The Measure of Erythropoiesis in Anemias
I. The Mixing Time and the Immediate Post-Transfusion Disappearance of T-1824 Dye and of Cr\textsuperscript{51}-Tagged Erythrocytes in Relation to Blood Volume Determination

By Max M. Strumia, Louise S. Colwell and Ann Dugan

IT IS OFTEN desirable to use the radioactive chromium method and the T-1824 dye method for the measure of the blood volume. Discrepancies in results from these methods have been reported by several observers.

Tagging of red cells with radioactive sodium chromate has become a preferred procedure for determination of the volume of the red cell mass and for the estimation of the survival of transfused red cells. The formulae which are generally applied for the calculation of these values from radioactivity data can be expressed as follows:

\[
\text{Red Cell Volume} = \frac{\text{Total Red Cell Radioactivity Transfused}}{\text{"Immediate" Radioactivity/ml. of red cells}}
\]

\[
\text{Apparent } \% \text{ Survival of} = \frac{\text{Radioactivity/ml. of Recipient's Red Cells at any time X 100}}{\text{"Immediate" Radioactivity/ml. of red cells}}
\]

It is evident that the validity of the preceding formulae depends essentially on the accuracy of the "immediate" post-transfusion radioactivity or 100 per cent value. The blood sample or samples for the determination of this value must obviously be secured after complete mixing of the labeled cells but before any considerable number had disappeared from circulation. This interval of time may be properly referred to as "mixing time."

Similar considerations apply to the determination of the plasma volume by the use of diazo dye T-1824 or Evans Blue.

The purpose of this study is to present data on the mixing time of transfused Cr\textsuperscript{51} tagged red cells and of dye T-1824, their disappearance from the vascular space, and the relationship of these phenomena to the measure of blood volume. A method of evaluation of data will be proposed which yields substantially identical results with the Cr\textsuperscript{51} and the T-1824 technics.

METHODS AND PROCEDURE

The method for tagging of red cells with radioactive sodium chromate* is one pre-
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viously outlined, with the following exceptions: (1) with the high specific activity now available, the concentration of chromium metal to which the cells are exposed is below two micrograms per milliliter of red cells; (2) samples of blood obtained after the transfusion of Cr\textsuperscript{51} tagged red cells are counted in flat bottom test tubes in an amount sufficient to contain approximately one milliliter of red cells (2 ml to 5 ml depending on the hematocrit). The blood is centrifuged and the supernatant plasma is removed. A correction is made for variation in the red cell column over one milliliter. Standards are counted in one milliliter volume.

The specific activity of the recipient's red cells is expressed in counts per minute/ml of cells, calculated by dividing the radioactivity of the cells counted by their volume:

\[
c. p. m./mL. \text{ of cells} = \frac{c. p. m. \text{ in cells counted}}{\text{volume mL. of cells counted}}
\]

The selection of the 100 per cent value used in the calculation of the blood volume is discussed later.

For the determination of the dye-plasma volume 5 ml of 0.5 per cent solution of T-1824* are injected with 5 ml of saline used to rinse container, syringe, and needle. Filter 6225 is used in a Klett colorimeter for reading of the dye concentration in the samples of serum.

The following procedure has been adopted for the collection of blood samples after the injection of Cr\textsuperscript{51} labeled cells and/or T-1824. The subject is fasting from solid foods but drinks 500 ml of water or fruit juice one hour before the study. One per cent novocaine is injected subcutaneously, and a suitable vein in each arm is entered with a 19 gauge needle attached to a clean, dry, ten milliliter syringe, and the tourniquets are released. After a blank sample is obtained, 10 ml of Cr\textsuperscript{51} tagged blood in a calibrated syringe and/or 10 ml of the measured amount of dye is injected through the same needle. The time of injection is generally 7 to 10 seconds and is determined with a stop watch. At the end of the injection, a second stop watch is started for the purpose of timing the postinjection samples. Ten milliliters of blood are collected from the opposite arm as nearly as possible at 1, 2, 3, 5, and 10 minutes, occasionally at 30 and 45 seconds and 20 and 30 minutes. After each sample is collected, the syringe containing the blood is detached and replaced with a clean syringe. The plunger of the collecting syringe is held firmly so that blood does not enter the syringe before the timed period. The blood samples are transferred to labeled tubes double oxalated for the Cr\textsuperscript{51} samples and plain tubes for the T-1824 samples. No difficulty is experienced with the blood clotting within the needle during the first five minutes. After this period, samples are obtained by independent venipunctures. All syringes and needles used are siliconized.

