The Use of DFP^{32} as a Red Cell Tag with and without Simultaneous Tagging with $Chromium^{51}$ in Certain Animals in the Presence or Absence of Random Destruction

G. S. EADIE, WIRT W. SMITH, and IVAN W. BROWN, Jr.

From the Departments of Physiology and Pharmacology and of Surgery, Duke University, Durham

ABSTRACT DFP^{32}, used to label erythrocytes *in vitro*, combines with cell constituents in two stages, the first almost immediate and involving tributyrinase inactivation, the second slower (more than 40 minutes) involving cholinesterase inactivation. Raising the DFP concentration increases the amount irreversibly bound, but increases even more the immediate post-transfusion elution, and DFP is unsuited for investigating erythrocyte viability of stored samples. *In vivo* tagging by intramuscular injection is satisfactory and normal survival curves are linear since the sample tagged has normal age distribution of cells in absence of random destruction. Here DFP^{32} curves are easier to interpret than Cr^{51} curves. In sheep, chromium elution occurs at two different rates producing a rapid initial drop followed by a slower one of about 3 per cent daily.

Random destruction alters cell age distribution. New equations are derived for cases in which this is constant both with and without chromium elution; they were applied satisfactorily to dog and sheep blood. Analysis of such curves is difficult; approximate values for random destruction rates can be obtained though not potential life spans. Chromium curves can be analyzed only with the help of DFP^{32} or similar curves, and yield little additional information. DFP^{32} and chromium can be used simultaneously to provide controls.

INTRODUCTION

Red cell survival is most frequently investigated at the present time using radiochromium, a method with many advantages from a technical point of view. Blood is labeled with ease, and measurement of radioactivity in the circulation after transfusion is not difficult since Cr^{51} emits gamma radiation

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of relatively high energy. Exact interpretation of results, however, presents problems, mainly because of "elution" of chromate from tagged erythrocytes causing a daily loss of a percentage of the radioactivity still remaining. This rate of loss, although constant from day to day, unfortunately varies from one individual to another. In addition there is the steady daily loss due to disappearance of cells which have reached the end of their life span, the number of these being the same each day under normal circumstances. Pathological conditions introduce new causes of cell disappearance at rates varying with the severity of the disease, and in most cases resulting in loss of erythrocytes without relation to their age. All this complicates interpretation, but even in the simplest case, *viz.* in which red cell destruction can be attributed entirely to senescence, difficulties in interpretation resulting from elution are evident. Thus when radiochromium is used, decrease in radioactivity in the blood is no longer linear, and the disappearance curve is described by the equation originally derived by Sheets *et al.* (1)

$$y = y_0(1 - t/T)e^{-kt} \qquad (1)$$

in which y is the radioactivity count on day t corrected for decay, y_0 being the count for day zero, the day of transfusion. The average potential life span of the erythrocyte is T days, and the fraction of chromium lost daily by elution is k. Data can be fitted satisfactorily and without bias to this equation by the method of Deming (2), but the procedure is very laborious, particularly since it is a method of successive approximations, and may require five or more repetitions to reach stable values. In carrying it out an electronic digital computer is almost a necessity. A value for T may, of course, be obtained by assuming one for k or *vice versa*, but in either case the calculated value is biased to an extent which depends on circumstances. Since normal variations in both T and k are fairly large, this procedure is not to be recommended. If, however, the value of T is known from some other source, the objection vanishes, and one may determine the elution rate for chromium. Methods of the Ashby type based on differential agglutination or hemolysis can sometimes be used to determine T, but only when donor and recipient belong to certain blood groups; autotransfusion is excluded. Moreover these methods are tedious and time-consuming. In 1954 Cohen and Warringa (3) showed that P^{32}–labeled DFP (isoflurophate) is irreversibly bound to erythrocytes, and disappears from the circulation only when the cells themselves leave. By injecting a small amount of the drug intramuscularly they could produce adequate labeling of the subject's red cells, and were able to follow their disappearance. Under normal conditions this was linear just as it was with the Ashby methods. These observations were confirmed by Mayer and Lee (4), and by Bove and Ebaugh (5). The latter extended

the method by using *in vitro* labeling, but found that there was a rapid initial loss of radioactivity lasting as long as 10 days, and they did not calculate life spans. The early rapid loss of radioactivity was attributed to elution. Previous labeling with DFP did not affect Cr^{51} survival curves, but it was not known whether all the cholinesterase in these cells had been inactivated.

