Distribution of Blood (Fe59) and Plasma (I131) Volumes of Rats Determined by Liquid Nitrogen Freezing

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Distribution of Blood (Fe^{59}) and Plasma (I^{131}) Volumes of Rats Determined by Liquid Nitrogen Freezing

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A standard method is described which gives reproducible blood volume values for rats and their various organs and tissues.

THERE has been no complete organ blood volume study for any animal, nor any definitive total body blood volume determination for the rat. Thus, an extensive study was undertaken to determine the normal red cell and plasma volumes of the whole rat and for each of its major organs or tissues. This study is a prerequisite to investigations concerned with the effect of various experimental procedures upon blood distribution in the rat.

Kaliss and Pressman[1] reported the plasma and blood volumes of a limited number of mouse organs, as determined with radioactive iodoproteins, but the given values varied considerably from animal to animal. These variations were attributed in part to variable blood loss during the process of dissection. Recently Friedman[2] determined the plasma volumes for seven mouse organs utilizing I^{131} albumin as a plasma label. Hemorrhage and anaesthesia were avoided by freezing the mice in liquid nitrogen prior to dissection.

Although several blood volume studies have been made for the rat, the results have been quite variable, ranging from 4.3 ml./100 Gm., Griffith and Campbell[3]; to 7.98 ml./100 Gm., Beckwith and Chanutin[4]. Using the Fe^{59} tagged cell method, Berlin and associates[5] reported the blood volume of the rat to be from 3.63 to 5.81 ml./100 Gm. and Sharpe, Culbreth and Klein[6] reported a mean value of 4.95 ml./100 Gm. The utilization of Fe^{59} cells probably provides the most reliable method of those available for determining red cell volume. However, when this method is used alone it does not give an accurate picture of the total blood volume. It is generally believed that the cell dilution method results in an underestimation of the true blood volume whereas plasma dilution methods are thought to lead to an overestimation (Sjöstrand[7]). This discrepancy has been attributed to hematocrit differences in the small and large vessels (Chaplin, Morrison, and Vetter[8]).

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METHODS

The animals used included both male and female rats of the Sprague-Dawley strain, weighing from 180 to 250 Gm. The females were in the diestrous stage of the estrous cycle as determined by the method of Blandau, Boling and Young.[9] Experiments were standardized with respect to time of day, since differences in time influenced the blood values of certain organs, (liver, intestine, kidney and lung). Males were used between 3 and 5 P.M. and females between 7 and 9 P.M.

The isotope dilution method was used for both the red cell and plasma volume measurements. In one series of rats red cell volumes were determined by using erythrocytes tagged with Fe^{59}. In a second series the plasma volumes were measured by using I^{131} tagged human serum albumin. Human albumin was selected as a satisfactory plasma label after comparing the results from using this compound with those obtained using rat plasma protein labeled in vitro with I^{131} or in vivo with C^{14} glycine and with S^{35} methionine. The tagged human albumin presented advantages in availability, in ease of assay and in higher specific activity. Careful observation, including temperature recordings, gave no evidence of the rats having an allergic reaction to the human protein.

Erythrocytes were labeled in vivo by administering to each donor rat 120 μc. of Fe^{59} Cl_3, (40

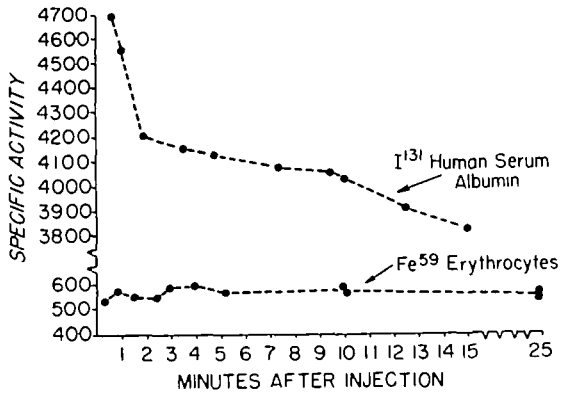

FIG. 1. Specific activity of blood after intravenous injection of Fe^{59} erythrocytes and I^{131} albumin.

