Evaluation of Blood Volume Measurement Techniques

By JOHN W. REMINGTON, PH.D., AND CARLETON H. BAKER, PH.D.

Our studies on the seeming loss of plasma from the circulation after transfusion and the mobilization of plasma often seen after hemorrhage or drug infusions have been handicapped by a large variation in measured responses between animals. Conversations with other workers on blood volume regulation have indicated that such variability is not uncommon. The necessity of using large groups of animals to test the effect of any experimental change makes for tedious research. It also raises the question as to the quantitative accuracy of the techniques used for measuring blood volume. We have, therefore, made several consecutive injections of T 1824 and Cr-tagged red cells in the same dogs to measure the ability of the two techniques to reproduce the same volume. This was then followed by a study of the ability of the two to follow known reductions in blood volume produced by stepwise hemorrhage.

We have shown that after an injection of tagged cells into dogs under morphine-sodium pentobarbital anesthesia, the blood activity level falls to a fairly stable value in about 10 minutes in splenectomized animals and in 20 to 30 minutes in intact dogs. Variations of individual samples, when corrected for any hematocrit change, about the average plateau value have a standard deviation of 3 per cent. Since the hematocrit technique shows a standard deviation of 2 per cent, errors attributable to an uneven distribution of tagged cells in the circulation would appear to be small.

Injected T 1824 does not, of course, reach a stable concentration after mixing is completed, but continues to disappear through the capillary membranes at what has been claimed to be an exponential rate. If the rate of this loss can be clearly established, from a semilogarithmic plot of concentration values one could extrapolate back to the time of injection and thus obtain a reliable plasma volume. Past studies in this laboratory have frequently produced dye-disappearance curves which show transient increases in concentration long after the "mixing period" is claimed to be over. If such deviations mark a protracted mixing time, an extrapolated slope constructed on the basis of the available points is robbed of its theoretical justification and becomes an empirical device only. Dye-concentration curves obtained in the present study show such deviations, coming at various times in the 70-minute sampling period, to be the rule rather than the exception. Plasma volume estimates based on the drawing of a best-fit slope leave much to be desired as far as quantitative reproducibility is concerned.

Methods
The dogs weighed from 11 to 22 Kg., and were selected as the largest and apparently the healthiest of the animals available to us. They had been on an outside run with free access to an adequate water supply for about a week before their use. In many cases, the spleens were then removed and the animals kept in individual cages for one to two weeks more. Two types of experiments were done. First, animals were given three or four consecutive injections of T 1824 and tagged red cells, separated by a sampling period of 80 minutes. Three anesthetic regimens were used. Most commonly we followed our usual procedure of giving 10 mg./Kg. morphine sulfate followed by an intravenous injection of 15 to 20 mg./Kg. sodium pentobarbital. Of 24 dogs so prepared, 11 were normal and 13 were chronic splenectomized. A second group of six dogs (four intact, two splenectomized) was given 200 mg./Kg. sodium barbital intraperitoneally (no morphine). Two full hours were allowed to attain a stable anesthesia before the first blood volume determination was begun. All anesthetized dogs were placed on their backs on an animal board and lightly tied for the duration of the experiment. A last group of 10 splenectomized dogs was given no sedation or anesthesia. They were gentle dogs, but not table trained. Six were kept on their sides on the animal board for
the duration of the experiment (310 minutes), but they became restless before the work was completed. The last four were allowed to remain on the floor except when samples were taken. We have not been able to distinguish between the results obtained with the six and the four.

In this first type of experiment, only one incision was made over the femoral artery and vein of one leg. Injections were made into the vein, using syringes weighed before and after delivery. At 10, 20, 30, 50, and 70 minutes after injection, samples of 3.5 ml. were uniformly taken from the artery into 5 ml. syringes, fitted with 24 G needles, the barrels and needles of which had been wetted with isotonic heparin solution. Any samples showing hemolysis or lipemia, which were rare, were discarded.

The tagged red cells were made from the animal's own blood, as described previously. The T 1824 used was in a 0.15 per cent solution, as freshly made up with distilled water from an ampule of 0.5 per cent dye solution (Lilly).

