Blood Flow Measurements With Radionuclide-labeled Particles

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Injection of indicators of different types into the intravascular compartment has been applied to the study of the circulation for many years. Solid foreign particles have been used as indicators to measure distribution of organ blood flow, distribution of cardiac output, and the presence of shunting through various organs. The ability to label these particles with radionuclides and therefore render them easily detectable or quantitated has been a major recent advance. Particles of several different materials, sizes, and shapes have been used previously, but currently plastic microspheres have the widest usage.

In general, accurate measurements are possible with these particles if strict standards are established and the method is appropriately applied. As with any biologic method, poor techniques and inattention to details may result in inaccurate and misleading results.

BACKGROUND

The first studies of the circulation using particles were those of Pohlman[1] who injected starch granules into fetal pigs in order to trace the flow patterns within the fetal heart. Glass microspheres were used by Prinzmetal et al. to detect vascular anastomoses in human hearts[2] as well as in various organs in rabbits.[3] Neutron bombardment was subsequently used to produce radioactive glass microspheres by converting the sodium within the glass to radioactive ^{24}Na.[4-6] Ceramic microspheres labeled with various radionuclides were injected into the circulation to produce localized tissue radiation[7] and were also applied to studies of the circulation.[8,9] Both glass and ceramic microspheres were considerably heavier than red cells and sedimented rapidly; lighter plastic microspheres, which could be easily labeled with many different radionuclides, have been developed recently.

Macroaggregates of albumin, which could be metabolized, were labeled with radioactive iodine and were used to study the human circulation,[10,11] but they had the disadvantage of wide variation of particle size; subsequently spherical albumin microspheres with more uniform size and shape were developed.[12,13] These microspheres also had the major advantage that they were metabolized, and the nuclide label could be excreted. The historical development of the use of these different types of microspheres in the study of the circulation is well summarized by Wagner et al.[14]

Recently, a new type of microsphere has become available. These are made of Dextran and come as a kit ready for nuclide labeling in the laboratory (Tracer Sephadex, Pharmacia Fine Chemicals, Uppsala, Sweden). A major advantage is that they have a relative density of about 1.12 g/ml, which is quite close to that of red cells (1.098 g/ml). The distribution of sizes in any specific batch is also considerably less than with other types of microspheres. However, they have not yet been as extensively investigated as the plastic microspheres.

RADIONUCLIDE-LABELED PLASTIC MICROSPHERES

The most commonly used nuclide-labeled microspheres are made of an inert plastic, and these will be discussed in detail. These insoluble "carbonized plastic tracer microspheres" (available from the Nuclear Products Division of the 3-M Company, St. Paul, Minn.) have a specific gravity of about 1.3 compared with about 1.05 for that of whole blood. They are currently available from stock in sizes of 2-4 to 50 μ diameter, labeled with 7 different gamma and 2 beta emitter nuclides. Different sizes and additional nuclide labels are available on special order. The nuclide is incorporated into the plastic and therefore the number of counts per minute (cpm) determined will be related to sphere volume. It is important to remember that flow measurements are made by the

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distribution of the microspheres and that the radionuclide label only provides a relatively simple means by which the number of microspheres can be calculated accurately. It is therefore essential that size and shape distribution of injected microspheres be fairly constant. Although sphere size and shape, specific activity, radionuclide label purity, and leaching are all checked by the manufacturer, we recommend that all newly delivered batches of microspheres be rechecked carefully. Once the appropriate size microspheres to be used for a particular study is selected (see below) the following quality check should be performed.

The microspheres are delivered sterile in multiple injection vials as a dry powder or suspended in either a 10% or 20% Dextran or isotonic saline solution with 0.19% by volume polyoxyethylene 80 sorbitan monooleate (Tween 80), a detergent, added to the solution to prevent aggregation of the microspheres. Sterility of the solution should be maintained by preparing the rubber stopper of the vial with a suitable iodine-containing solution prior to any aspiration. Since Tween 80 in a concentration of greater than 0.05% may adversely affect the circulation, the effects of the suspending solution and any additives should be assessed if the microspheres are to be used for repeated physiologic measurements. This is accomplished by measuring arterial blood pressure, blood gases, or other hemodynamic variables during the injection of these substances.

Although the microsphere size (diameter) range as specified by the manufacturer usually is met, this should be checked and any fragmentation or disruption of the microspheres noted. A microscope with suitably calibrated objectives and eye pieces is used; a micrometer grid is incorporated into one of the eye pieces. We examine the 15 and 9 μ diameter microspheres that we use through a 10x eye piece and a 43x objective, which affords

