A COMPARATIVE STUDY OF RED CELL VOLUMES IN HUMAN SUBJECTS WITH RADIOACTIVE PHOSPHORUS TAGGED RED CELLS AND T-1824 DYE [1, 2]

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In recent years, total blood volume and red cell volume have become an important measurement in experimental and clinical investigation of the hemodynamics of shock, burns, hemorrhage and fluid replacement.

The vital red method of Keith, Rowntree and Geraghty (1) introduced in 1915 was utilized by Robertson and Bock (2) during World War I to study blood volume in wounded soldiers. In recent years the Gibson and Evans (3) modification of Gregersen's T-1824 dye method has been widely used to study traumatic shock clinically (4, 5).

The measurement of the red cell volume by T-1824 plasma dye is determined indirectly from the centrifuge hematocrit value. It is generally agreed by most investigators that this value overestimates the red cell volume. Chapin and Ross (6) found a positive error of 8.5% in the centrifuge hematocrit. Gregersen and Schiro (7) showed that an average of 4.2% of dye and plasma remained in the erythrocyte mass when blood samples containing T-1824 or Brilliant Vital Red were centrifuged. Furthermore, it has been shown that the hematocrit of a peripheral vessel of the dog does not reflect the hematocrit of all of the vessels of the body (8).

The results of whole blood transfusions in the treatment of shock, burns, chronic infections and other surgical problems have stimulated interest in the total red cell volume as well as in the total blood volume. Consequently, an accurate method for measuring total red cell volume has been the objective of many investigators in the past few years.

The availability of radioactive isotopes opened a new approach to the study of red cell volume. Radioactive phosphorus (P^{32}) and two radioisotopes of iron (Fe^{55} and Fe^{59}) have received the widest application in this type of investigation.

In 1940 Hahn *et al.* (9) first described a method of determining red cell volume by means of radioactive iron tagged red cells. They found that radioactive iron administered orally to dogs became incorporated into their erythrocytes and that the "tag" remained as long as the cell existed intact. Tagged red cells obtained by this technique were used to measure the red cell volume of another animal by the isotope dilution method. Shortly thereafter, the same workers did a comparative study of the red cell and plasma volumes by the radioiron and dye method in dogs (10). Elaborating on the original technique of Hahn, Gibson *et al.* (11, 12) and Meneely *et al.* (13) used radioiron tagged red cells to determine the red cell volume in humans.

In 1940 Hahn and Hevesy (14) obtained P^{32} tagged red cells by injecting this isotope in a rabbit and allowing the animal to incorporate this material into its red cells. These tagged red cells were then used to measure the red cell volume of a second rabbit by the isotope dilution technique. Hevesy and Zerahn (15), a short time later, showed that red cells could be tagged with P^{32} *in vitro* after two hours incubation at 37° C. Hevesy (16) demonstrated that inorganic P^{32} upon entering the red cells is rapidly incorporated into organic phosphate compounds and that the loss of these compounds from the red cells is negligible at the end of one hour. Nylin (17–20) has utilized P^{32} tagged red cells to study blood volume,