**EXPERIMENTAL RESULTS.**

(a) **Normal Subjects.** All subjects used in these experiments were normal, young volunteers, mostly males. In all instances the chromated cells were autotransfused within one hour of collection, except in the series shown in table 2.

Figure 1 presents radioconcentration values from 194 observations in 45 subjects. Complete mixing required one minute or more from completion of transfusion and in all but one subject was completed in less than five minutes. In most subjects complete mixing occurred between the second and the third minute. Complete mixing is assumed to have been reached when the radioactivity values remain constant within the limits of accuracy of the procedure involved (± 3 per cent). Similar results have been obtained with the transfusion of fresh compatible blood in normal individuals.

* Warner-Chilcott Laboratories, New York, N. Y.
The constancy of values of radioactivity between 3 and 11 minutes post-transfusion is worthy of notice. A much different result is noted when serum density values are similarly plotted after intravenous injection of T-1824 dye. A total of 481 observations were made in 100 subjects. Study of individual curves indicates that after reaching a maximal density the dye concentration invariably declines. According to the time required to reach maximal concentration and to the rate of decline, all subjects may fall into one of three groups.

Group A — Normal Mixing. Eighty-six per cent of subjects fall into this group (fig. 2). Maximal concentration of the dye is reached between the ex-
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Figure 3.

Figure 4.
treme limits of 1 and 5 minutes, mostly between 2 and 3 minutes. Dye concentration following the maximal value declines slowly, so that the mean of all values at 10 minutes is 96 per cent.

Group B — Fast Mixing. This group comprises 8 per cent of subjects, (upper graph of figure 3). In these subjects maximal dye serum density is reached within 1 minute and 15 seconds thereafter, the dye concentration falls rapidly, so that at 10 minutes post-transfusion the mean concentration is only 90 per cent.
Group C — Slow Mixing. In this group, which comprises 6 per cent of all subjects, the highest serum density is reached slowly, after 9 minutes (lower graph of figure 3).

Mean values of the mixing time and serum density in the three groups is compared to the radio concentration of Cr$^{51}$ tagged autotransfused red cells in figure 4.

Individuals tend to show the same type of dye-mixing curve in subsequent observations. Figure 5 shows two mixing curves of dye injected in the same individual at an interval of several months. In either case there is a very rapid mixing time followed by a rapid decline in the concentration. Figure 6 shows
Figure 8.

Figure 9.
two normal dye-mixing curves obtained in another subject, also at intervals of several months.

Contemporaneous determination of the mixing time in the same subject with chromated red cells and dye T-1824, in 14 normal subjects shows that the maximal concentration of tagged red cells and of dye T-1824 occurs in every instance within 2 minutes of each other (fig. 7). Generally, individuals who have a rapid red cell mixing time also show a rapid mixing time of the dye (see graph S.H. of figure 7). The same is true of individuals with a slow mixing time, of which graph R.E. is an example.

When the period of observation is extended to 30 minutes (fig. 8), it will be noted that both the normal and the “fast” group show a continuous steady decline of the dye concentration.

(b) Patients. The determination of the blood volume by the dye method was carried out in 86 patients who were, for the most part, convalescing or being prepared for an elective operation (fig. 9). It is evident that in this group only 44 per cent of subjects show the mixing pattern which has been designated as “normal.” Fifty per cent of the patients show the “rapid” mixing pattern and 6.0 per cent the “slow” pattern.