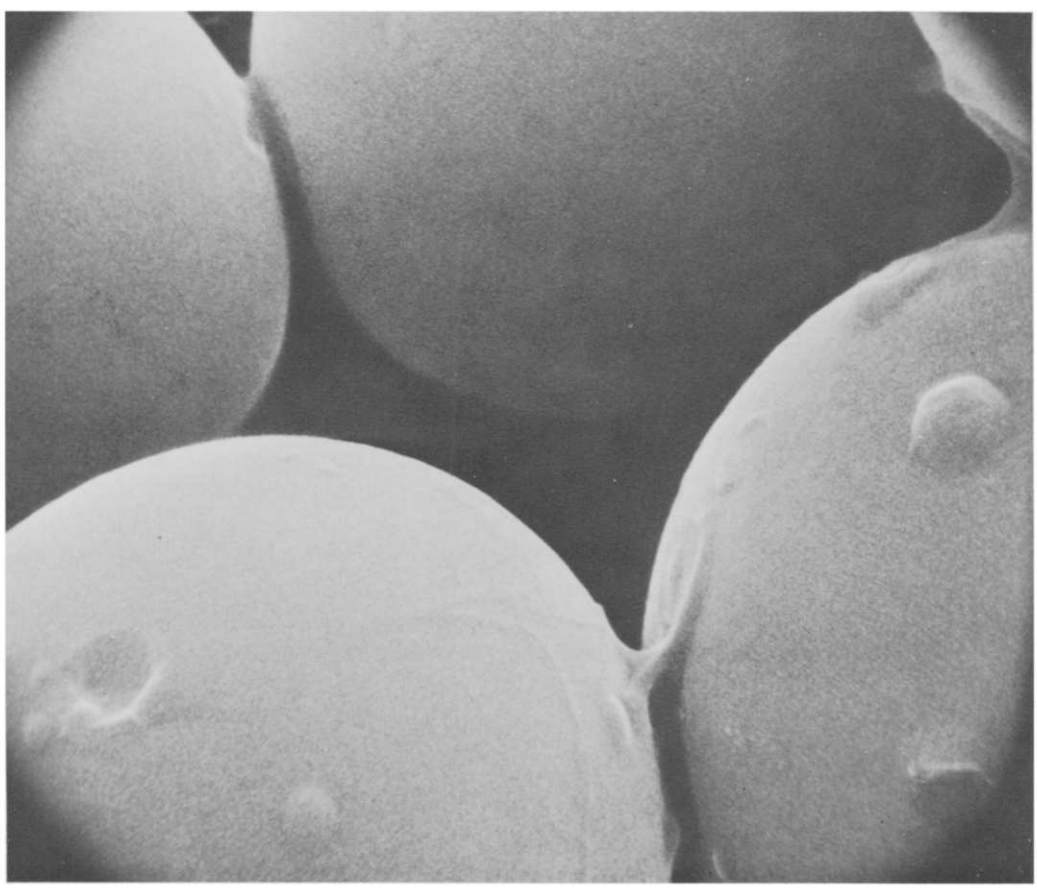

Fig. 1. Scanning electron micrograph of 8–10μ diameter "tracer-labeled microspheres" showing the permanent plastic bridging that may produce significant aggregation.

simple calibration of microsphere size. Several drops of the microsphere suspension, obtained from a well-shaken vial, are placed on a slide and covered with a cover slip. The slide is scanned systematically and all aberrant microspheres and aggregations noted. Measurements are made on 100-200 microspheres and a histogram drawn that demonstrates the average size and the range of distribution in size of the microspheres. Criteria for rejection of a specific batch will depend on the size of microspheres. The acceptable range will obviously be larger for 50 μ diameter microspheres than for 15 μ diameter microspheres. For 15 μ diameter microspheres, we reject the batch if the size range is greater than 10 μ, if the average size is 5 μ greater or smaller than the size ordered, or if there are more than 1% of damaged or hemispherical microspheres. Aggregations of microspheres generally can be broken up by vigorous shaking or placing the vial in an ultrasonicator for 1 min. Additional Tween 80 may be required but must be used with caution because of its possible harmful effects. Occasionally, the microsphere aggregation is due to plastic bridging between the microspheres, as shown in Fig. 1. These aggregations are obviously permanent, and if numerous, the microspheres cannot be used. The introduction into and growth of bacteria or fungi in the vial also increases the likelihood of aggregation. Resterilization of the vial and changing the suspending solution will reverse this.

Nuclide specificity of each batch of microspheres is determined by observation of the output display of a suitably calibrated multichannel pulse-height analyzer. The spectrum of the new batch, counted for a suitable length of time, is examined for extraneous peaks or other unusual spectral manifestations. At the same time, counts in the supernatant suspending fluid are checked by the following procedure. After mixing the vial contents well and, when necessary, placing the vial in an ultrasonicator for a period of 1 min to assure complete dispersion of the microspheres, a small volume of the microsphere mixture (0.05-0.1 ml) is withdrawn into a disposable tuberculin syringe and mixed well with 20 ml of 0.5% Tween 80 in isotonic saline in a centrifuge tube. This is then centrifuged at 1100 *g* for 20 min and the sample counted to obtain total activity as well as spectral distribution. The supernatant fluid is carefully removed through a Millipore filter and counted again. Nonmicrosphere supernatant fluid radioactivity greater than 0.2% of the total radioactivity is not accepted, and the batch of microspheres is returned to the manufacturer. Extreme care must be taken when separating the supernatant fluid from the microspheres, since it is easy to disturb the centrifuged microsphere pledget. Leaching is checked by resuspending the microspheres in 20 ml of the same solution and performing the same procedure at repeated intervals of 2-4 wk while the microspheres are being used for experimentation. If there is a significant increase in the percentage of counts in the supernatant fluid, the microspheres can be washed and checked again. In our experience, the only nuclide that occasionally undergoes leaching is ^{125}I. If after several weeks or months of storage the supernatant fluid has a count greater than 0.2% of the total, the microspheres to be used for a specific experiment should be carefully washed to remove all supernatant fluid, and thereby all nonmicrosphere counts, and then resuspended.

The specific activity (i.e., the number of counts per microsphere) is then determined. This procedure involves streak application of 200-300 microspheres onto 1 sq cm pieces of mm graph paper. If too many beads are streaked onto the graph paper it is often hard to count them accurately; if too few microspheres are counted in the scintillation counter, the sample may not be representative of the batch. With practice, it is usually quite easy to obtain 5 pieces of graph paper appropriately streaked for each batch. The pieces of graph paper are placed under a light, and after they have dried, the suspending solution of Dextran acts as an adhesive holding the microspheres in place. Glossy cellophane tape is placed over the microspheres to make a temporary mount on a slide which is placed under the microscope. The magnifications used are 25x for 50 μ microspheres, 60x for 15 μ microspheres, and 100x for 9 μ microspheres. The microspheres are streaked in such a manner that at the appropriate magnification the streak is not wider than the field of view; this makes counting much easier. The number of microspheres on each piece of graph paper is counted three times. The graph paper is then placed flat on the bottom of a counting vial and the total number of radioactive counts for each piece of paper determined in a gamma counter. The average number of microspheres and the

counts per minute for each piece of graph paper are obtained, and the counts per microsphere may then be calculated. The counts per microsphere for all five pieces of graph paper are averaged, and this value is used for that particular batch of microspheres. A half-life plot is made for each batch so that the specific activity at any particular time thereafter can be determined. The wider the size range of the microspheres the greater will be the counts per microsphere variability determined from the pieces of graph paper; this is one of the main reasons for rejecting microspheres with wide size ranges. The microspheres may now be stored for subsequent use.

PRINCIPLES INVOLVED IN THE USE OF MICROSPHERES

Standard indicator dilution techniques have been modified to measure the fractional distribution of blood flow by injecting a suitable indicator into the circulation and measuring its distribution within various organs during the initial transit.[15,16] This technique has been further modified by using microspheres as the indicator. The microspheres are trapped in the arteriolar or capillary vascular system on the first circulation after injection. This entrapment of the microspheres within tissues essentially accomplishes the integration of indicator dilution curves by physical impaction in the microcirculation.

In order to use microspheres to measure accurately the flow distribution to or within an organ, several important factors have to be considered. Immediately after injection into the circulation, the microspheres must be well mixed and evenly distributed within the blood stream so that the concentrations reaching all branching sites are the same. If distribution of blood flow within a specific organ is to be measured, uniform distribution of the microspheres throughout the cross section of the vessel may be important, and microsphere distribution should approximate red blood cell distribution. Complete entrapment of the microspheres in the vascular beds downstream from the site of injection is essential during the first circulation, and the microspheres must remain entrapped until counted. Injection and entrapment of the microspheres must have no effect on the general circulation nor on local organ hemodynamics. This is particularly important if serial observations are to be made. These various considerations will be discussed separately.