μg. of Fe) in two intraperitoneal injections. Donor blood was obtained by cardiac puncture after a minimum period of 2 weeks following the iron administration. At this time there was no measurable activity in the plasma. The injection dose for each rat of the red cell dilution series was 0.2 ml. of whole blood having an approximate activity of 5×10^5 cts./min.

The injection dose for each rat of the plasma dilution series was .2 ml. containing approximately 2 μc. of I^{131} and 10 μg. of albumin.

Injections of the labeled cells or plasma were made into the great saphenous vein under light ether anaesthesia. The injection procedure consumed approximately 1 minute, using a tuberculin syringe and a no. 25 gage needle. The syringe was enclosed in a holder having a control device to standardize the injection volume. Duplicate aliquots were diluted in volumetric flasks and 1 ml. portions were used to measure the activity of the injection dose. It was found that by using the syringe as described, the desired volume could be delivered with less than 1 per cent error. Three minutes were allowed for mixing of plasma and 15 minutes for mixing of cells within the blood stream. These times were selected after determining the dilution curves subsequent to intravenous injection of tagged albumin and erythrocytes (fig. 1).

Freezing Technique. At the completion of the mixing period the animals were submerged horizontally in liquid nitrogen, disturbing them as little as possible in the process. This freezing procedure was chosen so as to produce a rapid stasis of the blood and to make possible dissection of the organs for assay without blood loss. Initially, isopentane cooled to minus 140 C was tried as the freezing medium, but immersion in liquid nitrogen was found to freeze the animals more rapidly.

The time required for freezing the body fluids at different depths was determined in a separate series of rats by recording the body temperature changes with copper-constantan thermocouples implanted in the tissues. Thermocouple voltages were recorded with a Leeds-Northrup Speedomax type G recorder. Electrocardiograms were obtained during the freezing process from some of these same rats. Cardiac potentials were recorded from copper plate electrodes implanted subcutaneously on either side of the thorax.

Control studies were made with 8 rats weighing 200 to 225 Gm., in an attempt to determine whether or not there was a significant redistribution of blood as a result of freezing. These animals were anesthetized with Nembutal. Four received I^{131} albumin intravenously and the other 4 were given red cells tagged with Fe^{59}. Each rat was then immobilized in a close fitting wire cylinder and placed in a carriage above the scintillation counter. The surface activity was then determined from 4 segments along the longitudinal axis of the I^{131} recipients and from 6 segments of the Fe^{59} series. As the radiations from each segment were counted the cylinder enclosing the animal was rotated slowly by means of an attached motor. The shielding and detector were so arranged that each segment assayed was approximately 1.5 cm. wide. After these initial counts, each animal, still enclosed within the cylinder, was frozen in liquid nitrogen. The counts were then repeated using the same arrangements as were used initially. It was assumed that intravascular blood shifts due to freezing would be reflected as a difference in the counting rate of the segments before and after freezing. The recipients of I^{131} were frozen within 10 minutes after the injection, in order to minimize the escape of albumin into the tissue spaces. For this reason only 4 segments of these animals could be counted. After counting, the frozen animals were sectioned transversely through the center of each segment in order to determine what organs were over the center of the detector. Since the animals were of uniform weight and were mounted uniformly for counting, the corresponding segments of all rats tended to be uniformly related to the underlying organs.

Sample Preparation. After freezing, the rats used for the blood studies were wrapped in aluminum foil and placed in individual plastic bags to prevent dehydration. They were stored at −5 C until dissected. Dissection was carried out as rapidly as possible using instruments cooled with solid CO_2. All organs were removed in the frozen state without blood loss. A standard dissection technique was followed for all rats. The skin samples were obtained from the outer part of the right thigh and were without hair, but included the loose subcutaneous tissue. The entire muscle mass of the right thigh and leg, including the vessels and nerves, served as the muscle sample. The bone sample included the femur and tibia of this same leg, freed of soft tissue. The entire liver was used after removal of the inferior vena cava. The segment of the small intestine chosen was from the