Discussion

Barring immediate loss of radioactivity, destruction, or segregation of red cells, there should be little difficulty in determining the time when tagged red cells are completely mixed with the mass of circulating blood following transfusion.

It is generally recognized that radioactive sodium chromate is an ideal tag of red cells and permits an accurate and reliable method of determination of the volume of circulating red cells. The validity of the measurements of the plasma volume with the dye T-1824, however, has been the subject of much discussion. Large discrepancies in the total blood volume measured with tagged red cells-hematocrit and the T-1824 plasma volume-hematocrit methods have been reported by many investigators.

Although mixing time plays an essential role in the interpretation of the data pertinent to blood volume, it has been rather arbitrarily set by some investigators.

Gibson and Evans using T-1824 found, immediately after the injection, a rapid decline in the concentration of the dye, followed by a slower gradual fall. The rapid initial decline in concentration was considered due to progressive dilution rather than to loss of the dye from circulation. These investigators considered mixing time complete in 7 to 12 minutes.

Gregerson and Rawson found the average mixing time of T-1824 in normal subjects to be 9 minutes, and advises taking a single specimen at 10 minutes.

Barnes et al. considered mixing time complete in 4 to 8 minutes. Wasser and Mayerson allowed 10 minutes for mixing.

From the observations of Gibson and Evans and those of Gregerson and Rawson it has been assumed that the decline of dye concentration in the first part of the curve represents incomplete mixing, but slow removal from the
circulation. During the second phase the dye is completely mixed and removed at a slow, regular rate.

According to the observation of Ferrebee et al. the rapid initial decline of the dye-albumin complex is attributable not to dilution but to extravascular loss. Similar considerations are applicable to other methods of tagging albumin, such as radioiodinated serum albumin and chromic chloride.

The attempts to establish a single and fixed postinjection interval of time, at which samples of dye-plasma are obtained to determine the plasma volume or the extrapolation of data to 0 time, are based on the assumption that the mixing time and the rate of apparent loss of dye are similar in all subjects.

The data shown in the present investigation indicate that such is not the case.

The work of other investigators points to another possible mechanism of apparent early loss of tagged albumin from circulation, in addition to loss by mixing with the entire mass of circulating blood and to extravascular loss. This mechanism depends upon the functional dualism of blood volume: the volume of “actively circulating blood” and the “total volume of blood in the vascular system.”

This concept, based on the observation of Jager, is that in small vessels the red cells flow in the rapidly moving central portion of the stream while there is a slow moving, cell-free layer of plasma adjacent to the vessel walls.

There is much evidence emphasizing the role of the “marginal, cell-free, sluggish” plasma in relation to mixing phenomena and measure of blood volume.

Ebert and Stead found that blood in minute vessels has a lower red cell content than in larger vessels.

Hahn et al., using T-1824 and Fe tagged red cells in dogs, conclude that the dye under certain experimental conditions measures only the “rapidly circulating plasma volume” and not the volume of “sluggishly” moving plasma in cell-free films. This “sluggish” plasma is estimated at 20 per cent of the total.

Barnes et al. found that in man the red cell volume calculated from the dye-plasma volume and the hematocrit is on the average 13 per cent greater than that estimated from the Ashby method, with individual variations as high as 18 per cent. The same authors give as reason for the discrepancy the presence in small vessels of an excess of “marginal plasma” and as a consequence an unequal distribution of plasma and red cells throughout the circulating blood.

Miller likewise relates the mixing curve to the “sluggish” plasma.

Wasserman and Mayerson reject the concept of a sluggish, slowly mixing plasma, and consider the plasma as measured by radioiodinated albumin as a “definite entity.”