Shortly after removal, and after thorough mixing, the hematocrit value of each sample was determined in triplicate, using capillary tubes spun for 10 minutes at 13,000 r.p.m. The centrifuge and the specific technique have been described previously. The tubes were read with an International CR Reader. No correction for trapped plasma was considered necessary. A 1 ml. aliquot of whole blood was then pipetted into a test tube for the determination of radioactivity, as described previously. The rest of the sample was then centrifuged, and the plasma pipetted off. The plasma specific gravity was determined by the falling drop technique, and the T 1824 concentration was determined in undiluted plasma, using microcuvettes and a Beckman DU Spectrophotometer. The read optical density (O.D.) of each sample was corrected for any change in plasma specific gravity away from the value shown by the blank sample taken just before the injection, subtracting 1.0070 from each specific gravity before making the correction by simple proportion. The corrected O.D. values were then plotted on semilogarithmic paper, and the slope of best fit drawn by the same investigator for all animals. The reference standard was made by adding 5 microliters of the dye used for injection to 1 ml. plasma taken from the blank sample.

Plasma volumes were calculated from: (a) the O.D. value obtained by extrapolation of the disappearance slope to the time of injection; (b) the O.D. value shown by the 10-minute sample alone; and (c) the average O.D. value of the 10-, 20-, and 30-minute samples. At the end of the 70-minute period, the total sample loss was divided into plasma and red cell loss according to the respective hematocrit values. After subtracting the plasma loss from the initially determined plasma volume, the difference was in turn corrected for any plasma specific gravity change (which was usually quite small in amount) to give the expected new plasma volume. The difference between the second plasma volume determination and this expected value was then expressed in percentage of the latter. The expected cell volume represented simply the value given by the initial determination less the cells removed in the samples.

In the second type of experiment, 24 chronically splenectomized dogs under sodium pentobarbital anesthesia had one femoral vein and artery exposed as above and the other femoral artery exposed for bleeding. The aortic pressure was recorded by optical manometer from the ascending aorta by metal sound advanced through a carotid artery, and the central venous pressure was recorded by saline manometer through a plastic tube advanced through a jugular vein into the thoracic cavity. From the recorded aortic pressure pulses was calculated the cardiac index, expressed in ml./min./M². The total peripheral resistance was calculated as the mean aortic pressure in mm. Hg divided by the cardiac index. The zero level for the venous manometer was taken as that of the apex beat.

Results and Discussion

Cell Volume Reproducibility

The ability of successive injections of tagged red cells to measure the expected cell volume did not appear to be influenced by the anesthesia (table 1) and the reproducibility appears quite acceptable. The difference in mixing time between intact and splenectomized dogs observed before was seen here, but with five samples it was not difficult to recognize the plateau level. For all 34 dogs and the 84 attempted checks against the expected cell volume, the standard deviation was 4 per cent. This means that in any single animal, whether or not it was splenectomized, a second determination had 19 chances in 20 of being within ± 8 per cent of the first. The size of this variability was not set by any increase in the variation of individual samples from the mean with successive runs. This reproducibility is definitely better than that found previously for 17 dogs in which total cell volumes were calculated from two samples only.
Table 1
Reproducibility of Cell Volume Determinations

<table>
<thead>
<tr>
<th></th>
<th>1 C.V.* (cc./Kg.)</th>
<th>2 C.V./H (%)</th>
<th>3 Expected C.V. (cc./Kg.)</th>
<th>4 Expected C.V./H (%)</th>
<th>2-4t (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium pentobarbital</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11 intact dogs</td>
<td>1st 35.7</td>
<td>40.8</td>
<td>87.6</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>2nd 34.4</td>
<td>41.5</td>
<td>82.9</td>
<td>33.0</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>3rd 35.4</td>
<td>41.4</td>
<td>85.8</td>
<td>34.3</td>
<td>3.2 ± 1.7</td>
</tr>
<tr>
<td>7 splenectomized dogs</td>
<td>1st 25.3</td>
<td>34.6</td>
<td>73.2</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>2nd 24.3</td>
<td>35.8</td>
<td>68.0</td>
<td>24.6</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>3rd 23.1</td>
<td>35.6</td>
<td>64.9</td>
<td>23.9</td>
<td>—4.3 ± 1.7</td>
</tr>
<tr>
<td>Sodium barbital</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6 dogs</td>
<td>1st 32.0</td>
<td>42.4</td>
<td>75.5</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>2nd 31.7</td>
<td>42.7</td>
<td>74.3</td>
<td>31.0</td>
<td>2.1 ± 1.5</td>
</tr>
<tr>
<td></td>
<td>3rd 30.9</td>
<td>42.2</td>
<td>73.2</td>
<td>30.3</td>
<td>2.2 ± 1.6</td>
</tr>
<tr>
<td></td>
<td>4th 29.6</td>
<td>39.0</td>
<td>70.0</td>
<td>29.5</td>
<td>0.4 ± 1.6</td>
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<tr>
<td>Unanesthetized</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 splenectomized dogs</td>
<td>1st 25.5</td>
<td>34.6</td>
<td>73.8</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>2nd 25.2</td>
<td>34.0</td>
<td>74.2</td>
<td>24.8</td>
<td>1.6 ± 0.9</td>
</tr>
<tr>
<td></td>
<td>3rd 24.5</td>
<td>33.4</td>
<td>73.3</td>
<td>24.1</td>
<td>1.6 ± 1.3</td>
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<tr>
<td></td>
<td>4th 23.8</td>
<td>31.7</td>
<td>75.1</td>
<td>23.4</td>
<td>1.6 ± 2.0</td>
</tr>
</tbody>
</table>