EVENNESS OF MICROSPHERE MIXING

In order to measure organ blood flow, the microspheres must be well mixed at the site of the injection and their concentration in all arteries downstream from the site of injection should be similar, except for minor random variations. Distribution of the microspheres will then be similar to the distribution of blood to the organs. The two main factors that will affect this uniformity of concentration are the site of injection and the number of microspheres injected.[17] To obtain adequate mixing before the first major arterial branching, the microspheres should be injected as distant as possible from that point. When measuring the distribution of systemic blood flow, injection should be into the left atrium;[17-19] however, injection of the microspheres into the left ventricle usually has allowed for accurate measurement.[17,20,21] If blood flow to more distal organs, such as the kidneys, is being studied, injection into the root of the aorta may suffice. The best site for injection should be evaluated before commencing any study. If mixing is even and sufficient microspheres are injected, there should be only random differences in concentrations of microspheres between different arteries; and if the experiment could be repeated in an identical manner on numerous occasions, the variability should approximate a Poisson distribution.[17] Based on this assumption, it is possible to calculate the number of microspheres required in any organ or segment of an organ to express the accuracy with which flow can be determined with varying degrees of precision. For example, to have a distribution variability within 10% of the mean distribution at the 95% confidence level, 384 microspheres must be present in the organ, or portion of organ. In order to be accurate within 5% of the mean, 1536 microspheres need to be present. These calculated numbers of microspheres required for accurate measurement were confirmed experimentally in dogs, sheep, and lambs.[17,22] It is, therefore, essential that when measuring flow the number of microspheres injected be carefully calculated to satisfy these criteria for the organ or portion of organ with the lowest flow that is to be measured. In general, the limits of variability decrease as the number of microspheres present is increased, as predicted from the binomial distribution. Some nonrandomness of distribution exists in most circumstances, presumably related to incomplete mixing of the microspheres. As long as

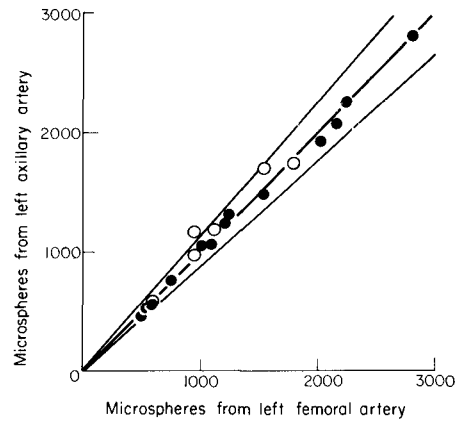

Fig. 2. Comparison of the numbers of microspheres in 10 ml of blood withdrawn simultaneously from the left axillary and left femoral arteries after injection into either the left ventricle (•) or the arterial perfusion line during total cardiopulmonary bypass (○) in rhesus monkeys.[26] Line of identity ± 10% is shown. $\hat{y} = 0.972X + 49.1$; $r = 0.993$; $Sy \cdot x = 18.55$.

at least 384 microspheres are present in each sample, this does not lead to major errors.

Uniformity of mixing as reflected by the concentrations of microspheres in major arteries has been examined under a variety of conditions in many different animal species, but should be evaluated anew for species or experimental designs not examined previously. In addition, it is advisable to compare concentrations in the major arteries supplying the upper and lower parts of the body, since the anatomical configuration of the aortic arch may create preferential streaming in certain animals, such as sheep. The even distribution of microspheres, injected at several different sites, has been examined by comparing microsphere concentrations in arterial blood obtained simultaneously from several different arteries both in normal and abnormal physiologic circumstances. These studies include comparison of two umbilical arteries in fetal lambs;[23] carotid and femoral arterial concentrations in rabbits,[24,25] dogs,[17] and sheep;[17] axillary and femoral arterial concentrations in monkeys[26] (Fig. 2); left and right carotid arterial concentrations in dogs[17] and sheep;[17] renal and femoral arterial concentrations in monkeys;[27] left and right femoral arterial concentrations in rabbits,[24,25] dogs,[17] and sheep;[17] and peripheral arterial and abdominal aortic concentrations in dogs.[17] Comparisons of concentrations of microspheres at several different points in a physical model have also been made.[28]

Comparisons of the number of microspheres per gram of tissue in the left and right cerebral hemispheres or the left and right kidney are easy to perform and also give an idea of evenness of distribution at the site of origin of the arteries supplying these regions. Whenever possible these procedures should be performed on every study as they provide a useful simple internal check of the adequacy of mixing. These comparisons have been made in fetal and neonatal lambs,[28,29] rabbits,[24,25,30] monkeys,[31] rats,[20] and sheep.[17] In addition, the fractional distribution of blood flow between two different areas of an organ, such as the kidney, has been compared to assess streaming and distal mixing of 15 μ diameter microspheres.[32]

The evenness of mixing of the microspheres can be assessed by injecting two or more different batches of microspheres a few minutes apart. If mixing is adequate, the patterns of distribution should be quite similar for each injection. This type of analysis has been performed in fetal lambs,[28] dogs,[18,33] monkeys,[27,31] rabbits,[24,30] rats,[20] and sheep.[22]

The measurement of the distribution of blood flow to major areas as described above is not significantly affected by the size of the microspheres used or the radial distribution of the microspheres within the cross section of the major arteries.[17,21,26,34] However, for measuring blood flow within specific organs, distribution of the microspheres in a manner similar to that of red blood cells is more important. Katz et al.[35] reported that the intracortical distribution of blood flow to the kidney was different when measured by different sized microspheres, presumably due to differing rheologic characteristics. However, McNay and Abe[36] did not find these differences. Domenech et al.,[37] in examining the relative blood flow to the inner (subendocardial) and outer (subepicardial) layers of the myocardium, showed that the size of microsphere injected was crucial in assessing the regional distribution of blood flow (Fig. 3). They found that when 50 μ diameter microspheres were injected into the left ventricular cavity, the concentration of microspheres in the subendocardial layers of the myocardium was significantly greater than in the subepicardial region of the myocardium. This could lead to the conclusion that coronary blood flow is greater to the inner than to the outer myocardial layers. However, as shown in Fig. 3, this disproportion between inner and outer myocardial distribution decreases progressively as microsphere size is re-

