 100 cc of 3% citrate would receive 3 g, which also corresponds to about 0·04 g/kg. Particularly in view of the fact that the transfusions were given 'as rapidly as possible', this paper provides further strong evidence of the safety of citrate.

In the paper, no reference is made to the previous use of citrated blood for transfusion. Todd & White (1911) seem to have taken it for granted that it was the obvious choice. The present author finds it frustrating to recall that in 1940, when he was introduced to these two scientists over a cup of tea and received a reprint of their work, he did not realize that he had lost forever the opportunity of finding out how they came to use citrate.

Transfusion of citrated blood in humans

The first transfusion of citrated blood given to a human seems to have been performed by Hustin (1914), who wrote that he thought of trying citrate as an anticoagulant because he knew from 'Gengou's thesis' that citrate had 'dispersal properties' and that it could stop a suspension of mastic from gelling. From experiments, he concluded that 0·2% citrate would prevent blood from clotting for 30 min. In the course of his experiments, he noted that red cells to which citrate had been added oxygenated themselves less well than red cells in sodium chloride, and drew the conclusion that citrate should be replaced, at least partially, by a non-toxic substance 'qui retarderait également' coagulation; he chose glucose and fixed on a glucose–saline solution. He first transfused citrated dog blood to a dog, then citrated human blood to a dog; finally, in March 1914, he transfused blood from one man to another. Concerned with the question of priority, Hustin (1934) later commented that one of the accounts which he published in 1914 (he published the identical text in three journals) was listed in the *Index Medicus* of 14 October 1914 but was not mentioned in the American press.

The next to publish was Agote (1915a). After getting colleagues to test citrate in animals and after receiving a large dose intravenously himself without untoward effects (Alcorta, 1989), on 9 and 14 November 1914 he supervised the transfusion of two patients, each of whom received 300 g of blood mixed with 3 g of sodium citrate. These details were given to *La Prensa*, the leading daily newspaper in Buenos Aires, and appeared in *The New York Herald Tribune* on 15 November 1914 (Agote, 1915a). Two months later, Lewisohn (1915a) published a short paper reporting that he had found blood mixed with citrate (0·2%) to be non-toxic in dogs, and that he had successfully transfused two patients. He mentioned Hustin's (1914) work and said that Weil had also transfused citrated blood successfully, but he did not mention Agote. Agote (1915b) believed that his claim to priority was being overlooked, but, as Rosenfield (1974) pointed out, Lewisohn had been interested for some years in using citrate in human transfusion and publication of his preliminary results was probably precipitated by the newspaper articles recounting Agote's work.

Weil (1915) found that guinea pigs or dogs could be practically exsanguinated and then rapidly restored by the transfusion of citrated blood; no unpleasant reactions were observed. Human patients were treated in the same way, using amounts ranging from 10 to 350 cc of citrated blood. Blood was aspirated by syringe from a vein, immediately mixed with citrate (1 cc 10% citrate to 10 cc blood) and injected into the patient using a syringe and stopcock. The blood was fresh in some instances; in others it was 3–5 d old; there was no apparent disturbance of any kind except a transient rise in temperature and polyuria.

In 1915, Lewisohn (1915b) published a second paper in which he described 22 transfusions given to 18 patients. Sometimes, as much as 1000 cc was transfused, but more often 500 cc; 0·2% citrate was found to prevent clotting for 3 d. Lewisohn (1915b) stated that he could find no reference to previous work on citrate, although he came across Hustin's (1914) paper after he had completed his toxicity experiments in dogs. He pointed out that Weil's (1915) suggested dose of 1% citrate was practicable only for transfusions not exceeding about 250 cc (untrue!) and that, on the contrary, Hustin, considering that a concentration of 0·2% was too small, had recommended the addition of an equal volume of glucose/saline to citrated blood, rendering his method impractical when, for example, 1000 cc of glucose–saline was added to 1000 cc citrated blood. In conclusion, Lewisohn (1915b) considered that his own method of using 30 cc of 2% citrate for 300 cc of blood was the best, and this was generally accepted.