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TABLE I

Composite data of 40 plasma and red cell volumes done simultaneously

Exp. no.	Date	Age	Wt. in kgm.	Clinical diagnosis	Plasma volume by T-1824	Av. venous ht.	Blood volume by T-1824	R.C.V. T-1824	R.C.V. P^{32}	Blood vol. P.V. R.C.V. P^{32}	Ratio R.C.V. by P^{32} / R.C.V. by T-1824	Body ht.	Ratio Body ht. / Av. V. ht.
10	5/26/48	38	72.6	Post-op. appendectomy	3085	48.2	5960	2875	2004	5089	0.697	39.3	0.815
11	5/28/48	58	57.3	Post-op. herniorraphy	3330	40.9	5577	2247	1720	5050	0.765	34.0	0.831
12	5/31/48	18	55.9	Post-op. appendectomy	2975	40.3	5440	2465	2089	5064	0.847	41.2	1.024
13	6/2/48	37	68.2	Bilateral ing. hernia	3290	47.9	6319	3029	2965	6255	0.978	47.4	0.989
14	6/4/48	67	54.6	Infected wrist	2862	43.0	5002	2140	2008	4870	0.938	41.2	0.958
15	6/7/48	30	81.5	Post-op. appendectomy	4940	40.1	8250	3310	2378	7318	0.718	32.4	0.807
16	6/9/48	56	70.5	Post-op. appendectomy	2658	45.3	4859	2201	1563	4221	0.710	37.0	0.816
17	6/10/48	67	69.1	Diabetic ulcer of toe	2615	40.2	4379	1764	1450	4065	0.821	35.6	0.885
18	6/14/48	34	72.7	Stab wound-pneumothorax	2597	49.1	5100	2503	1755	4352	0.701	40.3	0.820
19	6/15/48	36	54.6	Post-op. herniorraphy	3018	42.8	5270	2252	2115	5133	0.939	41.2	0.963
21	6/18/48	68	63.6	Fecal fistula	1675	61.5	4350	2675	1540	3215	0.576	47.9	0.779
22	6/23/48	34	53.6	Post-op. herniorraphy	2800	40.1	4670	1910	1885	4685	0.987	40.2	1.002
23	6/24/48	17	57.7	Post-op. herniorraphy	2700	49.4	5330	2630	2050	4750	0.779	43.2	0.875
24	6/28/48	47	45.4	Postpneumonic empyema	2390	26.6	3300	910	664	3054	0.730	21.7	0.817
25	7/1/48	18	63.0	Lacerated rectum	2795	45.7	5143	2348	1789	4584	0.762	39.0	0.854
26	7/2/48	28	69.7	Post-op. appendectomy	2772	49.6	5502	2730	1679	4451	0.615	37.7	0.760
27	7/6/48	47	42.0	Postpneumonic empyema	2418	28.6	3385	967	781	3199	0.807	24.4	0.853
29	7/13/48	20	62.7	Post-op. appendectomy	2361	47.5	4486	2125	1690	4051	0.795	41.7	0.878
30	7/14/48	20	63.2	Post-op. appendectomy	3245	45.6	5960	2715	1988	5233	0.732	38.0	0.834
31	7/16/48	26	81.8	Osteo of hip	2923	49.2	5750	2827	2339	5262	0.827	44.5	0.905
33	7/21/48	64	46.8	Post-op. exploratory lap.	2565	51.0	5238	2673	1925	4490	0.720	42.9	0.841
35	7/26/48	16	61.1	Post-op. appendectomy	2401	50.1	4802	2401	2291	4692	0.954	48.8	0.974
36	7/27/48	30	67.1	Gun shot wound of chest	2634	42.3	4562	1928	1771	4405	0.919	40.2	0.951
37	7/28/48	37	67.3	Ischio-rectal abscess	2776	49.4	5480	2704	2117	4893	0.783	43.3	0.877
38	7/29/48	60	65.0	Buerger's disease	2345	46.8	4409	2064	1698	4043	0.823	42.0	0.897
39	7/30/48	37	58.0	Diabetes; inf. of foot	3228	37.1	5129	1901	1695	4923	0.891	34.4	0.927
41	8/3/48	18	58.0	Post-op. appendectomy	2722	49.6	5405	2683	2330	5052	0.868	46.1	0.929
42	8/4/48	64	55.8	Squamous ca. of mouth	3170	44.2	5681	2511	2113	5283	0.841	40.0	0.905
43	8/5/48	42	91.5	Post-op. hysterectomy	2492	44.4	4483	1991	1698	4190	0.853	40.5	0.911
44	8/6/48	25	68.8	Post-op. herniorraphy	2690	48.8	5256	2566	2392	5082	0.932	47.1	0.965
45	8/10/48	78	49.3	Ca. of stomach	2619	40.9	4431	1812	1433	4052	0.791	35.4	0.866
46	8/11/48	29	75.9	Cellulitis of foot	2717	47.2	5150	2433	1775	4492	0.730	39.5	0.836
47	8/12/48	42	55.8	Inguinal hernia	2146	46.6	4022	1871	1695	3841	0.906	44.1	0.946
48	8/12/48	48	51.0	Osteo of mandible	2685	34.9	4126	1441	1004	3689	0.697	27.2	0.779
49	8/13/48	40	72.7	Fractured hip	3228	43.5	5710	2482	1984	5212	0.799	38.1	0.876
50	8/17/48	65	70.5	Ca. of colon	3569	41.6	6103	2534	2034	5603	0.803	36.3	0.874
51	8/18/48	25	63.6	Post-op. appendectomy	2934	48.5	5698	2764	2286	5220	0.827	43.8	0.903
52	8/18/48	24	70.0	Infected hip	3123	47.1	5906	2783	1829	4952	0.657	36.9	0.783
53	8/19/48	38	63.5	Fractured ribs	2394	51.0	4884	2490	1783	4177	0.716	42.7	0.837
54	8/19/48	20	55.7	Renal calculus	2571	45.6	4723	2152	1624	4195	0.755	38.7	0.849
					2811	44.8	5131	2320	1848	4659	0.800	39.4	0.880