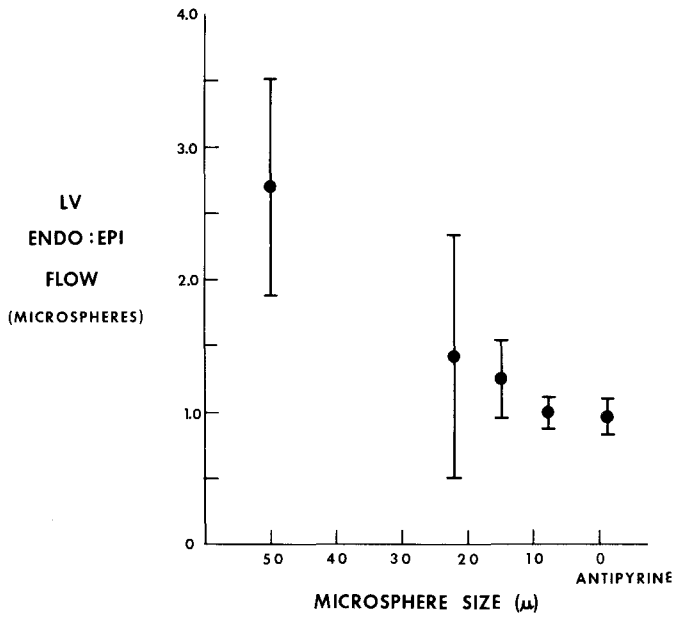

Fig. 3. Ratios of coronary blood flow (ml/100 g/min) distributed to the inner (subendocardial, ENDO) and outer (subepicardial, EPI) layers of the left ventricular (LV) free wall as measured by radionuclide-labeled microspheres of different diameters and by antipyrine.

duced. When microspheres of 9 μ diameter or 1-10 μ diameter filtered through 8 μ Nuclepore filters (General Electric) were used, distribution was similar in both layers and similar to that obtained by using radioactively labeled antipyrine.[38] From these data, it was apparent that small microspheres, which approximate red cell diameter, should be used to assess the distribution of flow within the myocardium.[38] Similar requirements may be necessary for many other organs. Phibbs et al.[39,40] studied the possible mechanisms involved in these differences in distribution patterns between large and small microspheres. They injected microspheres of different diameters into the left ventricle or ascending aorta of rabbits and then very rapidly froze the previously exposed femoral arteries. They found no evidence of sedimentation of microspheres in the range of 7.5-80 μ diameter. However, the smaller the microsphere the more closely they approximated the distribution of red blood cells within the cross section of the femoral artery. As seen in Fig. 4, microspheres of 60-80 μ diameter concentrated centripetally, whereas the microspheres of 7.5-10 μ diameter were fairly evenly distributed throughout the total cross-sectional area of the vessel. Thus, when radial distribution is uneven, as with the larger microspheres, small branch arteries with proportionately low blood flow might receive disproportionately fewer microspheres for that flow. These factors must be considered carefully when selecting the appropriate size of microsphere for the particular measurements desired. The evenness of distribution displayed by the smaller microspheres must be weighed against the increasing potential for incomplete entrapment, which occurs with the smaller microspheres (see below).

TRAPPING OF MICROSPHERES

A further prerequisite for the use of microspheres to measure distribution of blood flow is that all microspheres are entrapped in the peripheral microcirculation and that no significant number bypass the organs. Since the diameters of capillaries[41] and arteriovenous anastomoses[42] may vary between organs as well as between animal species, sphere size must be selected carefully to assure that a significant fraction of injected micro-

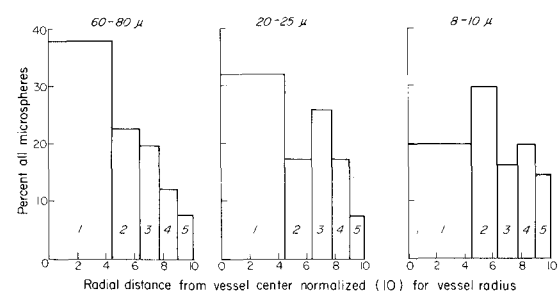

Fig. 4. Histograms depicting the radial distribution of microspheres of three different diameter ranges in the femoral arteries of rabbits.[40] The radius of each artery has been arbitrarily normalized to 10 units. The cross-sectional areas (1, 2, 3, 4, 5) of the arteries are equal.

spheres does not pass through the organs into the venous system. It is essential that all organs under study be tested for nonentrapment under all experimental conditions. The difference in diameter between capillaries and arteriovenous anastomoses can be used to measure the relative flows through each separately by injecting microspheres of different sizes. For example, if microspheres large enough to be trapped by both capillaries and arteriovenous anastomoses are injected, total organ blood flow can be measured. When smaller microspheres, which are trapped by capillaries but not by the arteriovenous anastomoses, are used then both nutrient and arteriovenous anastomotic flows can be calculated. In practice, most measurements probably represent a value somewhere between nutrient and total flows. Since few 15 μ diameter microspheres (now the most common size used) fail to be trapped and since most arteriovenous anastomoses are greater than 25 μ,[42] it is probable that flow through arteriovenous anastomoses in most studies reported has not been significant. However, it is important to recognize that alterations in experimental conditions may open up significant numbers of arteriovenous connections. Thus, conditions known to alter these communications, such as increased environmental temperature and anesthetic agents,[18,20,21,43] must be carefully checked. Under most conditions, only a very small fraction of injected microspheres is not trapped and therefore recirculates; however, it is necessary to establish acceptable criteria for the amount of nonentrapment for each experimental condition and each species.

Several different techniques have been used to assess the magnitude of shunting in various organs. The most direct method to measure the proportion of microspheres passing through a specific organ is to collect the total venous drainage and compare the number of microspheres in the drainage to the number lodged in the organ.[4,6,17,33,35,37] However, this requires extensive surgical procedures and has to be done under anesthesia. A second method involves counting the number of microspheres in the organ or organs which receive the venous drainage of the organs under investigation for nonentrapment. For example, the passage through the gastrointestinal microcirculation of microspheres injected either directly into a mesenteric artery or into the aorta distal to the origin of the hepatic arteries would account for a significant number of microspheres lodging in the liver.[4,44] In the study of the fetal circulation, the percentage of microspheres, injected into the umbilical arterial circulation, that lodged in the liver would reflect the amount of nonentrapment in the placenta.[23,45] More commonly used as an index of nontrapping in the peripheral microcirculation is the proportion of microspheres injected into the arterial circulation that are lodged in the lungs.[18,20,24-26,28,30,31,43,46-49] If the microspheres are injected into the left atrium or ventricle, the contribution of the bronchial arterial circulation must be considered. Injection of the microspheres into the descending aorta below the origin of the bronchial arteries obviates this problem, but this method does not evaluate the possibility of significant passage of microspheres through arteriovenous anastomoses in tissues supplied by vessels originating from the ascending aorta. This technique also requires that any nontrapped microspheres lodge completely within the organ receiving the venous drainage and do not pass through that organ as well. The failure of microspheres larger than 15 μ in diameter to pass through the lungs has been confirmed in several species;[8,18,20,22,30,33,49,50] this has been shown for the liver as well.[30,44] Smaller microspheres, i.e., those less than 10 μ in diameter, will pass through the lungs in significant numbers (±20%).[19,50]

A third method involves obtaining a sample of blood from a vein draining the organ in question, the right atrium, or a pulmonary artery and comparing the number of microspheres in that sample with the total number injected or the total lodged in the organ.[19,21,22,24-26,31,36,43,51] However, since Creasy et al.[49] have shown, by the use of external counting, that microspheres may continue to appear in the venous circulation for up to 15 min after injection into the left ventricle, this technique may not be accurate unless the sample is collected for as long as 15-20 min. Archie et al.[19] have discussed in detail the quantitation of nonentrapment in organs by obtaining, simultaneously, samples of blood from both the venous drainage and arterial supply of an organ. They also compared the use of a venous withdrawal sample and collection of total venous effluent as related to both the kidney and brain and showed an excellent correlation between the two techniques for measuring the number of nontrapped microspheres.

