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## II Survival studies

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**SYNOPSIS** Platelet survival was determined using untreated and siliconed glass bottles and plastic bags (Fenwal) for collecting and storing blood. The platelets were tagged *in vivo* with  $P^{32}$  in six polycythaemic patients undergoing treatment with  $P^{32}$ . The results showed that fresh ACD blood collected in untreated glass, siliconed glass, and plastic gave the same recovery of platelets in the recipients. The use of EDTA (Fenwal formula) as anticoagulant gave results inferior to those obtained with blood using ACD as anticoagulant. Even after storage up to 24 hours in untreated glass bottles (ordinary bank blood) a satisfactory recovery of platelets was observed. After storage for 72 hours the recovery was less but not negligible.

We have tried to solve part of the problem of preserving platelets for subsequent transfusion and for use in extracorporeal circulation by determining the survival of platelets in blood delivered in different containers, using ACD and EDTA as anticoagulant and storing the blood for periods of varying length before transfusion. It is difficult to determine the survival of thrombocytes but it is possible by using platelets tagged with various isotopes. A survey of the methods used was recently published by Aas and Gardner (1958). Tagging is usually performed *in vitro*, and the rather rough treatment to which the platelets are exposed during tagging has some influence on the results obtained, consequently labelling *in vitro* is not ideal for an investigation in which a relatively small difference in survival time might indicate superiority or inferiority of one container compared with another. We therefore used platelets tagged *in vivo* with  $P^{32}$ . We used blood from patients with polycythaemia who were treated with  $P^{32}$ . The technique employed is very similar to that used by Adelson, Rheingold, and Crosby (1957) in dogs and human beings. It is well known that tagging is maximal in about a week after the administration of the drug (Adelson *et al.*, 1957). Using blood from patients with polycythaemia gives another advantage, namely, the possibility of drawing several pints of blood from the same donor at very short intervals. The various 'pints' drawn from

the same donor contain the 'same' platelets marked in exactly the same way *in vivo*, thus affording an ideal opportunity for comparing the effect of storage on platelets in various containers before transfusion.

### MATERIALS AND METHODS

**DONORS** Patients with polycythaemia undergoing treatment with  $P^{32}$  were used as donors. They were given about  $5 \mu$ .  $P^{32}$  by mouth and after an interval of five to seven days bleedings were started, 500 ml. of blood being drawn on each occasion, and the bleedings were continued daily for three to six days, depending on the clinical and haematological condition of the patients. Six patients were included in the study, and delivered a total of 24 'pints' of blood. Two patients delivered six bottles each, two four bottles each, one three bottles, and the last donor only one. All the bleedings were performed either by the head nurse of the blood bank or by one of the authors (F. K-N). Automatic shaking was used during bleeding into glass bottles.

**RECIPIENTS** It was felt that it was undesirable and unnecessary to use 'normal' recipients, so recipients were patients from the Department of Radiotherapy undergoing heavy x-ray therapy for advanced malignant tumours, mostly carcinoma of the cervix uteri. The patients were all in a good clinical condition, but some of them had previously been transfused several times (Table I). The transfusions were preceded by screening for irregular blood-group antibodies (papainized cells and indirect Coombs technique) and by cross-matching

including the use of the indirect Coombs test. Most of the patients were investigated for platelet antibodies using Dausset's technique for complete (Dausset and Malinvaud, 1954) and Coombs consumption technique for incomplete antibodies. The Coombs consumption technique used was a modification of the technique elaborated by Moulinier (1955) and Steffen (1955). All the transfusions were given by one of the authors (F. K-N).

**BLOOD CONTAINERS, ANTICOAGULANTS, AND STORAGE BEFORE DETERMINATION OF PLATELET SURVIVAL** Untreated and silicined glass bottles and plastic bags (Fenwal) were included in the study. In the majority of the experiments ACD (Danish or United States Pharmacopoea, formula A) was used, but in three experiments EDTA (Fenwal formula) was employed. Most of the transfusions were given as fresh blood as quickly as possible after the bleeding, but in some experiments the platelet survival was determined after storage at 4°C. for periods varying between 15 and 72 hours. The exact composition of the various anticoagulants used is given in Part I.