organ volume and a number of other clinical problems.

The present study is a modification of Hevesy's technique for determining total red cell volume with P^{32} labelled red cells. The objectives have been (*1*) to develop a simple, rapid, accurate and easily reproducible method for measuring total blood volume by directly determining both plasma and red cell volume, and (*2*) to compare the results of the P^{32} and the T-1824 methods.

MATERIAL

Forty determinations were carried out on 38 subjects taken at random from the surgical services of the Medical College of Virginia Hospitals.[4] Thirteen were white males between 18 and 68 years of age; 23 were colored males between 17 and 78 years of age; and two were colored females 47 and 42 years of age. All but three subjects[5] were ambulatory. Their clinical diagnoses are shown in Table I.

METHOD

Approximately 15 cc. of blood are drawn from the antecubital vein of the subject and placed into a rubber stoppered vial containing a sterile solution of approximately 0.1 millicurie of radioactive phosphate and 0.1 cc. of .068 W/V solution of heparin[6] in saline. The P^{32} was obtained from Oak Ridge National Laboratory as phosphoric acid pH 3.0. It was originally buffered with 1 part 2.5% W/V sodium citrate solution and 9 parts M/15 Sorensen phosphate buffer solution but this was discontinued when it was found difficult to keep it pyrogen free. The vial is placed in an incubator at 37° C. and gently rotated every 10–15 minutes for two hours. Levi (21) has shown the distribution coefficient of labelled phosphate ions between corpuscles and plasma of equal weight to be approximately 1.0 at the end of this time.

After two hours incubation, the blood is centrifuged for three minutes at 3000 RPM and the supernatant plasma

[4] We are greatly indebted to Dr. I. A. Bigger, Professor of Surgery, and to the Surgical House Staff for their help in making available clinical material.

[5] Experiments No. 21, 31, and 52.

[6] 0.068% weight to volume heparin. One hundred mgm. of dried heparin in 14.5 cc. of saline; 0.05 cc. prevents coagulation of 5.0 cc. of whole blood.