Linear disappearance curves can occur only when the sample labeled consists of equal numbers of cells of all ages, and equation (1) was derived on this assumption. This condition is met only when disappearance is due solely to senescence. In menstruating females and in all known pathological processes involving abnormally great red cell loss, the older cells have been exposed to the destructive agency over a longer period than the younger ones, and are consequently fewer in number. In these cases equation (1) does not apply.

The object of this paper is to discuss the advantages and limitations of the use of DFP^{32} for following red cell survival when employed both alone and in conjunction with radiochromium under normal conditions and when cells are subject to steady destructive processes.

Methods

Sheep when acquired were treated with an oral dose of 13 gm. of phenothiazine as anthelmintic; throughout the experiment a trough containing a salt of this was always accessible. They were kept out of doors and allowed to graze; they were also fed a supplementary ration of hay and a mixture of grain and molasses.

Mongrel dogs weighing 15 to 20 kilos were maintained on a diet of Purina dog chow, supplemented with cooked horse meat and bone scraps.

Counting of both beta and gamma radiation was done in commercial well-type scintillation counters. When both types of radiation were to be counted specimens were placed in plastic tubes. It was found necessary, however, to keep all standards in glass tubes, for there was a slow but appreciable loss of contents from the plastic tubes no matter what type of seal was used.

Hematocrit readings were determined by centrifuging blood in Wintrobe tubes at 3,000 R.P.M. for 45 minutes using a No. 1 International centrifuge; the resulting figures were corrected for the 3 per cent of plasma which had been shown to be trapped under these conditions (6).

Uptake of DFP in Vitro

When erythrocytes are labeled by intramuscular injection of DFP^{32}, the amount of label taken up by the individual red cell is minute, but, because all erythrocytes in a sample are labeled, a satisfactory counting rate is obtained. This form of labeling, however, restricts the usefulness of the method to those few cases in which it is desired to follow the survival of the subject's

own cells. With *in vitro* labeling on the other hand, the tagged cells on reinjection are diluted by a great number of untagged cells, and the counting rate is reduced in proportion. It is therefore desirable to incorporate as much label as possible in cells tagged *in vitro*. Preliminary experiments on the rate of uptake showed that in the presence of 2×10^{-7} M DFP there are two phases, an initial rapid one not exceeding 1 minute in duration followed by a much slower one lasting more than 40 minutes. Two phases have also been reported by Bove and Ebaugh (5) who gave the duration of the first phase as less than 20 minutes. During the initial phase tributyrinase is inactivated, but cholinesterase is not, and the slower uptake probably represents combination of DFP with serine groupings such as those in cholinesterase (7) since it has been shown that inactivation of the latter enzyme by dilute DFP solutions proceeds very slowly (8). It was decided to adopt 45 minutes as a standard time of exposure of red cells to DFP since by this time uptake is very slow, and also because this length of incubation had already been adopted for chromate.

