insufficient for the complete formation of activator. Our data indicate that the ratios of proactivator to plasminogen are much more uniform than had been reported by Spritz and Cameron and agreed closely with the ratio obtained with a sample of purified plasminogen. The correlation coefficient of plasma plasminogen values compared with proactivator in our experiments was 0.86 with a P value of $<.001$, indicating a high degree of covariance. While these data undermine the experiments of Spritz and Cameron, which definitely suggested a proactivator other than plasminogen or plasmin, they do not, of course, rule out the possibility that other proactivators may exist.

Summary. When the concentration of SK was carefully controlled, a strong covariance relationship was found between the plasminogen and proactivator concentration in 12 normal human plasma samples. It is suggested that lack of such control had led earlier investigators to the erroneous conclusion that they had demonstrated the presence of a proactivator other than plasminogen or plasmin in human plasma. The euglobulin precipitate at pH 5.3 contained virtually all of the plasminogen present in the original plasma.

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Effect of Storage on Sheep Erythrocytes Used in Complement Studies. (30733)

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During investigations of the complement (C′) activity of humans with connective tissue diseases, it was observed that when erythrocytes from different sheep were employed, marked differences in the extent of immune lysis were obtained with a constant pool of human complement (HuC′). In addition, it was noted that progressive changes in the degree of immune hemolysis occurred during storage of an individual specimen of sheep red cells. Because sheep erythrocytes are an essential reagent in a number of serological and immunological test procedures, the present investigation was initiated to determine the effect of storage on the immune hemolysis, osmotic fragility and degree of spontaneous lysis of sheep red cells.

Materials and methods. Blood collection and storage. Nine healthy sheep (weight 225-325 lb) of mixed sex and breeding were bled aseptically by venipuncture. In each instance the blood was collected in an equal volume of modified Alsever's solution(1), pH 6.1, containing 20.5 g glucose, 8 g sodium citrate, 0.55 g citric acid, 4.2 g NaCl and sufficient distilled water to bring the volume to 1000 ml. The preserved blood specimens were stored in sterile 50 ml vaccine bottles at 3°C until tested. The osmotic fragility and susceptibility to immune lysis with a standard pool of C′ were determined at weekly intervals.

Immune hemolysis. Erythrocytes were washed and sensitized by the method de-

scribed by Kent and Fife(2). However, the washed suspensions were adjusted to contain 5×10^8 cells/ml (approximately a 2% suspension) rather than the 10^9 cells/ml as specified. Erythrocytes were sensitized with an equal volume of an optimal dilution of rabbit hemolysin. Sera from 47 healthy human males, age 18-25, were pooled to provide a single source of normal C′ for all experiments. The pool was divided into 0.5 ml amounts and stored at −60°C in a mechanically refrigerated freezer. Maintenance of this large stable pool of C′ permitted C′ titration at weekly intervals in a highly reproducible manner. A precise spectrophotometric method for C′ assay was employed(3) and C′ titers ($C'H_{50}$/ml) were expressed as the reciprocal of the amount of test serum needed to hemolyze 50% of the optimally sensitized erythrocytes in a reaction volume of 1.5 ml after 30 minutes' incubation at 37°C. Triethanolamine buffered saline (TBS) containing optimal concentrations of Ca^{++} and Mg^{++} was used as the reagent diluent (4). Susceptibility to immune lysis was determined on each lot of erythrocytes 1, 2, 3 and 4 weeks after collection.

The degree of spontaneous lysis was determined concurrently with C′ assays by combining 0.6 ml sensitized cells with 0.9 ml TBS and centrifuging at 1000 *g* for 5 minutes. Optical density of the supernate was read at a wavelength of 550 mμ.

Determination of osmotic fragility. Sheep erythrocytes preserved in modified Alsever's solution were washed simultaneously with those prepared for immune lysis. After the final wash, TBS (0.1457 M) was added to make a 20% suspension of the cells. The osmotic fragility of each sample was determined by two methods, namely the multiple tube dilution test modified from Dacie(5) and the continuous osmotic fragility curve or "osmogram" obtained by the technique of Shields and Allen(6). In the latter method 0.06-0.07 ml of the 20% red cell suspension was placed in a chamber formed by a dialysis bag stretched over a steel frame. The assembly then was immersed in a container of distilled water. As the water penetrated the membrane and diluted the internal suspension, progressive changes in optical density were photometrically measured and mechanically recorded.