**DETERMINATION OF PLATELET SURVIVAL** The principle of the method was determination of the activity of all isolated, washed platelets from an exact volume of plasma at different times after the transfusion. The method was a modification of the technique used by Aas and Gardner (1958). Exactly 19 ml. of blood was drawn into an untreated test tube containing 1 ml. of EDTA 5%. The contents were mixed; the haematocrit value (Wintrobe) was determined, and exactly 4.5 ml. of blood was transferred to four untreated test tubes (100 × 15/16 mm.) containing a mixture of 1 ml. EDTA 5% and 3 ml. dextran (Pharmacia) ( $M_w = 153,000$ ) in 0.9% saline. The contents of the tubes were thoroughly mixed and centrifuged at 175 g for 15 minutes. Exactly 20 ml. of the supernatant was transferred to silicined test tubes (100 × 15/16 mm.) and centrifuged at 1,000 g for 30 minutes. The supernatant was discarded, and the platelets were resuspended and washed once in 0.9% saline containing 1/10 by volume of triton 2% in 0.9% saline. The platelets were resuspended in 0.5 ml. 0.9% saline and quantitatively transferred to small flat metal cups (2.8 cm. diameter). The quantitative transfer was ascertained using 2 × 0.5 ml. saline for rinsing. The content (1.5 ml.) of the metal cups was air dried and then ready for the determination of the beta radiation. The weight of the dried content of a cup was 22 mg.

The thickness of radioactive substance is thus 3.6 mg./cm<sup>2</sup>. The self-absorption in the sample can be neglected as this small thickness. Using this method, the activity was determined in the blood container immediately before the transfusion and in the recipients one and three hours after the transfusion was finished and subsequently daily or on every second day during the following four to eight days.

The cups were placed in an automatic sample changer and successively brought under a Geiger-Müller tube which was provided with a mica foil 1.4 mg./cm<sup>2</sup> thick. All the samples were automatically placed in the same position under the counter; the geometrical conditions were thus

invariably identical. Four thousand pulses were counted and the corresponding time was automatically printed. The time could be read in hundredths of a minute. For the strongest samples a larger number of pulses was counted to determine the time with sufficient accuracy. The time was always five to 10 minutes. The error from reading the time is thus small, but the accuracy is influenced by the fact that the frequency of the current for the synchrotimer is constant only within 1%. Every sample was counted at least four times. Usually, counting was done at one-day intervals. In each counting, the calculated counts per minute were corrected for physical decay from the point in time at which platelet activity for a donor was first measured. Finally, the mean value of the corrected counts per minute was found.

Owing to the statistical fluctuation of the disintegrations the accuracy of 4,000 counts is about 1.5%. A mean value for several measurements was calculated, and the statistical error is therefore smaller than the figure given above. The resolution time of the Geiger-Müller counter is 100 μsec.; correction for losses due to the counter is thus less than 1%, even at the highest counting rates used in this investigation. On the whole, the accuracy of the activities observed was between 1 and 2%, which is sufficient for the present purpose.

The activity determined in the specimens was plotted on semilogarithmic paper against the time after the transfusion.

The platelet suspensions prepared were obviously not pure as there was slight contamination with red and white cells but it was generally found to be less than one red and one white cell per 1,000 platelets, and a control specimen containing five times as many red cells (but no platelets) had a negligible activity (one count/minute).

**DETERMINATION OF PLATELET YIELD IN RECIPIENTS** This figure is difficult to calculate accurately, but it was tried using the activity of the platelets in the bottle determined just before the transfusion, and the activity of the platelets in the first specimen drawn from the recipient one hour after the transfusion. (If the activity of the platelets from the patient was higher three hours after the transfusion, this activity was used.)