middle third. Its contents were removed. The extraneous blood vessels supplying each organ were severed as closely as possible to the organ and all excess adipose tissue was removed. The cardiac muscle included both ventricles freed from the atria, but excluded the enclosed frozen blood. Frozen blood samples in triplicate were taken, two from the heart and the other from the inferior vena cava. Most of the frozen organs or tissues were placed immediately after removal into weighed stoppered shell vials. However, the liver, lung and muscle were handled in a somewhat different manner. These were placed in standard weighing bottles. The organs were then minced with scissors and transferred in .5 to 1 Gm. aliquots (3 each) to shell vials. Without further treatment, radioactivity measurements were made in a well-type scintillation counter equipped with a crystal for gamma ray detection.

The 1 ml. aliquots of the injection dose were assayed at this time. The blood samples were counted at repeated intervals in order to correct for the decay factor, since these were the reference standards used for determining the blood content of tissues.

Calculations. Since it was not possible to achieve a satisfactory separation of the cells and plasma of blood after freezing, it was necessary to calculate the specific activity of cells or plasma from the activity of whole blood. For this purpose the hematocrit, the correction for trapped plasma in a packed column of red cells, and the density of whole blood were determined, using the heart blood from both male and female rats of the same weight range as that of the blood volume series. Hematocrits were determined by the method of Wintrobe[10] for 10 males and 10 females. The trapped plasma within the red cell pack was 4 per cent, as determined by measuring the amount of residual I^{131} plasma in the red cell column of Wintrobe tubes. The corrected average value for the hematocrit of 18 male rats was 41.5, $\sigma = 0.22$ and for 10 females 40.9, $\sigma = 0.3$. The value used for the density of rat blood was 1.056 which was the average of three separate measurements made on the pooled blood of 3 rats using a 10 ml. Guy Lussac bottle. This value agrees with that of Sherrington and Copeman.[11]

The formulas used for the blood volume determinations were:

General formula

$$\frac{\text{activity/Gm. tissue}}{\text{activity/mg. blood}} = \text{mg. blood/Gm. tissue}$$

Fe^{59} red cell dilution

$$(\text{mg. blood/Gm. tissue})\ \frac{(\text{Hct.})}{(1.056)} = \mu\text{l red cell/Gm. tissue } (RCV)$$

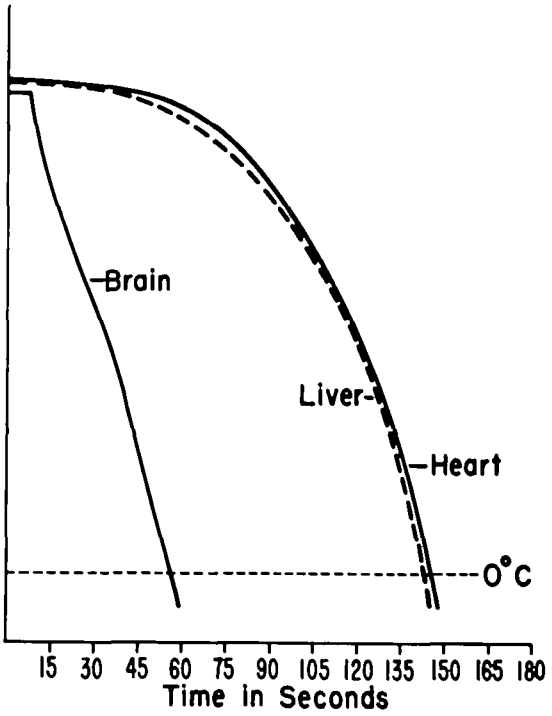

FIG. 2. Records of drop from body temperature to 0 C. within organs after immersing rats in liquid nitrogen.