To investigate the effect of DFP concentration on uptake, human blood was drawn into ACD solution, washed twice with Parpart's (9) buffered saline, pH 7.2, and made up to an estimated hematocrit reading of 50 per cent with the same solution. The cell suspension was then divided into six parts and placed in polyethylene centrifuge tubes. Varying amounts of DFP^{32} freshly diluted with Parpart's solution were added and all tubes were brought to the same volume. After 45 minutes at room temperature the tubes were centrifuged, the supernatant removed, and a measured amount of fresh buffered saline added. This was repeated until the supernatant showed a minimal and relatively constant radioactive count. There was a steady decrease in the amount of radioactivity in the supernatant fluid at a rate such that the number of counts in a wash was always the same fraction, *e.g.* one-half to one-quarter of the counts in the preceding wash, until minimal values were reached (Fig. 1). The fraction depends on the relative volumes of cells and supernatant, but is independent of the initial concentration of DFP, so that when this is low, minimal concentrations in the wash solution are reached with fewer washes. This probably means that the DFP within the red cell exists in two states, one loosely bound, rapidly reaching equilibrium with the suspension medium, and another much more firmly bound. The amount of the latter can be increased somewhat by increasing initial concentrations, but this also increases the number of washes necessary to remove as much as possible of the loosely bound DFP. The amount of this removed in this way was in most instances small in relation to that retained in the red cells, so that it was difficult to measure accurately the change on washing. Nevertheless washing is essential if an excessive drop in radioactivity in the blood in the first 24 hours following transfusion is to be avoided. However, because

of this initial elution DFP^{32} is unsuitable for use in investigating the immediate post-transfusion survival of erythrocytes.

In Vivo Survival of Erythrocytes Labeled in Vitro with DFP^{32}

A comparison with the Ashby method seemed to offer the simplest and most instructive test of the validity of the method. Accordingly, cells labeled with DFP^{32} *in vitro* were transfused and their survival followed both by the radioactivity and by a modified Ashby method using anti-A hemolysin (10). For this purpose 500 ml. of blood was withdrawn from a group O donor into 75 ml. ACD solution and centrifuged for 45 minutes. Plasma was removed and

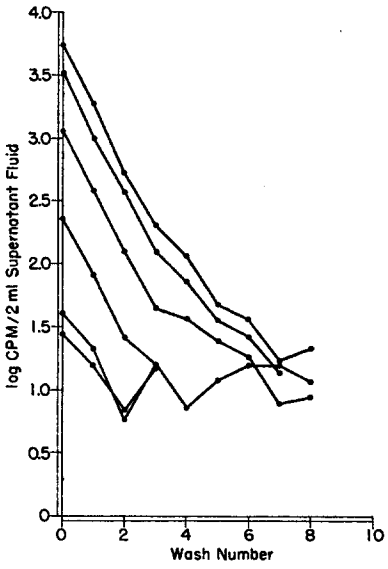

FIGURE 1. The rate of elution of DFP^{32} from cells after 45 minutes' exposure to varying concentrations. The ordinate gives the logarithm of the counts per minute in each wash. The figure for zero wash represents the concentration to which the cells were exposed.

stored in the cold while the cells were resuspended in saline containing 3 per cent of human serum albumin. After centrifuging and removal of the supernatant, 75 ml. of albumin-saline containing 130 μc. DFP^{32} was added and the cells, after thorough mixing, were allowed to stand at room temperature for 45 minutes with occasional gentle shaking. They were then washed twice with the albumin-saline, resuspended in their own plasma, and injected intravenously into a group A Rh-positive, healthy, young, male subject. Results are given in Fig. 2. It will be seen that survival curves obtained by the two methods are identical within the limits of experimental error if allowance is made for the initial elution of DFP. That this is elution and not cell destruction is shown by the Ashby counts, as the same cells were being followed by both methods. Elution from *in vitro* labeled cells cannot be prevented completely by any feasible system of washing, and may be

present even when the Cohn centrifuge is used for this. The linear character of the survival curves indicates that DFP in these concentrations does not damage cells in such a way as to lead to their disappearance from the circulation, and that once the initial period is past there is no further elution of the label.