In retrospect, it seems clear that none of the people involved in the introduction of citrate for human transfusion in 1914–15 realized how much work had already been carried out and that the idea of using citrate for human transfusion had been proposed as early as 1893.

Amounts of citrate used

One can begin by calculating the amount of citrate which would be required to prevent clotting if 1 mole of citrate bound one atom of calcium firmly. The atomic weight of

calcium is 40 and its plasma concentration is about 2·5 mmol/l (≈ 10 mg/dl). The molecular weight of trisodium citrate (dihydrate) is 294, so that a concentration of 2·5 mmol/l would correspond to 73·5 mg/dl plasma or, assuming a packed cell volume (PCV) of 0.4, about 44 mg/dl whole blood. However, citrate does not bind calcium firmly, and Sabbatini (1902) considered that a ratio of three molecules of citrate to one of calcium was necessary to prevent clotting. In a discussion of this subject at a much later date, Loutit & Mollison (1943) pointed out that with the disodium citrate–glucose solution which they had tested the concentration of citrate in the final mixture of plasma–diluent was well over 20 mmol/l and yet the occurrence of occasional clots was still a problem.

The amounts of citrate used by various early authors expressed as approximate molar ratios of citrate to calcium, making the assumptions described above, are as follows: Arthus (1902) 5:1; Sabbatini (1902) 4:1; Boycott & Douglas (1909) 10:1; Todd & White (1911) 20:1; Hustin (1914) ?:1; Agote (1915a) 6:1; Lewisohn (1915a) 5:1; and Weil (1915) 23:1.

Toxicity of citrate

An early investigation made it clear that citrate is rapidly metabolized. Salant & Wise (1917) found that 20 s after injecting about 50 mg/kg into dogs and rabbits only about one-third was left in the circulation. In human adults, toxic effects such as prolongation of the Q–T segment of the ECG and even cardiac arrest are observed only when the rate of citrate infusion exceeds about 0·04 mmol/kg/min, corresponding approximately to 1 l of citrated blood in 10 min. In practice, citrate toxicity is a problem only in special circumstances, such as in patients with liver failure (for references and discussion, see Mollison *et al*, 1997).

Spread of citrate transfusion as the preferred method

In 1915, on the eve of the widespread use of the citrate method, the two best methods of transfusion available were the multiple syringe and cannula method (Lindeman, 1913) and the four-way stopcock method (Unger, 1915). With both methods, the blood was transferred so rapidly that it was possible to transfuse large quantities without using anticoagulant. However, donor and recipient had to be in close proximity and at least two trained operators were needed. In writing about blood transfusion for war casualties, L. B. Robertson (a Canadian) mentioned citrate only as a possible method and evidently preferred to use cannulae with multiple syringes, flushed with saline as necessary (Robertson, L.B., 1916). When the same author wrote a second paper 2 years later, he was still cautious. 'The citrate method is by far the simplest but up to the present time there has not been sufficient evidence at the front to show that it is pre-eminently the method of choice over all others' (Robertson, L.B., 1918).

O. H. Robertson (1918b) had no such reservations: whereas other methods required one or two assistants, the citrate method could be performed by a single person. Robertson used a bottle with a capacity of 900–1000 cc; 160 cc of 3·8% citrate was used and marks made on the bottle at 660, 760 and 860 cc, corresponding to 500 cc blood, etc. (see Fig 1). He reported that 44 transfusions of 400–700 cc blood had been given to 38 patients at a casualty clearing station. Figure 1 shows the bottle used by O.H., Robertson ready for taking blood: one glass tube extends through a rubber bung to a level below the top of the citrate–glucose and is connected via a rubber tube (with a clamp) to the needle; another glass tube extends just through the bung and is connected to a Higginson's syringe; citrate is blown as far as the needle; after the needle has been inserted into the donor's vein, the syringe is reversed, mild suction applied and 500 cc blood withdrawn. Smaller bottles were used for taking 250 cc blood, two lots of this amount then being taken from a donor. When transfusing the blood, the Higginson's syringe was used to blow the blood into the recipient.