Berson and Yalow reinvestigated the subject of loss versus dilution of the dye by doing simultaneous determinations of the blood volume in human subjects with I labeled albumin and P or K tagged red cells injected intraarterially. The relatively rapid, early decline of tagged plasma is attributed to mixing, because a similar loss is noted in the tagged red cells. It is more likely that the early loss of red cells is attributable to damage due to washing. We do
# Table 1.

<table>
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<tr>
<th>Initials</th>
<th>Dye plasma volume ml.</th>
<th>Cr&lt;sup&gt;+&lt;/sup&gt; R.C. volume ml.</th>
<th>Absolute R.B.V.</th>
<th>Body hmtc</th>
<th>Venous hmtc</th>
<th>Body hmtc Venous hmtc</th>
<th>Dye-hmtc B.V.</th>
<th>Cr&lt;sup&gt;+&lt;/sup&gt; hmtc B.V.</th>
<th>Dye B.V. x 100</th>
<th>Cr&lt;sup&gt;+&lt;/sup&gt; B.V. x 100</th>
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Mean 0.93

S.D. 0.03
not wash the red cells and have seldom noted any decline of tagged red cells within the first 30 minutes post-transfusion in normal autotransfused individuals.

From the evidence available, we may conclude that a cell-poor, sluggish plasma does exist, and that this functional entity affects the mixing time of the dye.

The decline in the concentration of the dye following intravenous injection may then be attributed to two mechanisms: progressive dilution inside and progressive loss outside of the vascular system. The progressive dilution of the dye, in turn, occurs in two distinct phases: (a) mixing with the rapidly moving "central stream" containing red cells; and (b) slower diffusion into the sluggish, cell-free plasma films of the smaller vessels.

As a consequence, if time is allowed for complete mixing of the dye with the entire mass of blood, the resulting measure of the blood volume will always be larger than that based on tagging of red cells.

To compensate for the uneven distribution of plasma in the vascular system, correction factors have been proposed.

Even with such corrections, the variations obtained by simultaneous measures of the blood volume with plasma-hematocrit and red cell mass-hematocrit methods are too large to be acceptable. We must, therefore, admit that in addition to the diffusion of the dye-albumin into the erythrocyte-poor areas there is also a loss of dye outside of the vascular walls, as already pointed out.

From the results of our studies it is apparent that a subject with a rapid mixing time also has a more rapid extravascular loss of the dye in the early postinjection period.

In the present work, by "mixing time" of transfused Cr	extsuperscript{51} tagged red cells is meant the period of time measured from the end of the rapid transfusion of 10 ml. of blood to the moment when the radioactivity of the red cells reaches a value within 3 per cent of the highest radioactivity reading at any time. Data within 30 seconds from the end of transfusion are not taken into consideration for the reason given later.

The "immediate radioactivity," or "initial 100 per cent radioactivity," is the average of all values, from mixing time up to 10 minutes, which fall within ± 3 per cent of the mean of all values. This definition is valid only for autotransfused fresh cells, or for properly stored cells, in normal individuals, when the transfusion time is about 7 seconds.

For the dye T-1824 we have chosen the highest concentration reached at any time after the first thirty seconds from the end of the injection as the 100 per cent value upon which we base the calculation of the plasma volume.

As a justification of these assumptions, the total blood volume has been determined in 21 normal subjects simultaneously by the Cr	extsuperscript{51} method with fresh, autotransfused cells and the plasma dye-hematocrit method, using the procedure described (see table 1). The maximal difference from the absolute blood volume in the majority of cases is less than 5 per cent.

A similar study has been carried out in 19 normal, young males using auto-
TABLE 2

<table>
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<tr>
<th>Initials</th>
<th>Dye plasma volume ml</th>
<th>Cr\textsuperscript{4} R.C. volume ml</th>
<th>Absolute B.V.</th>
<th>Body hmtc</th>
<th>Venous hmtc</th>
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mean 0.93
S.D. 0.02
transfused or compatible blood stored for 21 days, Cr\(^{51}\) tagged, simultaneously with the dye-hematocrit method. Table 2 shows the results of this study. The maximal variation between any data from either of the two methods and the absolute blood volume is very small, 3.1 per cent, and the agreement in all instances is good.