The next series of experiments were designed to compare DFP^{32} with Cr^{51} under various circumstances. The first experiments were done with sheep. These animals were chosen for reasons irrelevant to the purpose of this paper, but this unintentionally afforded an interesting example of the use of DFP^{32}

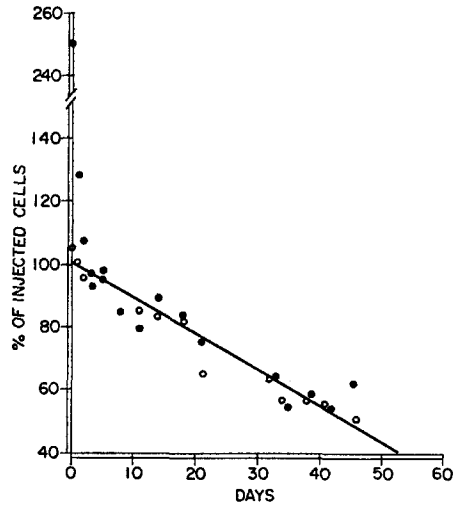

FIGURE 2. Survival of red cells followed by DFP^{32} (closed circles) and differential hemolysis (open circles). The ordinate gives the percentage of viable cells remaining on the day indicated.

in analyzing difficult survival curves. In the first experiments the points obtained with DFP^{32} again fell on a straight line with the exception of those on the first day or two whether cells were labeled *in vivo* or *in vitro*, the only differences being the smaller counting rates in the latter case even though 400 or 500 ml. of tagged blood was infused. The low initial counting rate is a definite disadvantage because of the rapid drop in the counting rate as a result of the relatively short half-life of P^{32} (14.3 days). This means that observations must be limited to the earlier part of the survival curve. This in itself has the disadvantage that even with linear disappearance, a long extrapolation is necessary to find the point of intersection with the base line; *i.e.*, the potential life span. The uncertainty consequent on this is shown by the widening of the 95 per cent confidence limits in the later part of the curve (Fig. 3). However, it is clear that the disappearance curve is linear, and it may be concluded that senescence was the only cause of cell disappearance

and that the sample tagged was normal with respect to age distribution. On the other hand the Cr^{51} data presented a different kind of difficulty. Because of the normal age distribution of cells and absence of random destruction, it was expected that the data would fit the equation of Sheets *et al.* (1). However, attempts to fit the data by the method of Deming (2) resulted in values for T, the potential life span, which were impossibly long—several hundred days—and incompatible with the values previously obtained. Using values for T obtained with DFP^{32} it was possible to eliminate the factor $(1 - t/T)$, *i.e.* the senescence effect, in the equation, and it then became apparent that

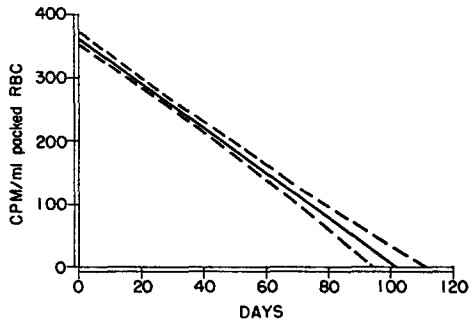

FIGURE 3. Survival of sheep erythrocytes followed by DFP^{32}; disappearance is linear, and the dotted lines show the 95 per cent confidence limits.

the elution rate, k, was not constant but dropped during the first week or so from a high initial value to a much lower but approximately constant rate.[1] Using a least squares value for k from the later points where it showed only random variation, and the value of T from the DFP^{32} curve, a Cr^{51} curve was calculated for the earlier period. These calculated values were then subtracted from the actual experimental values, and the differences were plotted against time on semilog paper (Fig. 4). Points were then seen to fall on a straight line within the limits of experimental error, indicating that the excess elution could be regarded as a separate simultaneous survival curve with the same potential life span, but a higher elution rate. Such a situation would occur if chromate combined two different kinds of groupings of red cell constituents from one of which it is eluted much more rapidly than from the other. No evidence was seen of more than two elution rates in any one of the four sheep studied. However, differences between individuals were marked (Table I). Two animals showed rapid elution rates of about 40 per cent daily for approximately half the chromium combined; others had slower