In the earlier studies using the distribution of

radionuclide-labeled microspheres to measure flow and flow patterns, 50 μ diameter microspheres were injected. There was no evidence of nonentrapment of these microspheres in any organs in fetal lambs.[28] Careful evaluation in rabbits[24] and monkeys[31] also revealed no significant passage of microspheres through tissues. The rabbits were studied when both normotensive and hypotensive, and with and without anesthesia, by collecting blood from both the superior and inferior vena cavae after injection of the microspheres into the left ventricle. In addition, the lungs were counted after injection into the descending aorta at the level of the diaphragm, and the fraction bypassing capillaries of the gastrointestinal tract was assessed by collecting the total portal venous return after injection of the microspheres into the left ventricle. Similar procedures were also performed in monkeys and no significant nonentrapment was present.[31] Injection of 50 μ diameter microspheres into the superior vena cava and the abdominal aorta in unanesthetized rats revealed no significant nonentrapment in the lungs and lower body, respectively.[20] Fifty micron diameter microspheres have also been shown not to bypass, in significant numbers, the systemic microcirculation in conscious dogs,[18] nonpregnant female rabbits,[46,49] and adult sheep.[52]

In contrast, a significant degree of nonentrapment in specific organs in several species has been shown with the more commonly used smaller (15 or 25 μ diameter) microspheres. Kaihara et al.,[18] using 15 μ diameter microspheres, found 5%–10% of microspheres injected into the systemic arterial circulation of conscious dogs trapped in the lungs. However, 8-10 μ diameter microspheres injected into the left ventricle or atrium generally showed less than 1% nonentrapment in the coronary circulation of dogs and sheep[17,38] and no significant numbers of microspheres greater than 15 μ in diameter bypassed the kidneys.[36,51] The proportion of microspheres injected into the systemic circulation of lambs that were not trapped was about 2%–6%,[19] and although this number is small, the error involved could be corrected for as described by Archie et al.[19] We have detected no significant nonentrapment of 15 μ diameter microspheres in fetal and neonatal lambs under normal conditions and following hypoxia or hemorrhage.[45,53] Nonentrapment of 15 μ diameter microspheres was also shown to be insignificant in rhesus monkeys.[26] Extensive investigation of the bypass patterns in rabbits has been reported by Warren and Ledingham.[30] They found that about 4% of 50 μ diameter microspheres injected into the systemic circulation bypassed the peripheral organs and were recovered in the lungs. Seven percent of 25 μ diameter microspheres and about 12% of 15 μ diameter microspheres were present in the lungs after injection into the systemic arterial circulation. They investigated the exact sites of nonentrapment and found no significant nonentrapment of any size microspheres in the gut, liver, kidneys, or lungs. More than 80% of microspheres of all sizes were not trapped in the ear, and as microsphere diameter decreased, an increasing percentage bypassed the hindlimb. Creasy et al.[49] showed that the degree of nonentrapment after injecting both 15 and 25 μ diameter microspheres into the descending aorta was not affected by ischemia or distal hypotension following inflation of a balloon in the aorta. They also showed that there was no significant difference in nonentrapment of 15, 25, and 50 μ diameter microspheres in the pregnant and nonpregnant rabbit. Hales has shown significant nonentrapment in the skin of sheep, particularly when exposed to increased ambient temperatures.[21]

EFFECTS OF MICROSPHERES ON THE CIRCULATION

The positioning of catheters for injecting the microspheres as well as for obtaining arterial blood samples may affect the circulation due to immediate local obstruction of arterial flow or subsequent thrombus formation. However, chronic implantation of a catheter in an artery is generally associated with the fairly rapid development of collateral circulation around the site of obstruction. For example, if one carotid artery is ligated, the distribution pattern between the left and right cerebral hemispheres is identical.[24,47] An unusual complication of chronic catheterization is perforation of the left ventricular wall; however, incomplete perforation with wedging of the catheter tip between the myocardial trabeculae is more common and may lead to local trapping of microspheres injected into the catheter. Calculation of myocardial blood flow in this instance would be impossible, since the microspheres present in the myocardial tissue do not all represent coronary arterial supply.

Acute circulatory changes may occur during, or shortly after, injection of the microspheres but are not frequent. Their incidence is related to the size of the animal, and therefore, to the cross-sectional area of the microcirculation, the number of microspheres injected, and the size of these microspheres. Aggregation of microspheres may produce significant circulatory alterations if large aggregations, even of small microspheres, occur. Warren and Ledingham[30] described a significant occurrence of complications in conscious rabbits injected with a fairly large number of 15 μ microspheres. Complications are less frequent in monkeys and rare in other species. The acute hemodynamic changes described[21,24,27,30] include transient hypotension, hypertension and bradycardia, a slight fall of cardiac output, and ventricular ectopic beats. Occasional transient neurologic signs, such as nystagmus, are encountered.[21,54] Arterial blood gases have not been affected systematically. Renal function is unaffected.[32,51] Sasaki and Wagner noted transient swelling of the feet in rats.[20]

The effects of microsphere injection on the distribution of cardiac output, organ blood flows, and hemodynamic variables also have been studied on a more chronic basis for periods of up to 14 days after the initial injection. In monkeys, 24 hr after the first injection, there were no significant changes in arterial blood pressure, peripheral resistance, heart rate, or cardiac output and its distribution.[27] In addition, arterial blood gases, hematocrit, lactate, and pyruvate concentrations were unaltered. Fetal lambs showed no changes in arterial blood pressure, arterial blood gases, or umbilical blood flow up to 16 days after the injection of microspheres.[28] The differences between distribution patterns of microspheres injected from 1 hr to 14 days apart have been studied in fetal lambs,[28] monkeys,[27] rats,[20] rabbits,[24,25] dogs,[18] and sheep.[22] No systematic differences have been demonstrated, although in rats the distribution to the kidney was higher in the second injection than in the first.[20] After a third or fourth injection of microspheres, greater variability occurred, although no consistent changes have been described.