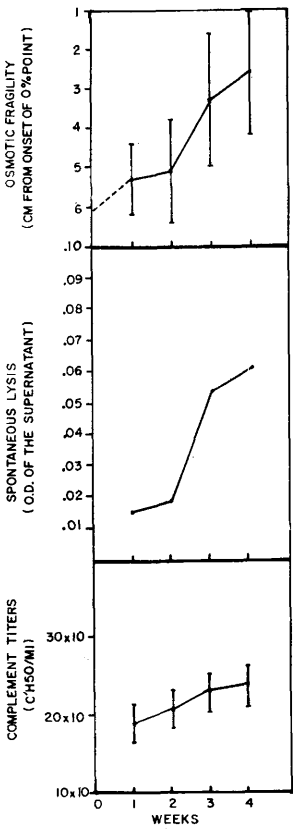

FIG. 1. Relationship between immune lysis, osmotic fragility, and spontaneous lysis of sheep erythrocytes during storage in Alsever's solution.

Results. Immune lysis. One week after collection of erythrocytes, the mean C′ titer obtained with 9 different lots of cells was 190 (S.D.M. ± 24) with individual values ranging from 154-222. Individual titers progressively increased with the age of the cells (Fig. 1) and after 3 and 4 weeks of storage the mean C′ titers increased to 231 and 242 respectively (Table I). This increase was shown to be statistically significant by paired test ($P < .01$). Seven of the 9 lots of erythrocytes continued to show a progressive increase in susceptibility to lysis by C′ during the fourth week of storage. The remaining 2 lots showed some decrease at this time but the change was significant in only one of the latter (Table I).

TABLE I. Effect of Storage on Erythrocytes from Individual Sheep.

Sheep	Complement titer (C'H50/ml) Storage 1 wk	2 wk	3 wk	4 wk	Spontaneous lysis (O.D. of supernatant) Storage 1 wk	2 wk	3 wk	4 wk	Osmotic fragility (cm. from onset to 0% point) Initial	Storage 1 wk	2 wk	3 wk	4 wk
A2	162	171	215	240	.030	.020	.095	.085	5.8	4.7	4.9	2.3	1.1
A28	154	187	232	262	.030	.020	.080	.090	6.2	5.6	4.4	1.4	.9
A36	197	207	260	240	.035	.015	.060	.055	5.9	5.0	6.3	3.1	1.7
A53	214	231	267	210	.006	.010	.020	.008	8.7	6.0	5.6	5.2	3.9
A20	222	N.D.	240	250	.010	.030	.070	.130	6.2	5.2	3.3	2.5	1.4
A45	176	212	200	211	.020	.030	.030	.055	5.8	6.4	4.1	2.1	2.5
A07	207	223	232	250	.000	.040	.085	.100	3.8	3.9	3.4	1.7	1.7
A25	200	225	240	285	.005	.002	.015	.020	5.4	6.1	6.5	5.8	4.5
A39	178	197	200	230	.000	.002	.020	.010	6.1	6.3	7.4	5.6	5.6
Mean	190	207	231	242	.015	.018	.053	.061	5.9	5.3	5.1	3.3	2.6
S.D.M.	±24	±24	±23	±25	±.012	±.013	±.027	±.040	±1.1	± .9	±1.3	±1.7	±1.6

N.D. = not done.

Osmotic fragility. It was readily apparent that the osmotic fragility of sheep erythrocytes progressively increased during the period of storage (Fig. 1). Although results obtained with the multiple tube test correlated well with the osmogram, the tube method did not provide data suitable for statistical analysis. By measuring the slope of the major curve and expressing the values as points indicating 0%, 50%, and 100% hemolysis, it was possible to provide data for statistics and for linear graphing (Fig. 2). By analysis of variance each value in the curve was significantly different from the baseline values by the third week ($P<.01$) and by the fourth week the significance was greater ($P<.001$). Subclass analysis of variance illustrated that the 0% point had the highest degree of significance. Use of this value for statistical comparison greatly increased the significance of the third week difference ($P<.001$) and the fourth week difference ($P<.0001$). Thus, the values obtained by this method especially of the 0% point, revealed a progressive and significant increase of osmotic fragility during storage. Spontaneous hemolysis also increased with storage of the erythrocytes and the changes paralleled those observed in tests of osmotic fragility. The various effects of storage on sheep erythrocytes were summarized in Fig. 1 wherein graphs of osmotic fragility values (calculated at the 0% point), immune hemolysis titers and spontaneous lysis values were presented. All data were plotted on the same time scale to illustrate the parallelism of the observed changes.