[1] Drury and Tucker (11) have described a rapid loss of radioactivity in the first day or two after transfusion of 5 to 10 ml. of chromium-tagged blood which they attribute to elution since the hematocrit reading was unchanged, and also since they could demonstrate a high rate of elution *in vitro*.

rates involving smaller proportions of the chromium. The slow rates varied from 2.2 to 3.5 per cent daily, and are definitely greater than the elution rate for human cells which is slightly less than 1 per cent a day on the average (10, 12). Absence of uniformity between different breeds of sheep is not surprising, since differences have been shown in the sodium and potassium contents of their red cells (13), and different hemoglobins have also been described (14). Several survival experiments on the same sheep showed the initial elution rate to be much the same at different times, the sheep showing

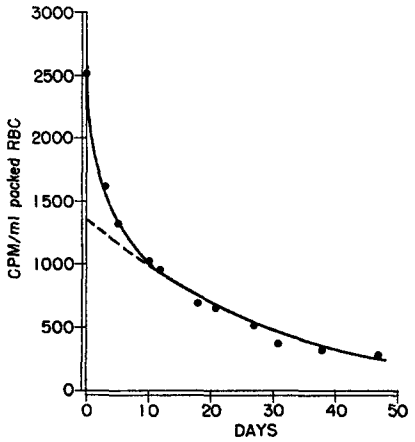

FIGURE 4. Survival of sheep erythrocytes followed by Cr^{51}, showing double elution curve. The solid line is drawn from the equation

$$Y = (1 - t/90.3)\ (1220e^{-0.429t} + 1361e^{-0.021t})$$

90.3 being the potential life span and 0.429 and 0.021 the elution rates.

a high rate in one experiment showing it in others, and *vice versa*. Unfortunately the sheep studied by us were accidentally killed before the hemoglobin was examined or the cellular sodium and potassium estimated.

A more complex situation arises when there is constant random destruction of red cells in addition to loss from senescence. Although random destruction, like elution, may remove a constant daily fraction of cells, equation (1) cannot be adapted to this situation since the age distribution of the sample labeled has been altered by the destructive process. Assuming that the daily rate of random destruction has been approximately constant since the oldest circulating erythrocyte entered the circulation, the following equation may be derived (see appendix):

$$y = (N/b)(e^{-bt} - e^{-bT}) \qquad (2)$$

in which y is the number of labeled cells (as indicated by the radioactivity count) surviving to day t, b is the daily rate of random loss, T the potential

life span in days, and N is the number of new cells entering the circulation daily, or, if a small sample of the circulating blood is being considered, N is the appropriate fraction of the total number formed daily. Suitable data were available from both dog and sheep experiments. On attempting to apply equation (2) to the data, it soon became evident that estimates of T, the potential life span, were subject to so great a standard error as to be of little value. In the case of the sheep it happened that two of the animals had been used in previous experiments, and that at that time random destruction

TABLE I

ELUTION RATES IN SHEEP AND DOGS IN THE PRESENCE AND ABSENCE OF RANDOM DESTRUCTION

	k determined in presence of random destruction by comparison of Cr^{51} and DFP^{32} curves	k determined in absence of random destruction as shown by DFP^{32} curves
Sheep		
S	0.0284	—
C	0.0304	0.0355
Sh	0.0292	0.0223
F	0.0306	—
R	—	0.031
T	—	0.026
Dogs		
B	0.0188	
G	0.023	
Sp	0.0204	

had been absent.[2] For these animals values of T were available, and, using them, it was possible to obtain estimates of b with a fairly small standard error (Fig. 5). Moreover, since the value of T is relatively indeterminate, it seemed probable that estimates of b might be relatively unaffected by errors in the value of T, and this proved to be the case. For instance, using a value for T of 130, b was estimated to be 0.0275, while if T was taken as 101, b was estimated as 0.0268, a difference of less than 3 per cent. In the case of the dogs 100 days was chosen as the value of T, and satisfactory curves were obtained. A close estimate of b can thus be obtained even if T is only approximately known, and this is of considerable practical importance, since b measures the intensity of pathological hemolytic processes, while T probably reflects little more than some characteristics of the newly formed red cell as determined by hereditary factors, and is unaltered, so far as known at present, by disease.