In general, in most species 300,000-500,000 microspheres of 15 μ diameter can be injected into the arterial circulation without significant acute or chronic hemodynamic alterations. In fetal lambs, in which the umbilical-placental circulation accounts for 40%-50% of the combined ventricular output,[55] we have injected 5 batches of 15 μ diameter microspheres, each batch with more than 10^6 microspheres, without significant effects. In dogs, up to 21.6×10^6 microspheres of 7-10 μ diameter have been injected without apparent adverse effects.[56] Rabbits and monkeys appear to be more sensitive to microsphere injections, and in these two species, the total number injected may have to be reduced.

The degree of obstruction of the microcirculation is affected by the diameter of the microspheres; the larger the microspheres, the larger the vessel plugged and the greater the portion of the circulation obstructed. Thus, the number of microspheres injected must be reduced considerably when larger diameter microspheres are used. In smaller animals, fewer microspheres should be used even if small diameter microspheres are selected.

SELECTION OF SIZE OF MICROSPHERES

From the preceding discussion it is evident that careful selection of microsphere size is necessary depending on the type of experiment and the particular organ or portion of organ under investigation. In order to approximate as closely as possible the actual distribution of blood flow, smaller microspheres are preferred. In studying the distribution of flow within organs such as the heart, where the distribution of 8-10 μ diameter microspheres truly represents the distribution of flow within the myocardium,[38] nonentrapment of these small microspheres must be assessed carefully. Fortunately, in the study of myocardial flow there is no significant nonentrapment. However, in many other organs, a significant number of microspheres of even larger diameter are not trapped, and this must either be corrected for,[19] or larger microspheres used. In general, 15 μ diameter microspheres satisfy both the conditions of distribution similar to red cells as well as absence of significant nonentrapment. In selecting the appropriate microsphere size for a particular study, therefore, the localization of flow required has to be weighed against the possibility of nonentrapment. With the larger microspheres, since the total number injected has to be reduced, the statistical precision of the measurements also must be considered.

MIGRATION OR DESTRUCTION OF MICROSPHERES AFTER EMBOLIZATION

Madden et al.[57] have reported that in rats, 15 μ diameter microspheres were recovered in washings from the oropharynx, trachea, bronchi, stomach, and urinary bladder, in some instances, as soon as 15 sec after injection into either the right atrium or aorta. We have been unable to duplicate their observations in either monkeys or lambs.

It is important to ensure that the nuclide label is not lost from the microspheres if the animal is allowed to survive for a prolonged period after injection. The microspheres are insoluble in concentrated solutions of NaOH and HCl at room temperature. Patterns of distribution of cardiac output obtained up to 2 wk apart are not significantly different indicating that the microspheres initially injected have not been altered.[18]

PREPARATION OF ANIMAL TISSUE SAMPLES FOR NUCLIDE COUNTING AND MICROSPHERE DETERMINATIONS

Working with radionuclide-labeled microspheres poses special problems in tissue sample preparation because the microspheres are discrete units of radioactivity with a density of 1.3. As a result, in liquid media, the microspheres settle to the bottom of the counting vial, whereas in whole wet tissues or carbonized samples (see below) they are dispersed throughout the tissue in the counting vial. When samples of these different types of tissue or different mass are to be compared to each other, the relationships between them must be determined since the geometry of the samples affects the counts (see below). The only other alternative is to prepare all the samples in the same manner, but often this may be very difficult to accomplish. Usually, the samples from one study will include vials containing, separately, fresh or formalinized whole tissue, carbonized tissue (see below), and blood reference samples (see below). Any one type may not be present. To use these different sample configurations accurately, their nuclide counting efficiency relationships must be determined as described in the next section.

The reasons for the sample preparations will become clear when we discuss the calculations. The vials used for counting the tissues should all be of the same type, since glass and plastic attenuate counts differently, particularly when low energy nuclides such as ^{125}I are present. All samples of a particular tissue type should contain the same volume of tissue packed to a preselected uniform height (geometry) in the bottom of the vials.[58] The wet tissue may be prepared either fresh or in formalin (see below). In either case, the tissue is cut into small pieces to facilitate packing into the bottom of the vials. Individual organs are divided as equally as possible between vials so that the tissue height in each vial is about the same. This general principle should be followed for each tissue. A small amount of 10% formalin added to the tissue vial prevents air liquid or air tissue interfaces, which may alter the counts, and acts as a preservative for storing the vials.

Preparation of vials containing carbonized tissue is simpler, since the powder created by grinding up the carbonized tissue can be more easily packed to a consistent height in the counting vials. Carbonizing organs, particularly large ones, has another major advantage in that the total mass of tissue to be counted is markedly reduced. Aliquots of organs have been used, but this assumes completely even distribution of the microspheres throughout that organ. We prefer to count the entire organ. Prior to carbonizing of any tissues, absence of radioactivity loss at the temperatures to be used must be checked for each nuclide by counting a known quantity of labeled microspheres before and after exposure to the same conditions used when carbonizing. We have not found significant loss of radioactivity below 350°C. The tissues are placed in disposable aluminum pans and covered with 10% formalin for several hours prior to carbonization. This facilitates removal of the tissue from the pans after carbonizing as well as subsequent crushing and blending. The pans are then placed in a vented oven at 325°C for at least 24 hr. After cooling, the sample is ground or blended into a powder and distributed into vials to a height of 3 cm. This procedure should be performed in a vented hood and wearing a mask to prevent the inhalation of any of the fine powder. Care should also be taken not to lose any of the carbonized powder. If the last vial to be filled of a particular organ contains less than 3 cm of carbon, an appropriate amount of nonradioactive carbon is added to the vial. The lid is applied over a small amount of cotton, and the vial shaken for about a minute to ensure proper mixing. The excess carbon powder is wiped off the top and inner sides above 3 cm with the

cotton which is then packed down on top of the powder. With this method, sample consistency can be maintained throughout all the vials produced for the whole animal.

Blood vials are processed simply. The blood is always hemolyzed before counting to ensure that the microspheres settle to the bottom of the vial. Without hemolysis, the beads might be randomly dispersed throughout the thin layer of packed cells and consequently would not be completely on the bottom of the vial. As with tissue and carbonized samples, sample consistency is important.

As a result of these methods of sample preparation, an animal could be divided into three different types of samples, each consistent within itself. If the blood sample configuration is taken as the normal or most efficient standard, then the other two sample types can be corrected and compared to the blood sample type. During the calculations, the counts in the carbon and tissue vials are therefore related to counts in the blood vials. By doing this, all samples can then be compared without further adjustments.