I^{131} albumin dilution

$$(\text{mg. blood/Gm. tissue})\ \frac{(1 - \text{Hct.})}{(1.056)} = \mu\text{l plasma/Gm. tissue } (PV)$$

Combining I^{131} and Fe^{59} values

$$RCV + PV = \text{true blood volume } (TBV)$$

$$\frac{RCV}{TBV} = \text{tissue hematocrit}$$

RESULTS

The mean freezing time for the body fluids within the deepest part of the abdominal cavity, after immersing the rats in liquid N_2, was 2 minutes and 28 seconds. This was determined from 6 rats in the weight range of 220 to 245 Gm. The recording thermocouple was secured to the common bile duct proximal to the liver. Typical records of the temperature drop within animals so frozen are shown in figure 2. Thermocouples implanted elsewhere showed that the central part of the cerebral hemispheres was frozen within 1 minute and the subcutaneous

TABLE 1.—*Blood Values of Male Rats, and of their Organs and Tissues*

	Fe^{59} red cell measurements				I^{131} albumin measurements				Combined data		
	No. rats	Mean Blood mg./Gm.	σ, % of mean	RCV μl./Gm.	No. rats	Mean Blood mg./Gm.	σ, % of mean	PV μl./Gm.	Mean PV + RCV μl./Gm.	σ, % of mean	Hct
Total rat	16	52.0	1.3	20.4	13	63.5	2.9	35.2	55.6	2.3	36.7
Adrenal	12	119	5.9	46.8	10	344	1.7	191	238	2.5	19.7
Bone	11	14.2	2.5	5.58	14	63.1	1.1	34.9	40.5	1.7	13.8
Brain, fore & mid	12	23.6	2.6	9.27	14	40.4	3.0	22.4	31.7	2.8	29.2
Cereb. hemisph.	10	22.4	4.4	8.80	14	38.2	3.4	21.2	30.0	3.7	29.3
Thal. & midbrain	10	28.2	4.0	11.1	14	37.3	4.9	20.7	31.8	4.2	34.9
Hind brain	10	24.0	3.6	9.43	12	41.4	4.2	22.9	32.3	3.9	29.2
Cerebellum	10	31.4	2.8	12.3	13	46.1	3.9	25.5	37.8	3.5	32.5
Pons & med.	10	16.7	6.8	6.56	13	28.9	3.7	16.0	22.6	4.6	29.0
Cardiac muscle	10	181	3.3	71.1	14	344	1.7	191	262	2.2	27.1
Hypophysis	6	84.8	8.0	33.3	9	107	4.7	59.3	92.6	6.1	36.0
Kidney	17	73.0	7.1	28.7	14	180	5.7	99.7	128	6.3	22.4
Liver	15	209	3.1	82.1	14	339	2.3	188	270	1.6	30.5
Lung	15	456	3.3	179	13	613	1.7	340	519	2.3	34.5
Seminal vesicle	11	22.0	8.5	8.65	14	25.8	7.6	14.3	23.0	8.0	37.6
Skeletal muscle	17	21.3	3.1	8.37	14	31.4	3.5	17.4	25.8	3.3	32.4
Skin	12	18.5	4.5	7.27	13	21.4	3.2	11.9	19.2	3.7	37.9
Small intestine	13	22.7	6.0	8.92	13	45.1	6.4	25.0	33.9	6.4	26.3
Spinal cord	12	17.8	6.8	7.00	19	32.6	7.0	18.1	25.1	6.9	27.9
Spleen	13	219	3.7	86.1	14	151	3.6	83.6	170	3.7	50.7
Sub. max. gland	10	64.5	9.4	25.3	13	101	8.1	56.0	81.3	8.3	31.1
Testis	13	10.6	3.4	4.16	14	20.8	3.1	11.5	15.7	3.2	26.5
Thyroid	10	243	15.5	95.5	9	155	4.6	85.9	181	9.2	52.7