The platelet activity was determined in 20 ml. of 'plasma' produced from four test tubes, each containing 4.5 ml. whole blood (= A : 1 ml. EDTA 5% + 19 ml. blood) + 1 ml. EDTA and 3 ml. dextran. The haematocrit value was determined in A and corrected for added EDTA and trapped plasma (Mollison, 1956), and from this figure the plasma haematocrit value (H. pl.) was found. The exact volume of real plasma (x) in the 20 ml. was calculated using the following formula:—

$$x = \frac{H.pl. \times 100}{H.pl. + 89} \times \frac{20}{100}$$

From this figure and the determined platelet activity, the platelet activity per millilitre plasma was calculated for the donor (D) and the recipient (R). Then the total volume of plasma transfused (U), and the total plasma volume in the recipient (Y) were found, and the platelet yield was determined as  $\frac{R \times Y \times 100}{D \times U}$ . The total volume

of plasma (+ anticoagulant) transfused was calculated using the haematocrit value corrected for trapped plasma (Mollison, 1956) and using a total volume in the bottle of 500 ml. of blood plus the volume of anticoagulant added minus the volume taken for various tests. The total plasma volume in the recipient was found using the Wadsworth formula, which gives a total plasma volume =  $43.1 \times \text{weight in kg.}$  (Mollison, 1956). The plasma volume calculated was corrected for varying haematocrit in the recipients. Arbitrarily, a haematocrit level of 40% was used as the base line for the corrections. A difference in haematocrit of 1% was taken to be equal to 0.5 ml. plasma per kg. body weight using the diagram from Mollison (1956, p. 62).

The results of these calculations are shown in Table I.

### RESULTS

Table I only includes 21 of the 24 experiments performed. Three of the recipients were excluded because the activity found was only minimal. The recipients excluded were (1) Case II B who received fresh ACD blood from an untreated glass bottle. This patient had been transfused on five previous occasions, and she had had three children and no abortions. (2) Case III B received 24-hour-old ACD blood from a Fenwal bag. The transfusion was

stopped after 300 ml. because of a febrile reaction and chills. The patient had previously been transfused three times; she had had two normal pregnancies, (3) Case IV D received 15-hour-old ACD blood from a Fenwal bag. The patient had previously been transfused on three occasions, and she had never been pregnant.

As already mentioned, the results obtained with the 'pints' withdrawn from a single donor give a very good opportunity for comparing the recovery of transfused platelets in the recipients in relation to the various containers and anticoagulants used. The results obtained with the bottles of blood procured from the five different polycythaemic donors appear in Figs. 1 to 5 (Donor VI who only donated one 'pint' is excluded from these figures). No corrections have been made for varying plasma volumes in the recipients used and no corrections have been made for varying volumes of anticoagulant used in the different containers, all containing 500 ml. of donor blood. The results obtained in the various recipients all represent the platelet activity in equal volumes of plasma. No corrections for varying activity of the platelets from the donor have been made, but it appears from Table I that the platelet activity was