TABLE II

Protocol of a typical experiment

Experiment no. 50. August 17, 1948. Subject: S. D. Age 65. Wt. 70.5 kgm. Diagnosis: Carcinoma of colon

Time	Procedure	Sample no.	Sample data				Determinations	
			Whole blood	cc. plasma	cc. red cells	Ht. (%)	Optical density of plasma	Radio-activity (C/M of cells)
10:25 AM	15 cc. blood withdrawn							
12:15 PM	Incubation completed							
12:50 PM	Three saline washings completed							
12:55 PM	Control sample for dye blank	C	4.7	2.7	2.0	42.6		
12:56 PM	5.0 cc. 0.5% T-1824 injected							
12:58 PM	10.0 cc. of tagged cell suspension injected. Amount not injected placed in Magath tube	CC	5.25*	3.1**	2.2	42.3		3808
1:11 PM	Sample	1	3.98	2.29	1.69	42.5	0.382	387
1:19 PM	Sample	2	4.9	2.81	2.05	41.8	0.378	394
1:28 PM	Sample	3	5.1	3.0	2.1	41.2	0.373	385
1:43 PM	Sample	4	5.0	2.98	2.02	40.2	0.363	396
1:58 PM	Sample	5	5.1	3.0	2.1	41.0	0.355	391

For plasma dye volume and radio-activity analyses

Calculations by T-1824:

$$\text{Plasma volume} = \frac{500 \times 0.590 \times 4.958}{0.389} = 3569 \text{ cc.}$$

$$\text{Blood volume} = 3569 \times \frac{100}{100 - 41.6} = 6103 \text{ cc.}$$

$$\text{Cell volume} = 6103 - 3569 = 2534 \text{ cc.}$$

Calculations by P^{32}:

$$\text{RCV in cc.} = \frac{3808 \times 50 \times 4.23}{395} = 2034 \text{ cc.}$$

Difference in RCV by the two methods.

By T-1824 — 2534 cc.

By P^{32} — 2034 cc.

500 cc.

$$\frac{500}{2534} = .1973 \text{ or } 19.7\%.$$

* Red cells suspended in saline. ** CC of saline. 1:100 dilution.

removed with a sterile syringe and needle. The plasma is discarded and an equal volume of sterile iced physiological saline (0.9 W/V sodium chloride) added to the cells. The erythrocytes are resuspended in the saline, gently rotated and centrifuged again. Three washings, maintaining sterile technique, are done in this manner and the cells finally suspended in a volume of saline approximately equal to the volume of plasma discarded. After anesthetizing the skin with 1% procaine HCl solution, an antecubital vein is punctured with a 19 G. needle attached to a three-way stopcock. Five cc. of blood are withdrawn to serve as a blank for the plasma volume determination; then, sufficient physiological saline is allowed to run through the needle to be certain that it is securely in the vein. Five cc. of T-1824 dye, followed by 10.0 cc. of the tagged cell-saline suspension[7] are then injected from calibrated syringes. In each case, the syringe is washed three times with saline. At approximately 12, 23, 30, 45 and 60 minutes after injection, approximately 5.0 cc. of blood are drawn from an antecubital vein of the opposite arm without stasis and placed into a 6.0 cc. Magath centrifuge tube containing 0.05 cc. of 0.068% W/V heparin in saline which has been dried by heating in an oven. The remainder of the tagged cell-saline suspension not injected is placed in a similar tube and denoted as the control cells. All tubes are centrifuged at 3000 RPM for 45 minutes in a size 2 International centrifuge equipped with a No. 240 head, the distance from the axis of the centrifuge to the bottom of the cup being 20.0 cm.

After determining the hematocrit (not including the buffy coat) the supernatant plasma is removed and the concentration of dye measured on a Coleman Junior Spectrophotometer set at 620 mμ. To each Magath tube is added an amount of 0.1% W/V sodium carbonate solution exactly equal in volume in cubic centimeters to the volume of packed cells; thus giving a 1:1 dilution of red cells. These are shaken vigorously until all cells are hemolyzed.

The control cells are further diluted as follows: 0.1 cc.