TABLE 2.—*Blood Values of Female Rats and of their Organs and Tissues*

	Fe^{59} red cell measurements				I^{131} albumin measurements				Combined data		
	No. rats	Mean Blood mg./Gm.	σ, % of mean	RCV μl/Gm.	No. rats	Mean Blood mg./Gm	σ, % of mean	PV μl./Gm.	Mean PV + RCV μl./Gm.	σ, % of mean	Hct
Total rat	38	49.3	1.1	19.1	25	60.7	1.7	34.0	53.1	1.5	36.0
Adrenal	39	144	2.8	55.8	32	379	2.9	212	268	2.9	20.8
Bone	32	12.6	3.3	4.88	24	49.9	3.0	27.9	32.8	3.1	14.9
Brain, fore & mid	37	24.7	1.4	9.56	31	36.8	1.4	20.4	30.1	1.4	31.8
Cereb. hemisph.	30	23.1	1.7	8.94	11	35.5	3.5	19.9	28.8	2.8	31.0
Thal. & midbrain	30	30.1	2.6	11.6	11	43.0	9.8	24.1	35.7	7.5	32.4
Brain, hind	30	25.4	1.9	9.83	27	38.8	2.6	21.7	31.5	2.3	31.2
Cerebellum	30	31.5	2.6	12.2	10	43.3	5.1	24.2	36.4	4.1	33.5
Pons & med.	30	18.2	3.8	7.05	10	28.9	7.6	16.1	23.2	6.1	30.4
Cardiac muscle	34	200	1.7	77.5	30	286	2.4	160	238	2.1	32.6
Hypophysis	29	69.4	7.4	26.9							
Kidney	40	76.1	3.8	29.5	29	222	2.4	124	154	3.0	19.2
Liver	38	201	2.9	77.8	22	334	3.3	187	265	3.1	29.3
Lung	36	508	1.4	197	31	551	1.6	308	505	1.5	39.0
Ovary	27	71.6	4.1	27.7	21	429	1.0	240	268	2.3	10.3
Skeletal muscle	37	18.9	2.3	7.31	29	27.8	3.2	15.6	22.9	2.8	31.9
Skin	36	16.3	3.6	6.31	31	21.3	2.2	11.9	18.2	2.8	34.7
Small intestine	33	20.1	3.9	7.79	29	48.0	3.8	26.9	34.7	3.8	22.4
Spinal cord	36	17.3	4.6	6.70	32	28.4	3.7	15.9	22.6	4.1	29.6
Spleen	38	161	3.9	62.4	27	127	3.0	71.1	134	3.4	46.9
Sub. max. gland	32	62.0	4.7	24.0	26	80.7	5.2	45.2	69.2	5.0	34.7
Uterus	27	56.2	6.9	21.8	22	60.3	4.3	33.7	55.5	5.4	39.2

tissue within 15 seconds after immersion. Electrocardiograms indicated that functional activity of the heart ceased within 8.5 seconds after immersion in liquid nitrogen.

The comparison of blood distribution within 6 segments of the body, before and after rapid freezing, revealed that 3 of the segments showed an increase and 3 a decrease in their relative content of blood after freezing. The regions of increase together with the average percentages are: heart and lungs 1 per cent, liver 4.2 per cent, and intestinal mass 7.4 per cent. The three segments showing a decrease are the head, the pectoral muscles and the pelvic muscles, with an average of 6.7, 12 and 5.6 per cent respectively.

The mean blood values, together with other data, for male rats and for each of 22 different organs or organ parts are shown in table 1. The corresponding values for female rats and for each of 21 different organs are given in table 2.

The mean blood volume for male rats was found to be 55.6 μl./Gm. (table 1). The corresponding value for female rats is 53.1 (table 2). In general there is a close agreement between the blood values per unit weight of corresponding male and female organs or tissues. The organ blood volumes range from a high of 519 μl./Gm. for the lung, (table 1) to a low of 15.7 μl./Gm. for the testis. Other organs having a high blood volume, listed in decreasing order, are the liver, ovary, cardiac muscle, adrenal, thyroid, spleen and kidney. In addition to the testis, other organs or tissues having a blood volume lower than that of the total rat per unit weight are skin, seminal vesicle, skeletal muscle, all divisions of the central nervous system, bone, and the small intestine.

The body hematocrit of male rats is 36.7 (table 1) and of female rats is 36 (table 2). The ratio of body to venous hematocrit is .884 for males and .880 for females. The tissue or organ hematocrits ranged from the high values of 52.7 and 50.7 respectively for the thyroid and spleen of males to the low values of 10.3 for ovary and 14 for bone. Other organs having low hematocrits include the small intestine, kidney and adrenal. The remaining organs or tissues have hematocrits not greatly different from that of the body as a whole.