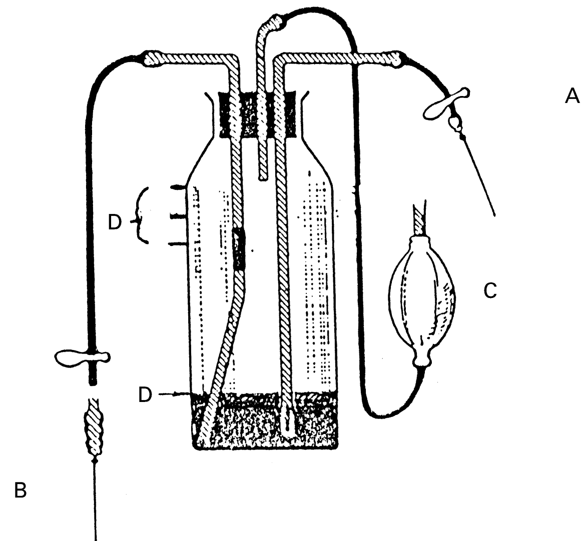

Fig. 1. Robertson's bottle for citrate transfusion (redrawn from Robertson, O.H., 1918b). (A) Line from donor. (B) Line to recipient. (C) Rubber bulb with valve (Higginson's syringe), supplying either negative or positive pressure. (D) Marks are made on the bottle (which has a capacity of 900–1000 cc) at 160, 660, 760 and 860 cc; citrate is added to the 160 mark and the donor is bled to one of the upper marks, corresponding to 500–700 cc blood.

Robertson's bottle was widely used. For example, Fleming (Alexander himself!) & Porteous (1919) reported that they had used it in 100 cases at a base hospital. Donors were bled of 700–800 cc, using 160 cc of 3·8% citrate. No serious problems were encountered, although there were occasional complaints of tightness in the chest due, presumably, to the rapidity of transfusion and relieved by stopping for half a minute.

DISCOVERY OF THE PRESERVATIVE EFFECT OF GLUCOSE ON RED CELLS

According to Robertson (1953), following the outbreak of the First World War, Peyton Rous sought a blood substitute that might be of use in emergency and, having concluded

that nothing would take the place of red cells, began research on methods of preserving them.

Rous & Turner (1916a) considered that, 'as red cells are almost totally impermeable to sugars', solutions of sugars might possibly act as colloids. They tried them as preservatives with various animal red cells and found that glucose and saccharose (sucrose) had the most marked effect in inhibiting lysis on storage, although laevulose, maltose and lactose were not far behind. The optimum preservative for sheep red cells was found to be 2·8% sucrose; glucose was 'fairly good' for washed sheep and human red cells, but the latter were best kept in sucrose–Locke's solution. Table IV of their paper shows that glucose and sucrose had very similar effects, e.g. at 20 d there was a faint trace of lysis with glucose but none with sucrose. They describe storing three parts of human blood with two parts 3·8% citrate [nowadays, the dihydrate of trisodium citrate is used, but Rous & Turner (1916a) used $2(Na_3C_6H_5O_7)\cdot 11H_2O$] and five parts of 5·4% glucose (a mixture later known as Rous–Turner solution) and say that the cells remained intact for 4 weeks.

In a second paper (Rous & Turner, 1916b), transfusion experiments were described. Rabbits were bled of substantial amounts (40 cc or more) of blood and transfused with a suspension containing an equivalent amount of stored red cells. When the blood had been stored for up to about 14 days, the recipient's Hb concentration was well maintained. For example, a total of 46 ml blood was taken from a rabbit and replaced with an equivalent amount of blood stored in citrate–sucrose for 11–12 d. The recipient's Hb concentration fell from 90% to 83% at day 3, but was 91% on day 4. Details of three other experiments with red cells stored in citrate–sucrose were given, two of storage for 11–15 d and one of storage for 23d. In this last case, it was concluded that all of the transfused red cells left the circulation within 2 d of transfusion. Rather surprisingly, no examples were given of transfusion experiments with red cells stored with citrate–glucose, although such experiments must surely have been carried out (see Robertson, O.H., 1918a). The authors stated that the preservative mixture they had found best was citrate–sucrose and that red cells preserved in it will remain in circulation and function so that the Hb concentration remains normal. Despite this statement, and for reasons which they did not give, they recommended that for the preservation of human red cells glucose should be used, i.e. the solution described in their first paper (see above). This was a fortunate choice as it later became evident that, unlike glucose, sucrose cannot be used metabolically by red cells.