TABLE I

#### PLATELET YIELDS IN 21 EXPERIMENTS

Container	Anticoagulant	Storage	Experiment No.	Bottle				Platelet Antibodies		Recipient				
				Haematocrit	Bottle Activity	Total Activity	Previous Transfusion Pregnancies	Complete	Incomplete	Haematocrit	Activity	Plasma Volume (ml.)	Total Activity	Recovery (%)
Glass	ACD (Danish)	Fresh	I A			61,000	0/0	+	—				25,500	42
			III C	49	5,003	223,000	1/10	Not investigated	40	424	2,680	132,000	59	
			V A	48	805	36,800	1/1	—	—	36	56	2,980	20,000	55
Glass (siliconed)	ACD (Danish)	Fresh	I B			51,000	0/0	—	—				37,800	74
			II D	47	680	30,400	1/3	—?	—	41	45	3,310	18,500	61
			IV A	53.5	2,974	127,000	1/1	Not investigated	35.5	176	2,280	47,900	38	
			V C	42.5	836	39,800	0/3	+++	—	34	68	3,100	23,400	63
Plastic (Fenwal)	ACD, Fenwal	Fresh	I E			51,000	0/1	—	—				17,400	34
			II F	47	645	25,200	0/0	—	—	44	48	2,900	17,300	69
			III D	43	4,920	198,000	0/2	Not investigated	34	206	4,230	101,300	52	
			IV C	49	3,060	118,800	3/0	Not investigated	40	235	2,240	65,500	56	
			V B	48	1,113	44,800	0/4	—	—	36.5	57	2,540	17,400	39
Glass	EDTA, Fenwal	Fresh	I C			52,500	2/2	Not investigated				13,900	27	
			ID			45,500	0/9	—	—				16,500	36
Glass	ACD (Danish)	24 hr.	VI	50	1,415	62,500	0/4	—	—	40	94	3,730	43,700	70
			IV B	49	2,617	116,000	1/13	Not investigated	40	124	2,570	39,600	34	
Glass (siliconed)	ACD (Danish)	24 hr.	III A	53	4,828	203,000	2/6	Not investigated	43	161	3,370	63,200	31	
			II A	55.5	688	28,700	0/1	—	—	39.5	14	2,310	3,950	14
Glass (siliconed)	ACD (Danish)	72 hr.	II C	53.5	625	26,700	4/5	—	—	44	25	2,580	8,450	32
			II E	45	770	30,500	0/3	—	—	27	10	3,500	3,950	13

I to VI are the sixth polycythaemic donors used. A to F are the different recipients who were transfused from the various donors.

Experiments using the six donations from donor I were all done with a slightly different technique working with the platelet activity per platelet both in the bottles and in the recipients. The calculations used, therefore, differed from those in the rest of the experiments. The haematocrit values are corrected for trapped plasma and added anticoagulant.

*Platelets in blood stored in untreated and siliconed glass bottles and plastic bags*

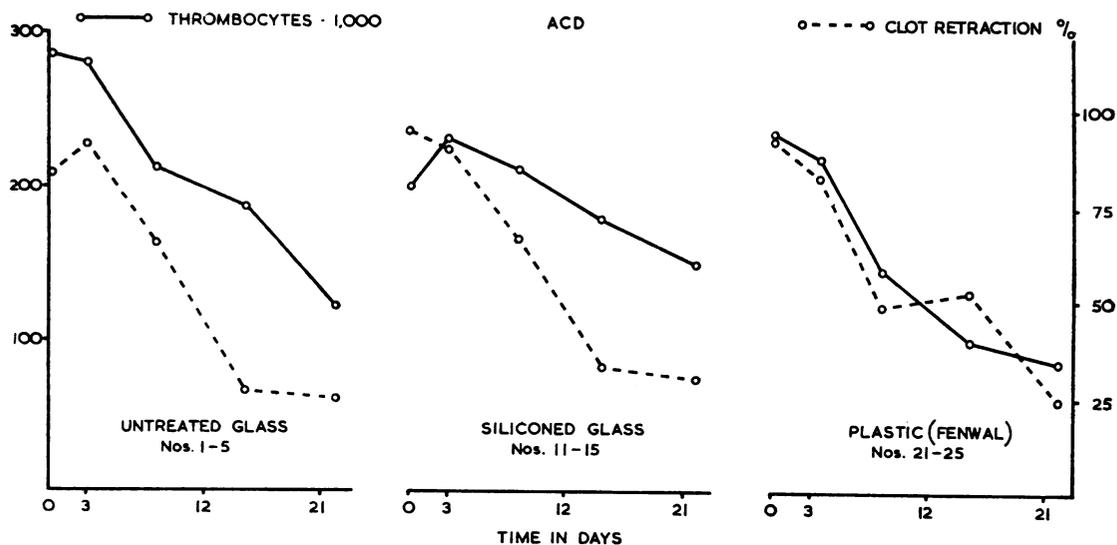


FIG. 1. *Thrombocyte survival in six recipients receiving 500 ml. blood from polycythaemic donor I treated with P<sup>32</sup>. All experiments were performed with fresh blood using varying containers and anticoagulants.*