[7] It is estimated that the total body irradiation from a single red cell volume determination is approximately 3.0 roentgen equivalent physicals (REP).

of the 1:1 dilution is made up to 5.0 cc. with distilled water, giving a 1:100 dilution. Then the radioactivity of the control cells and the other cells is determined in the following manner: Exactly 0.1 cc. of hemolyzed cell solution from each tube is carefully pipetted onto a copper planchet 1" in diameter and evenly spread by adding one drop of 10% aerosol OT [8] solution. These are dried at room temperature for approximately 30 minutes. The same pipette is used throughout but is carefully washed and dried between samples.

A Geiger tube and scaling circuit are used to measure the radioactivity of each sample. The Geiger tubes are of the self-quenching variety, have overall dimension of 3¾" × 1½" diameter and have mica windows ranging from 2.5–3.5 mgm./cm². These are shielded by 2" of lead and their background counts range from 25–35 counts per minute. They are standardized at regular intervals to determine changes in plateau and counting efficiency. The sample to window distance is 2 mm. and the overall geometric reproducibility is claimed by the manufacturer to be on the order of 0.1%. 4096 counts are totaled for each sample and the result expressed in terms of counts per minute.

CALCULATION OF RED CELL VOLUME

From the dilutions of the red cells and the radioactivity analyses the red cell volume is calculated as follows:

(*1*) RCV in cc.

$$= \frac{\text{C/M/cc.}^{9}\text{ injected} \times \text{cc. of red cells injected}}{\text{C/M/cc. recovered from subject}}$$

(*2*) C/M/cc. injected = C/M/0.1 cc. × 10 × 100

(*3*) C/M/cc. recovered = C/M/0.1 cc. × 10 × 2

(*4*) cc. of cell injected = cc. of saline suspension × hematocrit

Substituting in (*1*) therefore

$$\text{RCV in cc.} = \frac{\text{C/M/0.1 cc.} \times 10 \times 100 \times \text{cc. of red cells injected}}{\text{C/M/0.1 cc.} \times 10 \times 2}$$

$$\text{RCV in cc.} = \frac{\text{C/M of control sample} \times 50.0 \times \text{cc. of cells injected}}{\text{C/M test cells from subject}}$$

Table II is a protocol of a typical experiment.

To determine the technical error in pipetting and counting, 10 samples from a tube of hemolyzed cells were prepared and each sample counted 10 times. The coefficient of variation[10] ranged from a minimum of 0.36% to a maximum of 3.2% with an average for the 100 determinations of 2.2%.

RESULTS

The tabulated results of the 40 determinations are shown in Table I. The T-1824 dye method gave a mean red cell volume of 2320 ± 79 cc.; with the P^{32} method the mean red cell volume was 1850 ± 66 cc. These two mean values are significantly different ($d = 470 \pm 102$ cc., $t = 4.6$, $P < .01$). In every case, the red cell volume as determined by the P^{32} method was less than the comparable value obtained with T-1824 dye. The table shows the ratio of the RCV by P^{32} to the RCV by T-1824 for each case. The mean value obtained from these 40 ratios was 0.800 ± 0.153. None of the individual ratios fell outside the range of the mean ratio plus or minus two and a half times its standard error.

We assume the expression $\frac{\text{RCV by } P^{32}}{\text{PV} + \text{RCV by } P^{32}}$ to be the relationship of red cells to plasma in the entire body (Body ht.) The average body hematocrit of the entire series was 39.4 while the average venous hematocrit was 44.8. The average venous hematocrit is found by averaging the hematocrit of the six blood samples taken without a tourniquet in the course of the experiment. No correction factor is applied for the occluded plasma.

DISCUSSION

Determination of the red cell volume by means of tagged erythrocytes is based upon the following assumptions: (*1*) the tag remains essentially intact with the red cell for the time of the experiment, (*2*) the physical principle of dilution of tagged particulate matter is accurate for measuring changes in volume of similar particulate matter, and (*3*) all of the tagged cells become completely mixed with the untagged cells of the subject.