The normal source of the red cell's energy is the anaerobic breakdown of glucose to lactic acid, and Rous and Turner (1916a, b) were, of course, mistaken in their belief that red cells are almost totally impermeable to glucose, although they are impermeable to sucrose. The only sugars apart from glucose that can be phosphorylated by hexokinase are other hexoses (specifically fructose and mannose) and these can also support red cell metabolism during storage (Beutler, 1989).

When, at a later date, measurements of survival *in vivo* were made, using differential agglutination, retention of viability was found to be far better with glucose than with sucrose. For example, after storage for 3 weeks with glucose (Rous–Turner solution), some 50% of the red cells were still present in the circulation 1 month after transfusion, but after storage with citrate–sucrose for only 2 weeks survival was down to 50% after 1 week; in fact, although the rate of spontaneous haemolysis on storage is far lower with citrate–sucrose than with citrate alone, post-transfusion survival is much the same with the two solutions (Mollison & Young, 1941, 1942). It is a little surprising that the superiority of glucose over sucrose was not detected by Rous and Turner, but the methods they used for assessing viability – changes in Hb concentration and red cell count – were probably too inaccurate at the time.

When the survival of red cells stored in Rous–Turner (R–T) solution was later compared with that of red cells stored in the citrate–glucose solution used routinely by British transfusion services in the period 1940–43 (100 cc 3% trisodium citrate and 10 cc 30% glucose for 430 cc blood), R–T solution was found to be clearly superior: after 18–28 d storage, survival at 1 month was approximately twice as good as with the solution in general use (Mollison & Young, 1941, 1942). In fact, R–T solution was almost as good as acidified citrate–glucose solutions (Loutit *et al*, 1943). The superiority of R–T solution may be due to its relatively large volume of glucose and to the fact that autoclaved glucose is strongly acidic.

R–T solution has one serious drawback: because of its large bulk and high citrate content, the red cells stored with it can be transfused only after a certain amount of sedimentation has occurred and a substantial amount of the supernatant can be discarded. Although the solution was used by O. H. Robertson in 1917–18, in the Second World War it was replaced by citrate–glucose solutions of much smaller volume (see above), although only after the efficacy of glucose as a red cell preservative had been confirmed by red cell survival measurements (Bushby *et al*, 1940; Maizels & Paterson, 1940; Mollison & Young, 1940).

OSWALD H. ROBERTSON

Robertson was born in England in 1886, but his parents emigrated to California 2 years later (see Cushing, 1936; Allen, 1985; Hanigan & King, 1996). After going to California University, Robertson did his clinical training at Harvard.

After qualifying, he started research on pernicious anaemia, but in 1915 he was invited by Simon Flexner to the Rockefeller Institute, where he joined Peyton Rous's laboratory to begin experimental studies on anaemia and where he started a firm and lifelong friendship with Rous. In 1917, he enlisted in Base Hospital 5, the Harvard Unit, of which the Officer Commanding was Harvey Cushing. He completed work with Rous on experimental polycythaemia in rabbits before leaving.

In September 1917, the medical service of the Third Army requested technical help from Base Hospital 5 and Robertson went to work at no. 3 CCS in Grévillers near Bapaume. He was evidently familiar with the use of citrate and very soon after his arrival in France he published a paper describing a

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method which, as already described, was widely copied. In Keynes's (1949) account of the history of blood transfusion, Robertson was given the credit for introducing the citrate method to the British Military Hospitals in France. Keynes's opinion carries some weight as he himself 'used transfusion on a very large scale in France' during the last year or two of the war (G. Keynes, personal communication to the author in 1978).