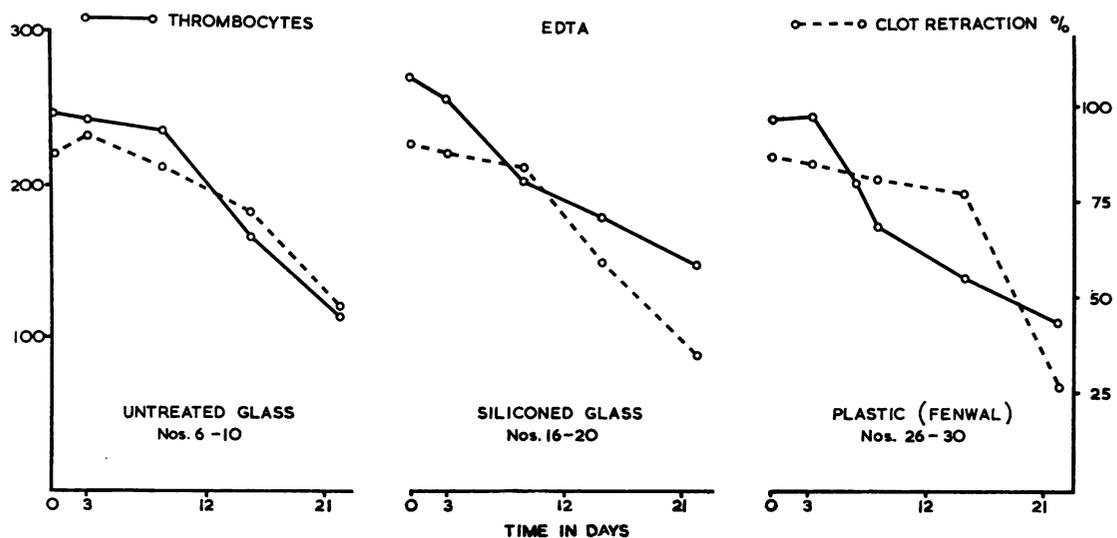


FIG. 2. *Thrombocyte survival in five recipients receiving 500 ml. blood from polycythaemic donor II using plastic bags, and untreated and siliconed glass.*

fairly constant during the experiments performed with blood donations from the respective donors, especially when it is taken into account that the volume of anticoagulant used is less in the Fenwal series than in the ACD (Danish) series. (The Fenwal ACD solution is 75 ml., the Fenwal EDTA solution 50 ml., and the Danish ACD solution used 150 ml.)

DISCUSSION AND CONCLUSION

The main conclusion which can be drawn from the experiments is that the container does not, to any appreciable degree, interfere with the recovery or the survival time of the thrombocytes if the blood is transfused during the first few days after the with-

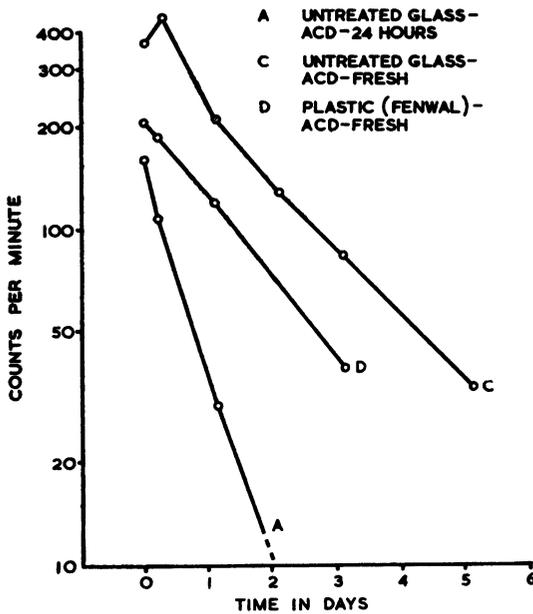


FIG. 3. Thrombocyte survival in three recipients receiving 500 ml. blood from polycythaemic donor III using a plastic bag and untreated glass.