[2] The reason for linear disappearance at one time and curvilinear disappearance at another is unknown; the most likely explanation seems to be infestation with intestinal parasites.

In the presence of random loss of label by elution as well as by cell destruction, the situation is much more complicated (Fig. 6). If k is the daily rate of elution, the appropriate equation (see Appendix, equation (16) is:

$$y = (N/b)(1 - e^{-b(T-t)})e^{-(b+k)t} \tag{3}$$

It is very difficult to solve this equation without more information than is afforded by a single survival curve. However, if two curves are available, *e.g.* one with chromate labeling, and one free from elution difficulties, *e.g.*

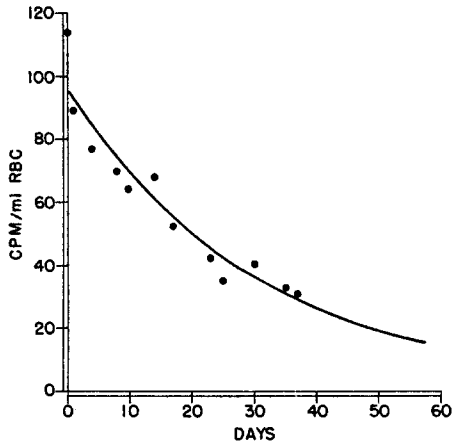

FIGURE 5. Survival of sheep erythrocytes in the presence of random destruction followed by DFP^{32}. The solid line was drawn on the assumption of a potential life span, T, of 101 days from the equation

$$Y = 683\ (e^{-0.026t} - e^{-0.626T})$$

DFP^{32} or Ashby, it is possible to obtain a fairly close estimate of k by dividing the experimental values obtained with chromium by the corresponding values from the other survival curve. The logarithms of the ratios are plotted against time and should form a straight line, the slope of which is k if natural logarithms are used. That this is so is shown in Fig. 7. Table I gives elution values obtained in this way. (For sheep, because of DFP elution, only slow rates of chromium elution could be calculated.) Agreement is satisfactory with values obtained in other sheep experiments. As far as we are aware the elution rate for the dog has not been previously determined.

The rate of chromium elution, however, is probably only of academic interest, and is more easily determined in the absence of random loss. The real interest of this experiment lies in the treatment to which the cells had been subjected. In the case of two sheep DFP^{32} was injected intramuscularly, thus labeling all cells. A small sample of blood was then removed, treated with a high concentration of non-radioactive DFP so as to inactivate not only

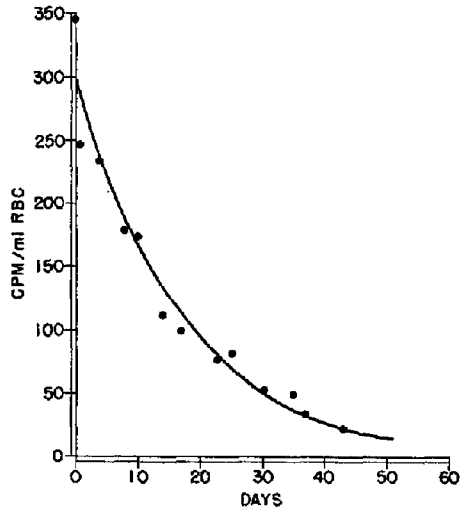

FIGURE 6. Survival of sheep erythrocytes in the presence of random destruction followed by Cr^{51}. The rate of Cr^{51} elution was determined by comparison with the DFP^{32} curve. The solid line was drawn from the equation