NUCLIDE COUNTING

Equipment Preparations and Calibration

We have used a 512-channel multichannel pulse-height analyzer (Searle Analytic Des Plaines, Ill.); however, any gamma-counting equipment with a multichannel pulse-height analyzer can be used. The following section will deal with the preparation and calibration of a multichannel system, but most of the information is applicable to 1-3 window spectrometers.

Preliminary preparation of the instrument involves voltage-peaking the detector, which requires a stable radionuclide source such as ^{137}Cs. The ^{137}Cs source is counted initially at a low detector voltage. The voltage is gradually increased until the count rate plateaus. This plateau is usually maintained for a moderately large increase in voltage, and then the count rate rises rapidly again as voltage is increased. The detector voltage should be set permanently at about the midpoint of the lower third of the plateau range. The next stage of instrument calibration depends on the exact nuclides to be used and, for which, pure sources of each nuclide are needed. Once the number and types of nuclides to be used have been determined, the range calibration can be computed; for example, when working with the 5 radionuclides ^{125}I, ^{141}Ce, ^{51}Cr, ^{85}Sr, and ^{95}Nb, a total energy range of 0-900 keV is used. To standardize the instrument, the ^{137}Cs gamma peak at 662 keV is adjusted with the gain control into channel 376. Increasing the gain moves the standard peak to the right, decreasing the energy range viewed by the instrument. This channel number is calculated:

$$\text{Channel no.} = \frac{\text{Peak energy of interest} \times \text{Total no. of channels}}{\text{Energy range}}$$

In this circumstance, each of the 511 channels (channel 0 is the time channel) of the instrument is calibrated to 1.76 keV/channel with a total range of 900 keV. When the four nuclides ^{125}I, ^{141}Ce, ^{85}Sr, and ^{46}Sc are used, the analyzer is calibrated to a total energy range of 0-1300 keV using the same technique. In this case, each of the 511 channels represents 2.54 keV. Fewer channels can be used, but 511 channels provide a very sensitive output of the complex spectra counted. The disadvantage of a large number of channels is the increased time taken for data output. This is solved in our system by using a multiple region of interest module that automatically integrates and dumps up to eight preselected regions of interest. Without such a unit, all of the channels of the spectrum must be dumped individually and the channel integration done by hand or computer.

The stability of the calibration is dependent on the high voltage power supply and the gains in the preamplifier and the pulse-height analyzer. Any voltage or gain fluctuation will alter the peak position. When stable equipment is not available a method must be devised for monitoring peak position fluctuations. Corrections for these fluctuations must be made in subsequent calculations, since variations in the standard peak position will produce changes in the peak areas (spectral distribution) of the nuclides. Our system of calculation depends on the ability to reproduce the response matrix of the analyzer each time samples are counted. Stability over the counting period negates the use of a monitoring system and results in a high accuracy and reproducibility in stripping the spectra.

Spectral Distribution

Once the energy range has been set and stability ensured, pure nuclides are counted individually to determine the most suitable region of

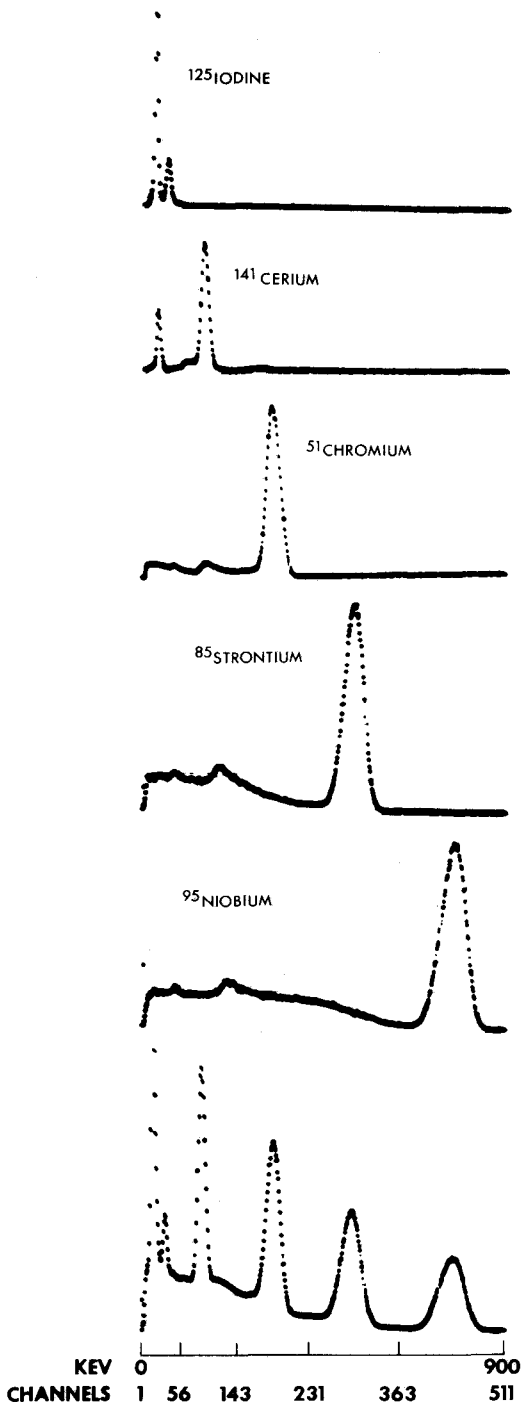

Fig. 5. Energy spectra of each of the five nuclides used, plus a combination of all five, displayed on the 512 channel pulse-height analyzer calibrated to 0-900 keV. Channel numbers indicate those channels used to establish appropriate regions of interest (ROI) for each nuclide (see appendix).

interest for each major peak (Fig. 5). Each nuclide is counted at several count rates, and the best region of interest around the peak is determined. Since the geometry and distribution of nuclide within a sample will affect count rates, the pure standards used for these calibration steps are prepared to simulate as closely as possible the conditions that will pertain when tissues under investigation are counted.

Since we are interested in a fairly wide range of count rates (10^3-10^6 cpm), peak stability is a major consideration. We therefore use wide continuous regions of interest extending from the center of the prephotoelectric peak trough to as close to background as possible on the high energy side of the peak (Fig. 5). For example, the region of interest for niobium-95 extends from channel 363 to 511 and for strontium-85 from channel 231 to 362.