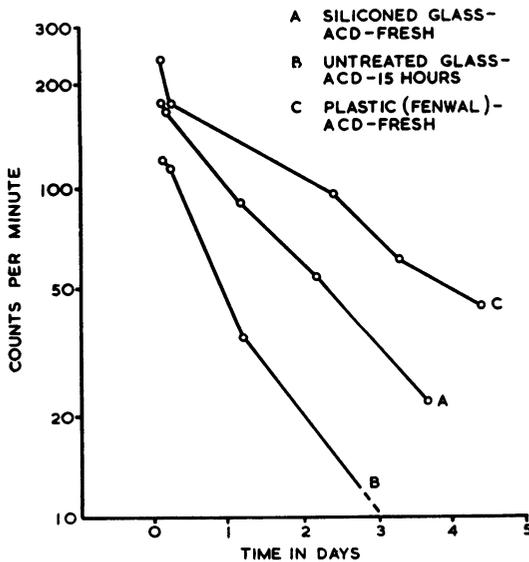


FIG. 4. Thrombocyte survival in three recipients receiving 500 ml. blood from polycythaemic donor IV using a plastic bag and untreated and siliconed glass.

drawal of the blood. Contrary to expectation neither siliconed glass nor plastic containers were necessary to secure a reasonable recovery of viable platelets.

In three experiments (Fig. 1, C, D, F) using EDTA (Fenwal formula) as anticoagulant we found that this anticoagulant was inferior to the ordinary ACD solutions. This finding was also contrary to our expectations as EDTA is to-day the anti-

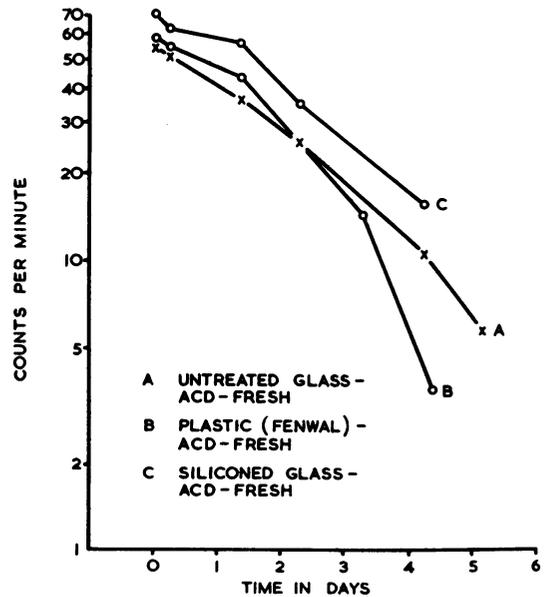


FIG. 5. Thrombocyte survival in three recipients receiving 500 ml. blood from polycythaemic donor V using a plastic bag and untreated and siliconed glass.

coagulant of choice in studies of platelets *in vitro*. It does not contain glucose, and it cannot be excluded that this may be of major importance. In all the EDTA experiments—and only in these experiments—did we find an initial 'dip' in the survival curves (Fig. 1, C, D, F), and this initial dip may be indicative of some damage to the platelets caused by the EDTA solution. EDTA is a very effective chelating agent, which may cause partly reversible damage to enzymes essential for the viability of platelets but this explanation is obviously only pure speculation. It should be mentioned that Adelson *et al.* (1957), who also determined the survival of platelets labelled *in vivo* with  $P^{32}$  using EDTA as anticoagulant, found an even more pronounced initial dip, but they made their first determination 30 minutes after the transfusion. In the present investigation the first determination was made one hour after the transfusion. The experiments with untreated and siliconed glass bottles and Fenwal

bags using fresh ACD blood gave almost identical results. There is some variation in the platelet recovery in the various recipients (Table I), but the averages from the three groups of recipients are almost identical, namely 52% for untreated glass bottles, 59% for siliconed glass bottles, and 50% for Fenwal bags. In contrast to these relatively satisfactory results, the three EDTA experiments gave an average of only 27%. The number of experiments is limited, but nevertheless it seems justifiable to conclude that the Fenwal-EDTA solution used in the 'platelet pack' is in no way superior to the ordinary ACD-Fenwal bag or an ordinary ACD glass bottle.