*C.V.* = Cell volume.
†Hematocrit value.
‡± 1 standard error.

by the hematocrit value. We have used the arterial hematocrit for this purpose. The original cell distribution space can be obtained by dividing the cell volume by the hematocrit (C.V./H) in table 1. If this space is taken to represent the total blood volume, then the standard deviation of the agreement between observed and expected volume is 3.0 per cent. The smaller deviation is in large part attributable simply to the larger value for blood volume, so that a given discrepancy represents a smaller per cent change.

Actually, because injected dye measures a larger blood volume than do tagged cells, it is claimed that the hematocrit value for the whole of the blood volume (particularly in splenectomized dogs) is usually lower than the arterial hematocrit. Correcting the C.V./H values given in table 1 by 1/0.9 would make them all larger and the sample loss relatively less, but the effect on the bookkeeping would be of almost negligible proportions. Contrary to expectation, the calculated total circulatory hematocrit did not remain constant through the successive determinations, but varied as the plasma volume error varied (see below). Correction of the C.V./H values for a changing total circulatory hematocrit worsened the fit with the expected value.

Plasma Volume Reproducibility

As in the past, we have not obtained with any consistency the orthodox type of dye-disappearance slope. For example, the O.D. values for the five samples of the first plasma volume determination for 59 dogs under pentobarbital anesthesia were expressed in per cent of the 10-minute value. The obtained curves group themselves into five patterns, as shown in figure 1. Only 17 cases showed a decreasing dye concentration with each successive sample. At various times after injection, the others showed an increased dye concentration. It should be emphasized that these were all arterial samples, drawn without stasis, and the O.D. value was corrected for any change in specific gravity of the plasma. In many of these curves, the drawing of any disappearance slope for purposes of extrapolation is a highly subjective affair. In the reproducibility studies, we have calculated plasma...
volume by extrapolation of the best-fit slope and, in a more objective manner, by use of the concentration at 10 minutes alone or of the average concentration shown by the 10-, 20-, and 30-minute samples.

The results obtained on 18 dogs are shown in Table 2. Successive dye injections gave plasma volumes greater than the expected values, the fit being worse with the third injection than with the second. If the trend is ignored, the standard deviation of the 36 attempted checks against the expected values, as determined by the extrapolated slope procedure, was 12.0 per cent or three times greater than that obtained with the tagged-cell injections.

Because the dye concentration at 10 minutes was usually above the best-fit slope, the average plasma volume based on its value was almost the same as that by slope extrapolation. As expected, the volumes calculated from the average concentration over 30 minutes were greater. With the second plasma volume determination, the relations of the three calculated volumes remained essentially unchanged, although the variability was greater with the two objective methods than with the slope extrapolations. The 50- and 70-minute points weighed heavily in the selection of the best single slope, and our attempt to achieve a more objective measurement on the basis of certain samples only was unrewarding. With the third determination, the disappearance slope had obviously steepened so that both the 10-minute O.D. value and the average O.D. value of the first three samples gave a greater plasma volume than did the extrapolated slope.

The steepening of the slope represents a factor which was overlooked at the time, although it should not have been. When, to determine the dilution volume of a second injection of dye, the spectrophotometer is nulled against a "blank" sample which contains dye remaining from the first injection, the ma-
The relative role of the two factors was considered by a series of experiments shown in figure 2. The spectrophotometer was nulled against undyed plasma at the start, and then not reset. For a first determination, samples were taken over 70 minutes as before, and a "blank" sample taken at 80 minutes. A new dye injection was then made, and the total dye O.D. read on samples taken over the second collection period. Four such injections were made on each animal. The uncorrected O.D. values are shown in figure 2B. The O.D. value of the "blank" was then subtracted from each of the postinjection samples to give the curve shown in figure 2A. These subtraction curves show the progressive steepening noted in the previous experiments when the spectrophotometer was nulled against the blank sample. Next, the best-fit slope of the first sampling period was projected over the time span of the next sampling period, so that each postinjection O.D. value had subtracted from it an expected value taken from this extrapolated slope. The difference is shown by the open circles of figure 2A.