$$Y = 319\ (1 - e^{0.0275(T-t)})\ e^{-0.0797t}$$

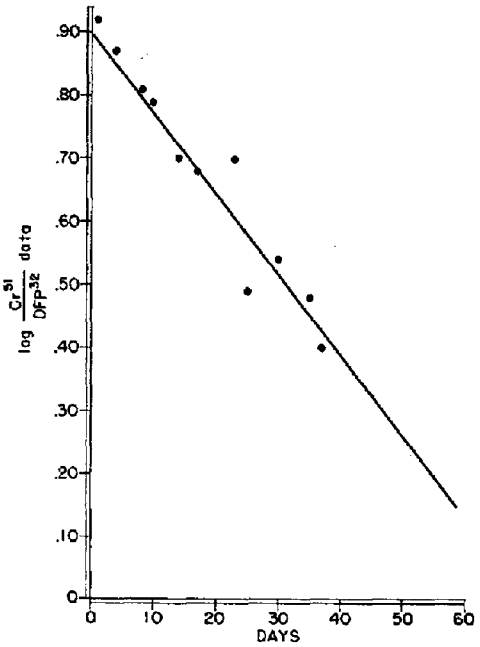

FIGURE 7. Sheep erythrocyte survival. Logarithms of the ratio of counts obtained by Cr^{51} and DFP^{32}. The cholinesterase of the cells labeled with DFP^{32} was inactivated. The slope is linear.

the tributyrinase, but also the cholinesterase, then labeled with rachromate and reinjected. Fig. 7 shows the logarithm of the ratio between Cr^{51} counts and P^{32} counts plotted against time. Here the points clearly fall on a straight line, and the slope yields a value of k very close to that obtained by other methods. In the case of the other two sheep red cells were removed and labeled with concentrations of DFP^{32} sufficient to inactivate cholinesterase. At the same time disappearance of the animal's own untreated cells was investigated by labeling a small sample of blood with rachromate. Results with these sheep were similar to those with the others, with the only difference

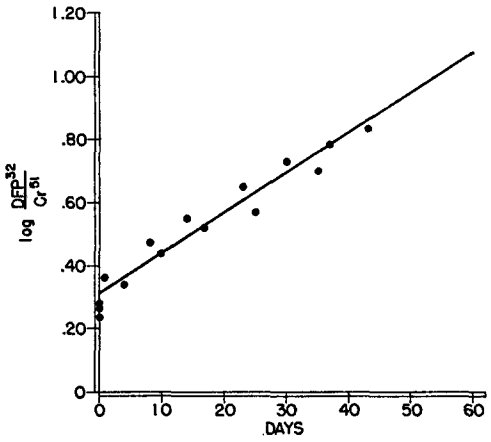

FIGURE 8. Sheep erythrocyte survival. Logarithms of the ratio of counts obtained by DFP^{32} and Cr^{51}. Cholinesterase of cells labeled with Cr^{51} was inactivated by non-radioactive DFP.

between the DFP^{32} and the Cr^{51} curves being the elution rate of chromium (Fig. 8, Table I). From this it follows that the survival of cells whose enzymes have been inactivated permanently by DFP is unaltered. This becomes clear if we consider the possible nature of damage to the cell from loss of cholinesterase activity. (*a*) The immediate viability of the cells may be affected, an effect similar to that which occurs with prolonged storage even under the most favorable conditions. The absence of an initial drop in the Ashby curves rules this out. (*b*) It may increase the rate of random loss or (*c*) it may decrease the potential life span; *i.e.*, it may increase b or decrease T. But in either of these cases changes would show up as a gradual change in the slope of the line shown in Fig. 8, which would no longer be linear; and the calculated value of k would be altered.