It is generally claimed that blood should be fresh when given as a source of blood platelets, for example, to thrombocytopenic recipients. What is meant by 'fresh' is usually not exactly defined, which often results in a demand from the clinicians for delivery of the blood immediately after the bleeding. This has several practical drawbacks and causes the blood delivered to be less safe because of emergency handling (cross-match, serological tests, etc.). It is reasonable to believe that the platelet recovery will be maximal if the blood is given immediately after the bleeding, but the last five experiments in Table I show that the platelet recovery was quite satisfactory after a limited period of storage (average

45% after  $\leq$  24 hours of storage in untreated glass bottles), and even after storage for 72 hours some platelet activity could be found in the recipients. It is reasonable to believe that the use of silicone for longer periods of storage may be of importance, as it causes less activation of coagulation factors; a platelet recovery of 32% in experiment II C (Table I), in which blood was stored for 72 hours in a siliconed bottle, is in accordance with this suggestion.

The results of the recovery studies obtained with the various containers and anticoagulants after different periods of storage are summarized in Fig. 6.

As a consequence of the results obtained we now use blood which is less than 24 hours old as fresh blood, and in the absence of acute bleeding we use blood less than 72 hours old for thrombocytopenic recipients. Where a very high recovery of platelets is really essential, e.g., for a bleeding thrombocytopenic patient we use, of course, fresh blood drawn immediately before the transfusion, as it must be assumed that even when the platelets are stored under optimal conditions at 4°C. their average survival time will decrease with the period of time during which they have been stored. For extracorporeal circulation we use blood drawn during the afternoon of the day before the operation, which with our very limited experience has been absolutely satisfactory.