That the radioactive tag remains essentially intact in the red cell for longer than one hour has been shown conclusively by Nylin (22) and confirmed by us. Figure 1 illustrates a typical experiment and shows the activity of the red cells as a function of time. Furthermore, radioactive analyses of the supernatant plasma of samples taken up to one hour after injection do not show sufficient activity to indicate any significant loss of activity from the tagged cells to the plasma. Aliquots of the plasma counted 50–60 C/M while our background was 25–35 C/M. The ratio of the counting rate of the cells to background was 10:1 to 15:1.

The validity of the second assumption was tested by an *in vitro* experiment designed to measure the

[8] Dioctyl sodium sulfosuccinate.

[9] C/M/cc. = counts per minute per cubic centimeter of red cells.

[10] Coefficient of variation $= \frac{S \times 100}{M}$. Coefficient of variation denotes the standard deviation as a percentage of the mean. N has already been taken into consideration in determining S (standard deviation).

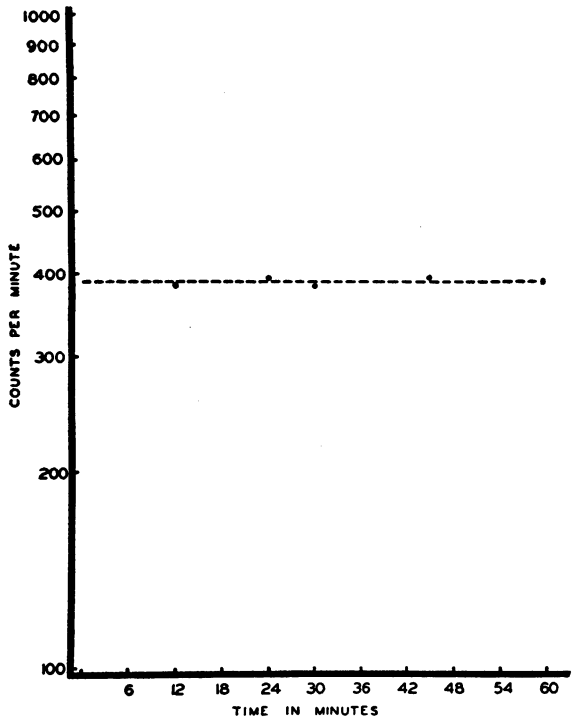

FIG. 1. COUNTS PER MINUTE CONTAINED IN RED CELLS AS A FUNCTION OF TIME

red cell volume of a definite quantity of blood. Tagged cells were introduced into a measured quantity of blood and thoroughly mixed by gentle rotation. At 12 minute intervals up to one hour, samples were poured off into Magath centrifuge tubes. The red cell volume by the isotope dilution technique agreed within 4% of the volume determined from the hematocrit corrected for the occluded plasma by Gregersen's factor of 0.96 (23) (Table III).

The third assumption is difficult to prove, but the constant activity of the red cells up to one hour after injection strongly suggests that it is valid.

It has been stated the red cell volume as determined by the isotope dilution technique is independent of the arterial or venous hematocrit (11). Although this statement is factually correct, the hematocrit does enter into the calculation of the red cell volume when the volume of tagged red cells injected is calculated. The volume of red cells injected is thus overestimated since no account is taken of the occluded saline when the hematocrit of this suspension is determined.

Correction factors have been proposed for occluded plasma (Gregersen's 0.96), but a correction factor for occluded saline has not been determined. Experiments are planned to determine this variable.

A similar error is introduced when the volume of red cells is hemolyzed with an exact volume of 0.1% sodium carbonate solution. However, since both control and test cells are treated alike this error cancels itself.

We assume that the packing factor of the tagged cell-saline suspension remains constant in each experiment. It is obvious that application of a correction factor here would make the red cell volume by the isotope dilution technique even lower than presented values.