Unexpectedly, in about half the cases, these open circles show variation about a constant value rather than a downward slope. In these cases, then, the disappearance rate was seemingly not changed by the injection of more dye, and the two successive disappearance slopes were essentially parallel with a linear scale plotting, but not with a logarithmic scale plotting. In the rest of the cases, the open circles did decline, but it still was not always clear that the logarithmic slopes were equal.

These results could mean that the disappearance slope taken for the first 70 minutes would not continue through the next 70 min-
The individual dye-disappearance curves for the four successive determinations for the six dogs of figure 2 under sodium pentobarbital anesthesia.

utes. It could also mean that these slopes are not necessarily exponential at all.

By subtracting the dye removed in the samples taken and correcting the dye concentration remaining in the dog for any plasma specific gravity change, the expected total optical density can be calculated. These expected values are given in figure 2 as points plotted at the time of injection, with a vertical line representing ± 1 standard error. A priori, we would expect the lower curves to give better quantitative agreement with these expected values than either of the upper curves. This was not necessarily true. No matter how calculated, with successive injections there was still the strong tendency for the dye concentrations to be lower than expected (or the plasma volumes to be greater). Of the three groups of dogs, the unanesthetized animals came nearest to showing parallel dye-disappearance slopes, yet the amount of plasma volume overestimation was greatest. There is no apparent clue, then, in the disappearance slope itself to the lack of plasma volume reproducibility.

The failure of reproducibility cannot be attributed, either, to a progressive elimination of the sort of curve irregularities shown in figure 1, which make the slope determination difficult and might give false values for the first plasma volume determination. The average O.D. values for the six dogs under sodium pentobarbital anesthesia given in figure 2A are shown in figure 3. The steepening of the curves already referred to is quite obvious after the fourth dye injection. Increases in O.D. values late in the sampling period are as common with the later dye injections as with the first one.

Hemodynamic studies on 24 dogs subjected to three bleedings. The transepts represent ± 1 S.E. of the difference from the initial value.

Volume Estimations after Hemorrhage

Control plasma (slope extrapolation) and cell volume determinations were made in 24 splenectomized dogs. The animals were then bled rapidly from the artery until the blood loss, when added to the previous sample loss, averaged 8.1 ml./Kg. After 10 minutes were allowed for circulatory compensations for the hemorrhage to be completed, a second injection of dye and tagged cells was made. The spectrophotometer was nulled against a plas-
Figure 5

Plasma volume (P.V.) and cell volume (C.V.) reductions in 24 dogs subjected to hemorrhage, expressed as per cent of the initial value. Correction of the P.V. for change in plasma specific gravity gives the result plotted as the line (P₀), with ± 1 S.E. depicted by the transept. Correction by the amount of hematocrit change gives the dotted line (Pₙ). The actual hematocrit and specific gravity changes are plotted below.

As shown in figure 4, the first two bleedings caused little change in mean aortic pressure but did produce cardio-acceleration, a rise in total resistance, and a decline in cardiac index and in central venous pressure. The last bleeding produced a fall in aortic pressure with less reduction in cardiac index, so that the total resistance decreased. These responses to hemorrhage are not unlike those obtained with smaller stepwise bleedings. 9

In figure 5 are shown the plasma and cell volume reductions accompanying the sampling periods and the bleedings (lumped together), as expressed in per cent of the initial value as obtained before the hemorrhage. The bleedings were often followed by rapid reductions in both plasma specific gravity and in arterial hematocrit (fig. 5). Except after the first bleeding, the changes in the two were of proportionate degree, indicating an inward shift of ultrafiltrate but little mobilization of whole plasma. The expected plasma volume shown as the solid line in figure 5 was corrected upward by almost the same amount if done on the basis of the specific gravity change (P₀), or of the hematocrit change (Pₙ).