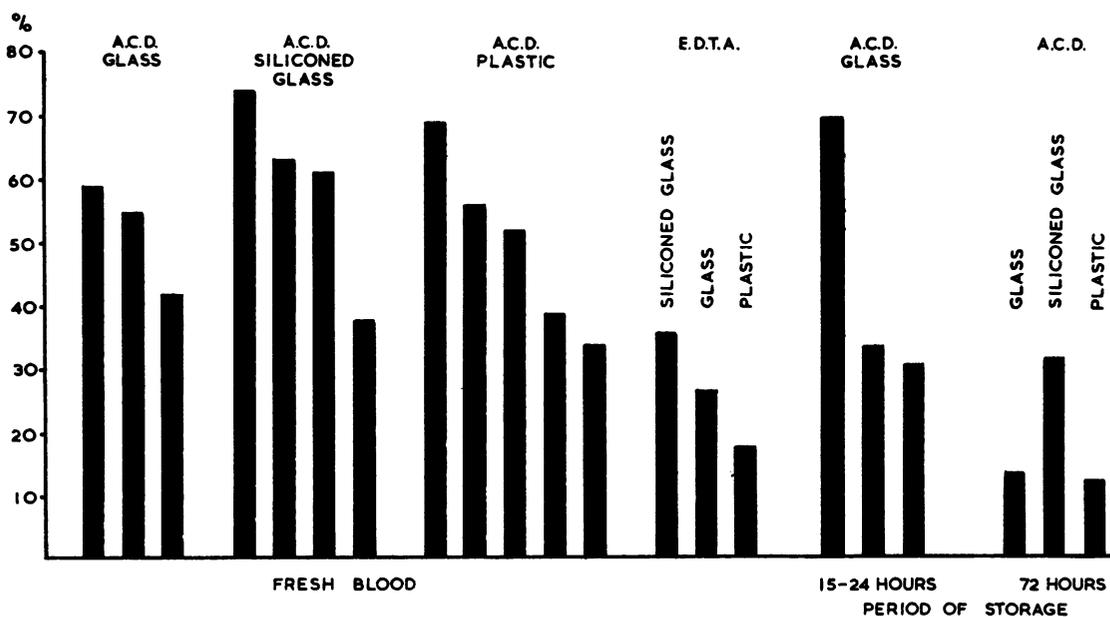


FIG. 6. The various columns represent the percentages of the transfused platelets found in the recipients. The first four groups represent the results obtained using fresh blood. The last two groups represent the results obtained when the blood was transfused after varying periods of storage.

It was not possible to find any explanation for the three failures in the experiments, but unfortunately no investigations were made for platelet antibodies in the three recipients. In experiment IIIB, the recipient suffered a febrile transfusion reaction. Neither erythrocyte nor complete leucocyte antibodies were found in her serum.

The results of the experiments reported are in disagreement with most previously performed work in this field, in which it is claimed that ordinary bank blood in untreated glass bottles is greatly inferior to that stored in plastic and siliconed equipment (Gardner, 1958; Stefanini and Dameshek, 1955; Tullis, 1953).

However, McIlvanie (1958) reported the results of an investigation in which he compared plastic bags, siliconed, and untreated glass bottles in transfusing fresh ACD blood to various patients and determined the platelet yield by counting the platelets. He found almost identical results for the various containers. The results he obtained with siliconed bottles were only slightly superior to those obtained with plastic bags and untreated glass bottles. The difference may not have been statistically significant, and thus McIlvanie's observations are in agreement with our results.

Most of the investigations in which superiority for plastic and siliconed equipment is claimed have been performed in America, where evacuated blood bottles are in common use. Such bottles cause more foaming during the bleeding, which, as pointed out by Stefanini and Dameshek (1955), may damage the platelets. However, this does not seem to be a plausible explanation for the poor results obtained with glass bottles, since even bleeding by gravity with automatic shaking causes some foaming.

Obviously, it cannot be excluded that the type of glass and silicone used may influence the results, but this problem was considered to be outside the scope of the present investigation.

It appears from Table I that some of the recipients had previously been transfused. Transfusions (and pregnancies) may cause iso-immunization against platelets, but it does not seem reasonable to assume that the transfusions given exerted any influence on the results obtained in a comparison of the various groups of containers.

In two of the recipients in whom the experiments were successful complete platelet 'agglutinins' were shown by Dausset's technique. Recipient IA had weak and recipient VC fairly strong agglutinins. These agglutinins were cold with an effect only at low temperature, and in spite of the presence of these agglutinins in the recipients normal platelet recovery and a normal platelet survival time were observed. These experiments are in agreement with two

experiments performed by van Rood, Eernisse, and van Leeuwen (1958, Figs. 1 and 3). These authors found a normal survival time in two patients with complete platelet agglutinins and no incomplete agglutinins. This is in harmony with the fairly frequent demonstration of complete, cold platelet agglutinins in normal subjects, and shows that the clinical importance of complete cold platelet agglutinins is still an unsolved problem.

A detailed discussion of the results obtained in relation to platelet life span will not be given, as this problem is outside the scope of the present investigation. It may, however, be stated that a  $T_{\frac{1}{2}}$  of two to three days was observed in most of the experiments in which fresh blood was used, while the use of stored blood resulted in some shortening (in most cases,  $T_{\frac{1}{2}}$  was about one day). The results obtained agree with those obtained by Adelson *et al.* (1957) who used a similar technique in two experiments. The limitations of the techniques in which isotope-labelled platelets are utilized will not be discussed, but reutilization and elution must obviously be taken into consideration. In using polycythaemic donors it must also be remembered that the life span of the platelets in these patients may either be decreased or increased (Alfos, Field, and Ledlie, 1959). However, these problems do not exert any influence on the results obtained in the present investigation or on the conclusion drawn in relation to the influence of the various containers on platelet recovery in blood transfusions.

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