Similar studies of simultaneously determining red cell volume with tagged cells and plasma volume with T-1824 have been conducted by Gibson *et al.* (11) and Meneely and his group (13) using radioactive iron. In a series of 40 normal males, Gibson found an average ratio of red cell volume by radioiron to red cell volume by T-1824 of 0.845. The standard error of the mean of his series was ± 0.11. The average ratio of the body hematocrit to average venous hematocrit for his series was 0.906. Meneely's study included 28 male patients and revealed an average ratio of red cell volume by radioiron to red cell volume by T-1824 of 0.809, with the standard error of the mean being ± 0.122. The average ratio of the body hematocrit to average venous hematocrit for his series was 0.887.

Mutual confirmation of the results of the radioiron and radiophosphorus techniques is suggested by the lack of a significant difference in their mean ratios.

Further evidence confirming the results presented here is found in the recent report of Reeve and Veall (24). These workers using a technique

TABLE III

Values of in vitro *experiment to measure known volume of red cells*

Determination no.	Volume of whole blood	Hematocrit in %*	Calculated volume of red cells (ht. ×0.96)	Volume of red cells by P^{32}	Per cent difference
1	1000 cc.	38.6	370	389	+4.1
2	1000 cc.	41.1	395	384	−2.5

* Average of five determinations.

similar to ours found the mean ratio of

$$\frac{\text{red cell volume by } P^{32}}{\text{red cell volume by T-1824}}$$

to be 0.87. Hematocrit values in their study were corrected by a factor of 0.95.

Correction of the hematocrit for trapped plasma was purposely omitted in the basic calculations (Table I), to facilitate a more fundamental comparison of methods. If Gregersen's correction factor (0.96) is applied to the hematocrits in Table I, the mean ratio of

$$\frac{\text{red cell volume by } P^{32}}{\text{red cell volume by T-1824}}$$

is 0.87.

Mayerson, Lyons *et al.* (25) report a mean ratio of $\frac{\text{red cell volume by } P^{32}}{\text{red cell volume by T-1824}}$ of 0.99. This unusually high ratio is probably due to (*1*) dissimilar basic techniques and (*2*) the use of a hematocrit correction factor of 0.915 (26).

The P^{32} method as described here is easily done and has many advantages over radioiron. It is necessary to produce a donor of radioactive cells when iron is used, but when P^{32} is used, the subject's own red cells may be conveniently tagged. Furthermore, the concern of blood type and Rh compatibility is eliminated. Separated isotopes of radioiron are difficult to produce in either the cyclotron or the chain reacting pile. Some long-life iron is usually present in the most carefully separated isotopes of iron. Concern has been expressed over the use of long-life isotopes such as Fe^{55} (half-life, four years) in human subjects. The use of P^{32} (half-life, 14.8 days) eliminates this concern. The preparation of blood samples containing iron for radioactive analysis is a complicated procedure. The red cells are wet ashed and the iron precipitated; the iron must then be redissolved and electrolytically deposited on copper discs in a special apparatus before the activity can be measured. Radioactivity analysis of P^{32} tagged red cells can be simply carried out on hemolyzed red cells by the method described.

CONCLUSIONS

1. A comparative study of the red cell and plasma volumes of 38 surgical patients as measured by P^{32} tagged red cells and T-1824 plasma dye is presented.

2. On the average, the red cell volume as measured with P^{32} tagged red cells is 20.0% less than the value obtained with T-1824 plasma dye.

3. This discrepancy is probably due to the difference in the hematocrit of blood in peripheral veins and blood in other vessels of the body and to the intrinsic error of the centrifuge hematocrit.

4. The body hematocrit on the average, is 12.0% less than the average venous hematocrit.

5. The hematocrit value enters into the calculation of the red cell volume by the isotope dilution techniques and probably results in slight overestimation of the red cell volume.

6. Statistically, results of the present study do not differ significantly from similar studies using radioiron tagged red cells.

7. The advantages of the P^{32} method over the radioiron method are outlined.

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