Transfusion of preserved red cells

When Robertson turned to blood storage, he chose what was then known as the 'Winchester' bottle, with a capacity of more than 1800 cc, because he used the large-volume preservative solution recommended by Rous and Turner: 500 cc blood was taken into a mixture of 850 cc of 5·4% glucose and 350 cc of 3·8% trisodium citrate. The citrate and glucose were autoclaved separately to avoid caramelization.

The red cells were found to settle rather slowly, a disadvantage as the blood could not be used at once. By the end of 4–5 d, the red cells had settled to 800–900 cc and could then be used because this amount of cell suspension does not contain more citrate than is given in an ordinary transfusion. The supernatant was siphoned off (using mouth suction to establish siphoning) and the volume made up to 1000 cc by adding 2·5% gelatine in normal saline. The blood cell suspension was poured through gauze into the giving bottle and warmed. It was then given under pressure, transfusing 500 cc blood in not less than 10 min. Only 'group IV', i.e. group O, donors were used to avoid the need for agglutination tests (Lee, 1917). Donors were chosen from men with trivial wounds or from those who had recovered from slight wounds. Also, donors with a history of malaria, trench fever or syphilis were excluded, although no tests were carried out.

Most transfusions were of blood kept for 10–14 d, but a few were of blood stored for as long as 26 d. Up to 1000 cc blood was transfused. In all, 22 transfusions were given to 20 patients. Cases were chosen as being likely to die without transfusion, but yet with a chance of recovery if given blood. The effects were considered to be fully as striking as with fresh blood; the length of time for which the blood had been stored appeared to have no influence on its beneficial effect, the improvement in those who received blood preserved for 3 weeks seeming to be just as marked as in those who received blood stored for much shorter periods. Robertson concluded that, probably, 4 weeks is about the limit for which blood can be kept for transfusion as red cells usually begin to disintegrate soon after this. Robertson does not seem to have envisaged progressive deterioration from the outset of storage but rather complete preservation for a period followed by rapid deterioration (rather as with milk which remains fit to drink until it turns sour). After transfusion, there was a marked improvement in the colour of recipients, their pulses became slower and stronger and their blood pressures rose by 20–40 'points'. Hb estimations were not helpful in assessing red cell survival as the Hb level often fell when fresh blood was used because of restoration of plasma volume. There was no evidence of haemolysis. Eleven out of the 20 patients were discharged to base in good condition.

O. H. Robertson (1918a) concluded that the 'chief value of this method lies in the convenience of having a large quantity of blood on hand for a rush'. He mentioned that, in a recent experience when supplies of stored blood ran out, much time was spent in finding suitable donors from among the trivially wounded as most such patients had been passed on from the CCS. Also, whereas 600 or even 700 cc could be taken from well donors, such amounts could not justifiably be taken from those who were exhausted.

Initially, Robertson took five units, stored them with ice and used them satisfactorily. He was then (November 1917) sent to another CCS (no. 48) near the front. At this time, he devised his ice box: a packing case lined with tin within a larger case, the gap between the two being filled with sawdust. Ice was placed at one end of the inner case. He had 20 pints in stock when the 'show' began: despite being in charge of the resuscitation ward, he gave six transfusions a day until the blood ran out; he then gave ordinary citrate transfusions. 'By the end of the war, his iced jars packed in sawdust were familiar to surgeons and regimental aid posts near the front line' (Hanigan & King, 1996).

At the time of the big German push in March 1918, O. H. Robertson came near to being overrun by the enemy (P. Rous, personal communication to the author, 1958), but he returned to the USA with the Harvard Unit in Jan 1919, at which time he was awarded the DSO by the British. In 1958, with Rous and Turner, he received a Landsteiner award from the American Association of Blood Banks for pioneering work with preserved red cells.

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