In subjects with a rapid mixing time, and rapid dye loss from circulation, the calculation of blood volume based on the standard method of extrapolation of the slope of disappearance to 0 time results in a significantly larger blood volume. Thus, if the dye concentration data on 7 normal individuals with fast mixing time are used to calculate the blood volume by the standard extrapolation method the results are 12.6 per cent higher than those obtained by the method here proposed (see table 3 and figure 8). In the case represented in figure 5, the blood volume based on the “maximal” dye concentration is 4349 ml., and with the extrapolation method, 4839 ml., a difference of plus 10.3 per cent.

In cases of normal mixing time and normal loss of dye from circulation, no significant difference is found between the results obtained with the two methods of estimating the blood volume (see table 4 and figure 8).

An objection has been raised concerning application of the method of calculation here discussed to blood volume in subjects with a slow mixing time; significant loss of dye by extravascular diffusion presumably could occur in these patients before mixing is complete. It has been calculated from data on 86 normal subjects with “normal” mixing time that the actual dye loss in 9 minutes, 40 seconds following injection of dye averages 3 per cent. Subjects with slow mixing time have a similar loss of dye. The loss involved, within the limits of time imposed, is relatively small. It is also noted that individuals with a slow mixing time are a minority, 6 per cent.

A word of caution is necessary concerning possible confusion of “circulation” time and “mixing” time.

The mixing curve of Cr\(^{51}\) tagged red cells seen in figure 10 shows considerable similarity to those obtained by Freinkel et al.\(^{28}\)

These investigators injected T-1824 dye with I\(^{131}\) labeled human serum albumin within a period of 1 second. When the injection was made in the subclavian vein and the samples were obtained from the femoral artery, thus measuring the heart and lung circulation time, maximal concentration of both ele-
ments occurred at 10 to 15 seconds after injection. Thereafter the concentration dropped rapidly, reaching the lowest concentration at about 25 to 30 seconds, after which it increased slightly.

In our experiments the injection time is longer (7 to 10 seconds), and this slower injection time is reflected in the lower peak of initial concentration (fig. 10). The circulation time from cubital vein to opposite cubital vein averaged a little over 15 seconds. In most of our experiments, sampling begins 1 minute post injection, and, therefore, the initial high concentration, marking the circulation time, is generally missed.

Delayed, high initial concentrations, representing long circulation time, have been noted in patients with severe cardiac insufficiency. From the work of Nylin it is reasonable to assume that in these patients the dye remains trapped in the dilated cardiac cavities for a period of time after the injection, before it becomes completely mixed with the circulating blood.

The measure of the blood volume in patients with cardiac dilatation requires great care and the data of individual patients should be analyzed to differentiate between prolonged circulation time and mixing time.

**Conclusions**

1. The mixing time of transfused tagged red cells and the mixing time of
injected dye T-1824 occur within two minutes of each other in a given normal individual, but vary considerably from individual to individual.

2. The decline in concentration of the injected dye after complete mixing also varies from one subject to another. In most normal individuals the maximal concentration of T-1824 dye occurs between 2 and 3 minutes postinjection. In this "normal" group, at 10 minutes post-transfusion the decline in the dye concentration averages 3 per cent. In 8 per cent of normal individuals, the mixing is "rapid," and the dye concentration reaches the maximum within one minute and 15 seconds. Thereafter, the dye concentration declines more rapidly, so that the loss at 10 minutes post injection averages 10 per cent. In the remaining 6 per cent normal individuals the mixing time is "slow," requiring 9 to 10 minutes.

Of 86 bed-patients, 50 per cent show the "rapid" mixing pattern.