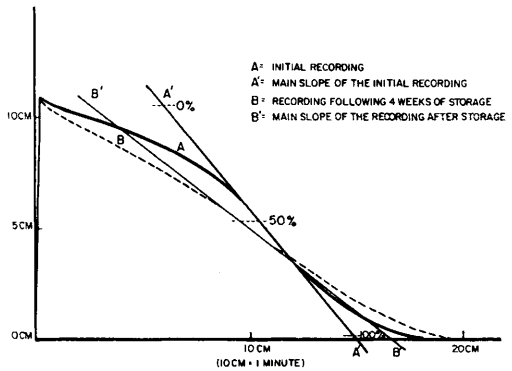

FIG. 2. Osmogram showing osmotic fragility of sheep erythrocytes before (line A) and after 4 weeks of storage (line B). Values closest to start of the test indicate increased osmotic fragility. Horizontal distances to the 0, 50, and 100% points represent amount of time required to reach the indicated degree of hemolysis.

Discussion. Other investigators(7-8) have shown that the osmotic fragility of human erythrocytes increases during storage in Alsever's as well as other acid-citrate-dextrose (ACD) solutions. Therefore, when progressive changes of C′ titers were noted in assays performed with the same lot of sheep erythrocytes after storage for various periods, consideration was given to the possibility that these variations might be related to an altered cell state which would be reflected in tests of osmotic fragility.

It is recognized that variables other than intrinsic changes in sheep cells during storage could influence C′ activity. Among these is the reduction of C′ titer that accompanies re-

peated freezing and thawing of the serum. The influence of this variable was minimized in the present experiments by freezing the C′ pool in appropriate aliquots and thawing each sample only a single time. Moreover, the findings are not indicative of C′ deterioration; a significant loss of C′ activity during the course of the studies would have resulted in reduced rather than increased immune hemolysis. In view of these considerations, it was apparent that the observed increase of C′ titer was due primarily to erythrocyte alterations induced by storage and not simply to changes of C′ activity *per se*.

With each lot of sheep erythrocytes studied, immune hemolysis progressively increased throughout storage and after the third week C′ titers differed significantly from the original values. Although individual lots of sheep blood consistently showed this increased sensitivity to immune lysis during storage, the rate of increase varied and thus precluded use of the initial levels for predicting the titers that eventually would be attained after prolonged storage. Nevertheless, the definite trends shown by all specimens in this study imply that sheep cells stored for more than 3 weeks may be unsuitable for complement studies and should be used with caution.

Regardless of the method used for appraising osmotic fragility (multiple tube or osmogram), all lots of sheep erythrocytes exhibited increased fragility during storage. Comparison of data related to the changes in osmotic fragility revealed a striking parallel to those associated with the alterations of sensitivity to immune hemolysis noted above (Fig. 1). Nevertheless, the individual value obtained in either the fragility studies or tests for immune hemolysis did not provide a basis for predicting what could be expected in the other test. Neither could the values given by a single method be extrapolated to predict the value that would be attained by the same procedure after further storage of the red cells.

Existence of a parallel relationship of increased fragility and immune hemolysis suggested that common factors played a role in both phenomena. However, the inability to correlate individual values suggests that still other factors may contribute to the variability of immune hemolysis obtained with different lots of sheep erythrocytes. In addition to further investigation of the causes of differences in susceptibility to immune lysis exhibited by various lots of sheep erythrocytes, a critical evaluation of the efficacy of various blood preservatives and investigations on the potential value of flash freezing of sheep red blood cells for use in serologic tests seem to be desirable.

Summary. Sheep erythrocytes stored for periods up to 4 weeks in modified Alsever's solution exhibited significant and progressive increases in susceptibility to immune hemolysis in assays conducted with a constant source of human C′. Moreover, the increased susceptibility to immune hemolysis closely paralleled the changes of osmotic fragility and a tendency to undergo spontaneous lysis. The findings suggested an intrinsic relationship between immune hemolysis and osmotic fragility. It was noted that cells stored longer than 3 weeks in modified Alsever's solution could be unduly fragile and hypersensitive to immune lysis thus rendering them unsuitable for serologic tests.

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