Discussion

The rapid freezing of small animals in liquid nitrogen appears to circumvent many of the difficulties normally associated with organ blood volume determinations. These difficulties derive from variations in blood distribution due to hemorrhage, anesthesia, method of killing the animal, and to shifts of blood resulting from manipulation of the animal during dissection. The observations reported here, which involved the use of more than 100 rats, show that the use of radioactive tracer methods combined with liquid nitrogen freezing provides a satisfactory approach to the determination of blood volume in individual organs and tissues. The greatest change in distribution of blood upon freezing was the decrease of 5 to 12 per cent noted in the skeletal muscles masses. This observation is not in complete accord with that of Gell,[12] who confined his measurements to the hind limb tissues and concluded that there were no fluid shifts upon freezing rats in liquid nitrogen. The observation that 250 Gm. rats are completely frozen within 148 seconds after immersion is in close agreement with the report of Gell.[12]

The present study is based on the premise that the 3 minutes allowed for mixing of I^{131} albumin in the blood stream minimizes error from leakage of the small protein molecule from the blood stream (fig. 1). If the blood concentration of albumin at 15 minutes after injection is used in calculating plasma volume, the resulting figure exceeds the 3 minute value by 10 per cent.

The differences in the blood volume values reported here for the rat and those of other investigators may be explained, in part, by differences in strain, sex and weight, of the animals used, and to differences in method. The present study reveals that males have a slightly higher blood volume than females of the same weight. Of the several studies of the normal blood volume of rats in the literature, none utilized both plasma and cell labels. However, the values given by Berlin and associates,[5] using cells labeled with P^{32} (4.59 ± .57 ml./100 Gm.) and those of Sharpe and associates[6] using cells tagged with Fe^{59} and Fe^{55} (4.95 ± .27 ml./100 Gm.), approximate those of the present study. Their values for hematocrits of rats, (45.8 ±

2.6 and 48 respectively) are considerably higher than those of the present study. Sharpe and associates[6] used rats weighing more than 300 Gm. This may explain the discrepancy in part.

Gibson and associates[13] used both a plasma and cell label for determining the distribution of cells and plasma in large and minute vessels of the dog. They reported blood volumes for several organs which do not agree very well with those of the present study. This is to be expected, however, since their assays were made subsequent to allowing blood to drain from the organs during sectioning and after removing all visible vessels.

Summary

The red cell volume, plasma volume and the tissue hematocrit have been determined for the frozen adult rat and for each of its major organs or tissues. The blood volumes are expressed in microliters per gram of organ or body weight. The isotope dilution method was applied using Fe^{59} tagged erythrocytes for the red cell determinations and I^{131} serum albumin for the plasma determinations. Animals were frozen in liquid nitrogen and organs were removed and assayed without blood loss.

These methods have provided standard reproducible organ blood volume values for the rat. It is believed that these values are more representative of normal than those of previous reports since the technics applied made it possible to avoid some of the difficulties previously associated with organ blood volume determination.

Summario in Interlingua

Le volumine erythrocytic, le volumine de plasma, e le hematocrite de histo esseva determinate pro congelate rattos adulte e pro lor major organos e histos individual. Le volumines de sanguine es exprimite in microlitros per gramma de peso del organo o del corpore. Esseva usate le methodo dilutional a isotopos. Le determinationes erythrocytic esseva executate per medio de etiquettage a Fe^{59}. Albumina seral a I^{131} esseva usate pro le determinationes de plasma. Le animales esseva congelate in nitrogeno liquide, e le organos esseva excedite e essayate sin perdita de sanguine.

Le methodo usate ha resultate in reproducibile valores standard del volumine sanguinee in le organos del ratto. Nos crede que nostre valores es plus representative del norma que le valores presentate in previe reportos, proque le technicas usate rendeva possibile evitar certes del difficultates previemente associate con le determination del voluminee de sanguine in organos individual.

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