The measured volumes were referred to these expected volumes (P₀), expressing the difference again in terms of per cent of the expected. As shown in figure 6, after the first bleeding the dye measured a volume 4 per cent greater, on the average, than the expected but the size of the standard error precluded any significance. After the second bleeding, the measured volume was but 1.5 per cent greater. After the last bleeding, however, the measured volume was significantly greater than expected and the standard deviation very large.

The tagged cells also overestimated the cell volume by about the same degree for the first
two bleedings but less (and without statistical significance) after the final hemorrhage. The error or red cell measurement was greater than with the reproducibility studies, but still was less than that of the plasma volume determinations.

It has been claimed\textsuperscript{10} that hemorrhage is accompanied by a trapping of red cells in some site not available to injected tagged cells. Such a trapping would produce the opposite to what was seen in these experiments. Or it might be that the similar directional changes of both cell and plasma volumes after hemorrhage would reflect a delayed mixing time. There is evidence that this is true as far as the cells are concerned. In the prehemorrhage determination, the cell volume was $100.8 \pm 0.4\%$ of the plateau level at 10 minutes. After the first bleeding, it was $97.4 \pm 0.6\%$ per cent at 10 minutes and $101.2 \pm 0.6\%$ per cent at 20 minutes. After the last hemorrhage, the average volume was $98.3 \pm 0.7\%$ at 10 minutes, the same at 20 minutes, and finally $100.5 \pm 0.6\%$ per cent at 30 minutes. But since the cell volume used in the bookkeeping was based on the plateau level, whenever it was reached, one could hardly say that the delayed mixing was really determining the tendency for the cells to overestimate the cell volume.

If the trend in cell volume and in plasma volume had a common physiological basis, then the data presented could be interpreted to mean that the error of reproducibility of the dye technique was actually better in these bleeding experiments than was true for the reproducibility studies done on unbled dogs. In both types of experiments, we are quite uncertain why the dye technique has behaved so badly. There is no clear evidence that the failure of closer agreement with the expected is directly related to the anomalous dye-disappearance curves which we find. In final analysis, the dye concentration is not as great 10 minutes after injection as we expect it to be. These results are opposite to those of Cruickshank and Whitfield,\textsuperscript{11} who found that succeeding dye injections gave less steep slopes and better dye recovery.

**Total Circulatory Hematocrit**

Considerable evidence has been presented that in splenectomized dogs the total circulatory hematocrit (TCH) bears the same relationship to an arterial or venous hematocrit through various procedures, such as bleeding and transfusions.\textsuperscript{7,8} The average ratio for a whole series of dogs showed but minor changes in the above experiments, but the value for an individual animal showed no necessary constancy. Hence, in the reproducibility studies, the standard deviation of the change in TCH/H ratio from the initial value (84 cases) was 5.2 per cent, which meant that subsequent determinations had one chance in 20 of being greater than 10.5 per cent of the first. In the hemorrhage experiments, after the first bleeding the standard deviation of the ratio from the initial was 7.8 per cent, after the second bleeding it was 10.6 per cent, and after the third it was 15.8 per cent. Since three variables are concerned in this ratio, it would be difficult to establish just which one was most responsible for the large variation, but certainly the plasma volume is most suspect. At least a variability of this order makes one hesitant about making physiological interpretations based on an alleged constancy of the ratio.

**Summary**

To assess the ability of an injection of Cr$^{51}$-tagged red cells and one of T 1824 to measure a standard part of the total blood volume, two types of experiments have been done. First, three or four successive injections were made, separated by the sampling period of 80 minutes, in both intact and splenectomized dogs under different anesthetic regimes. The tagged cells gave total cell volumes showing no trend away from the expected value, based on the first determination and the cells lost in the sample taken, and the standard deviation was but 3.0 per cent. There was no real difference in this error depending upon the anesthesia. The T 1824 measured plasma volume tended to become progressively greater than the expected value with each injection. This trend could not be corrected out on the basis of either a plasma specific gravity or a hemato-
crit change. The standard deviation was three times larger than for the cell volume. Part of this variability might be related to fluctuations in dye concentration away from the best-fit slope, as were quite common at various times during the sampling period. Yet, plasma volumes based on the concentration shown at any particular time (e.g., 10 minutes) gave slightly worse agreement with the expected. The second experiment involved measurements of cell and plasma volume after three successive bleedings. Here both volumes were greater than the expected, with the standard deviation of the cell volume still less than that of the plasma volume. Curiously enough, T 1824 could follow the changes in plasma volume in these bleeding experiments a little better than it could reproduce the same volume in essentially normovolemic dogs.

References
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