The time of the injection of the tagged red cells and of the dye must be short, preferably between 7 and 10 seconds.
3. Multiple determinations of the concentration of tagged red cells and of dye T-1824 should be taken at short interval immediately following injection, preferably at 1, 3, 5 and 10 minutes post-transfusion. For the measure of the blood volume with the dye T-1824, the highest concentration is chosen; for the Cr$^{51}$ tagged red cells method, the "initial 100 per cent radioactivity" is obtained by an average of all values, from mixing time up to 10 minutes post-transfusion, which fall within ± 3 per cent of the mean of all values. Special care in interpretation of results is required in individuals suffering from cardiac insufficiency.

4. Simultaneous determinations of the blood volume done with the Cr$^{51}$-hematocrit and dye T-1824-hematocrit procedures, applying the method of calculation here proposed, have resulted in variations of less than 5 per cent in 98 per cent of instances when compared with the "absolute" blood volume. In 80 per cent of the cases the variations were less than 3 per cent.

5. In the individuals who have a rapid mixing time, comprising 50 per cent of the bed-patients and 8 per cent of the normal population, calculation of the plasma and total blood volume by standard extrapolation method results in values 12.6 per cent higher than those obtained by the proposed method.

**Summario in Interlingua**

1. Le immixtion de transfundite erythrocytos a marcage isotopic e le immixtion de injectiones del colorante T-1824 require tempores non differente per plus que duo minutas in le mesme individuo normal, sed ab un individuo al altere, ille tempores varia considerabilemente.

2. Le declino del concentration del colorante injicite que occurre post que le immixtion es complete varia etiam ab un individuo al altere. In le majoritate del individuos normal le concentration maximal del colorante T-1824 occurre inter 2 e 3 minutas post que le injection ha essite effectuate. In iste gruppo "normal," le declino in le concentration del colorante attinge un valor medie de 3 pro cento al fin de un intervallo de 10 minutas post le injection. In 8 pro cento de individuos normal, le immixtion es rapilde, e le concentration del colorante attinge su maximo intra un minuta e 15 secundas. Postea le concentration del colorante descende plus rapidemente de manera que le perdita al fin de 10 minutas post le injection attinge un valor medie de 10 pro cento. In 6 pro cento de individuos normal, le immixtion es lente e require 9 a 10 minutas.

Inter 86 patientes allectate, 50 pro cento representava le typo de immixtion rapilde.

Le tempore usate in infunder le cellulas marcate e le colorante T-1824 debe esser breve, preferibilemente inter 7 e 10 secundas.

3. Multiple determinaciones del concentration de erythrocytos marcate e del colorante T-1824 debe esser effectuate a breve intervallos immediatemente post le infusion, preferibilemente post 1, 3, 5, e 10 minutas. Como mesura del volumine de sanguine per medio del colorante T-1824, le plus alte concentration es usate; in le caso del metodo a erythrocytos marcate per Cr$^{51}$, le "100 pro cento initial de radioactivitate" es obtenite per calcular le
valor medie de omne observationes ab le tempore del immixtion usque a 10 minutas post le transfusion in tanto que illos se trova intra ± 3 pro cento del valor medie general. Attention special debe esser prestate al interpretation del resultatos in individuos qui suifre de insufficientia cardiac.

4. Determinaciones simultanees del volumine de sanguine per medio del technicas a Cr⁶¹, hematocrite, e colorante T-1824 con hematocrite—con le application del methodo de calculation hic proponite—ha resultate in devia- tiones de minus que 5 pro cento in 98 pro cento del casos in comparation con le “absolute” volumines de sanguine. In 80 pro cento del casos le deviation amontava a minus que 3 pro cento.

5. In le individuos con immixtion rapide—i.e. 50 pro cento del patientes allectate e 8 pro cento del population normal, le calculation del volumine de sanguine total e de plasma secundo le methodos standard de extrapolation resulta in valores que es 12,6 pro cento plus alte que le valores obtenite per le methodo hic proponite.

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