Finally it may be observed that DFP^{32} and chromium have been used in two ways: one to label treated cells, *i.e.* those whose cholinesterase has been inactivated, and the other, as a control and to label untreated cells. DFP^{32}

used together with rachromate would seem to be of value in experiments of this type.

It is a pleasure to acknowledge the technical assistance of Mr. W. G. Curtis.

APPENDIX

Theoretical Considerations

Only those cases will be considered in which the pathological process causes red cell disappearance at a constant rate, independent of the age of the erythrocyte (random destruction). Such a loss might occur due to a stabilized infestation with intestinal parasites, a hereditary defect in the cell itself, or from some other cause. Disappearance from senescence will also occur. The number of cells, per unit of blood, which disappear in this way on any day will be fewer than the number N which would have reached this age in the absence of pathological destruction. If the daily rate of destruction is b, then

$$s = N \cdot e^{-bT} \tag{4}$$

The number of cells, y, per unit of blood dying from senescence in the first t days after labeling will then be ts which equals $t \cdot N \cdot e^{-bT}$. If n_0 is the number of cells tagged per unit of blood, and x is the number of these that have been destroyed pathologically in the first t days after labeling, then the number of cells remaining on day t

$$n = (n_0 - x - y) = (n_0 - x - t \cdot N \cdot e^{-bT}) \tag{5}$$

The rate of random destruction is, by definition, bn, and therefore

$$dx/dt = b(n_0 - x - t \cdot N \cdot e^{-bT}) \tag{6}$$

Integrating with the conditions that $x = y = 0$ when $t = 0$

$$n = n_0 e^{-bt} - (N/k)e^{-bT} + (N/k)e^{-b(t-T)} \tag{7}$$

But

$$n_0 = N \int_T^0 e^{-bt} \cdot dt = (N/b)(1 - e^{-bT}) \tag{8}$$

and

$$n = (N/b)(1 - e^{-b(T-t)})e^{-bt} = (N/b)(e^{-bt} - e^{-bT}) \tag{9}$$

This equation, however, can be applied experimentally only if there is no elution of the label. When this is present its rate k adds to that of the random destruction

so that if the rate is b before the day of labeling it is $(b + k)$ after. The following considerations will, however, apply to any case in which a new rate of random loss is produced on the day of labeling, as might perhaps occur on transfusion into a different environment.

In this case

$$s = Ne^{-b(T-t)}e^{-(b+k)t} \tag{10}$$

and

$$y = N\int_0^t e^{-(bT-kt)}\,dt \tag{11}$$

Integrating with the condition that $y = 0$ when $t = 0$

$$y = (N/k)e^{-bT}(1 - e^{-kt}) \tag{12}$$

and

$$n = n_0 - x - (N/k)e^{-bT}(1 - e^{-kt}) \tag{13}$$

The rate of random destruction is

$$dx/dt = (b + k)[n_0 - x - (N/k)e^{-bT}(1 - e^{-kt})] \tag{14}$$

By an argument similar to the one above it can be shown that

$$n_0 = (N/b)(1 - e^{-bt}) \tag{15}$$

and therefore

$$n = (N/b)(1 - e^{-b(T-t)})e^{-(b+k)t} \tag{16}$$

If the same blood is followed with Cr^{51} and simultaneously with DFP^{32} or by the Ashby method, the rate of chromium elution can be found by dividing the counts obtained by one method by those obtained with the other. For if n_1 is the count obtained in the absence of elution and n_2 the count with elution, from equations (7) and (16),

$$n_1/n_2 = (N_1/N_2)e^{kt} \tag{17}$$

On plotting the logarithms of the ratios obtained for each blood sample against time a straight line should be obtained whose slope is $(-k)$ and which cuts the Y axis at ln (N_1/N_2). When two different radioisotopes are used N_1N_2 must be multiplied by factors relating to the numbers of erythrocytes for each isotope.

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