By setting the instrument in this manner, spectral distribution shifts due to electronic fluctuations and count rate summation are minimized. The final regions of interest are determined at the differing count rates either by combining all five nuclides together or, more simply, by counting each individual nuclide sequentially and superimposing the stored spectra of each nuclide (lowest panel, Fig. 5). After these final five regions of interest adjustments are made, the percentage of each nuclide in each region of interest is determined. One of the major criteria for initial selection of a particular nuclide is that there is one well defined primary energy peak and a minimal number of counts above, or to the right, of that major peak (Fig. 5). If this criterion is met, the calculations by the spectral distribution stripping technique are much simplified, since that nuclide's highest energy peak is solitary, and the percentage of the total count represented by this peak can be calculated (Tables 1 and 2). For example, if a peak

Table 1. Percentages of Total Counts Per Minute of Each of the Five Nuclides in the Five Preselected Regions of Interest (ROI) in the Settled Microsphere (Blood) Configuration

	ROI				
Nuclide	A	B	C	D	E
^{125}I	99.99	0	0	0	0
^{141}Ce	23.87	76.13	0	0	0
^{51}Cr	11.56	13.46	74.98	0	0
^{85}Sr	15.79	21.18	8.77	54.21	0
^{95}Nb	12.56	19.47	15.70	13.02	39.26

Table 2. Percentages of Total Counts Per Minute of Each of the Five Nuclides in the Five Preselected Regions of Interest (ROI) in the Carbonized Tissue Configuration

Nuclide	ROI				
	A	B	C	D	E
^{125}I	99.99	0	0	0	0
^{141}Ce	22.78	77.22	0	0	0
^{51}Cr	12.67	15.92	71.42	0	0
^{85}Sr	14.64	22.90	9.51	52.94	0
^{95}Nb	11.12	20.29	16.23	12.63	39.73

area for a specific nuclide is in channels 144-231 (of a total of 511), that nuclide will not be found above channel 231. But, due to Compton interactions, this nuclide will have crossover (cross talk) counts in channels 1-143, which will have to be subtracted from the counts of any other nuclide that has a peak that falls in the lower energy group of channels. As a result, for any group of nuclides, there will always be a higher peak energy nuclide (to the right of the energy spectrum), free of any other nuclide, from which the calculations (stripping) can be started. The total amount of this nuclide is computed from the percentage of that nuclide in that "pure" peak area (Tables 1 and 2). The percentage of total counts of this nuclide in the other regions of interest in question (peak areas of the other nuclides) is also known, so that the amount of cross-talk counts can be stripped from each of the lower energy regions of interest. This leaves the next lower energy nuclide peak area "pure" to start the calculation process over again. The process is continued through each region of interest, resulting in each nuclide being allotted its correct number of counts of the total in the sample. The derivation of the equations used for calculating the amounts of each nuclide in a sample containing several different nuclides are presented in the appendix. It should be remembered that with this type of analysis the errors are cumulative. When a sample contains five nuclides, the amount present of the nuclide calculated first (^{95}Nb) is most accurate and that calculated last (^{125}I) is least accurate, since the relevant region of interest has undergone stripping four times.

Computer programs are available for calculating the amounts of nuclide responsible for each of many overlapping peaks.[59-61] Therefore, selection of a nuclide need not necessarily be curtailed by its peak overlapping that of another nuclide.

Coincidence Loss

Once the appropriate regions of interest have been selected, coincidence loss is determined. This procedure involves controlling all variables except increases in count rate. Geometry, one of the most influential variables, must be strictly controlled. Another important aspect of this determination is the energy range counted. With our equipment, coincidence loss is critical only when preparing the injection vials where the count rate range is normally 10^6-10^7 and occasionally as great as 2.5×10^7. The total number of counts in the range 0-900 keV is measured in each nuclide sample. When only the peak area of a particular nuclide is counted, the coincidence loss is very different, thus emphasizing that the energy range counted as well as the peak energy of a particular nuclide influences this determination. As a result, coincidence loss should be determined over the same energy range as is used for the samples to which the results of the calibration determinations are to be applied. Nuclide separation at high count rates is difficult and can only be accomplished when spectral distribution percentages are determined at those high count rates and corrections for coincidence loss are made.

The procedure we use was adapted from an unpublished course manual (used at University of California, San Francisco) written by Kenneth G. Scott, Ph.D. It involves the use of three samples counted separately and then simultaneously with count rates ranging from 75,000 to 125,000 counts/sec each. Geometry is controlled by using a special vial designed to hold the samples. A styrofoam adapter is placed in the bottom of a standard counting vial. Three holes are made in the styrofoam adapter equidistant from the center and from each other. The samples of microspheres are placed in Beckman plastic microcentrifuge tubes and centrifuged to pull the microspheres into the point of the tube. The holes and the microcentrifuge tubes are numbered so that whenever they are counted they are counted with the same geometry. For each different nuclide, each of three microcentrifuge tubes is counted separately three times for 1 min in its own adapter hole in the vial. Then all three are counted together for the same length of time (3 times at 1 min each). The following equation is used to calculate the resolving time in seconds. All counts must be converted to counts/sec for all these calculations.

$$t = 3/2 \left[\frac{1 - \left(\frac{M_{123} - 1\ Bg}{M_1 + M_2 + M_3 - 3\ Bg} \right)}{(M_{123} - 1\ Bg)} \right]$$

where M_{123} = all 3 tubes counted together in counts/sec; M_1, M_2, M_3 = each tube, respectively, counted separately in counts/sec; Bg = background count. Depending on the nuclides used, the magnitude of t ranges from 0.3×10^{-6} to 0.6×10^{-6}, with the smallest t related to the lowest energy nuclide (^{125}I).

To calculate the true count rate (R_{true}) for any particular nuclide, the following formula is used:

$$R_{true} = \frac{R\ observed}{1 - (R_{observed} \times t)}$$

where R_{true} = the real count rate in counts/sec; $R_{observed}$ = the observed count rate in counts/sec; t = resolving time factor for a particular nuclide in sec.

Counting Efficiency

The efficiency of gamma counting depends on sample height and volume (nuclide dispersion) as well as sample density and composition.[58] When several different types of samples are counted in a particular experiment, the total counts of each nuclide in each sample type must be adjusted to some norm in order to carry out a meaningful comparison. Since the comparative efficiency relationship is energy-dependent, this must be determined for each nuclide in each geometric configuration. As the efficiency for any nuclide is generally higher when the nuclide-containing tissue is concentrated at the bottom of the counting vial (with a 3-inch crystal, below 1 cm in height), we have chosen to select the settled microsphere configuration as our normal standard. Efficiency alterations that occur by using microspheres dispersed differently in tissue samples are always corrected to this standard configuration when comparing counts in different tissues. Details of tissue preparation techniques and the reasons for selecting different sample configurations have been discussed.

Since the settled microsphere (blood) sample configuration is considered to be the calculation standard or norm, the only information needed for computation is the spectral distribution of percentages (Table 1, Fig. 5) required for separating the various nuclides. For carbonized samples,

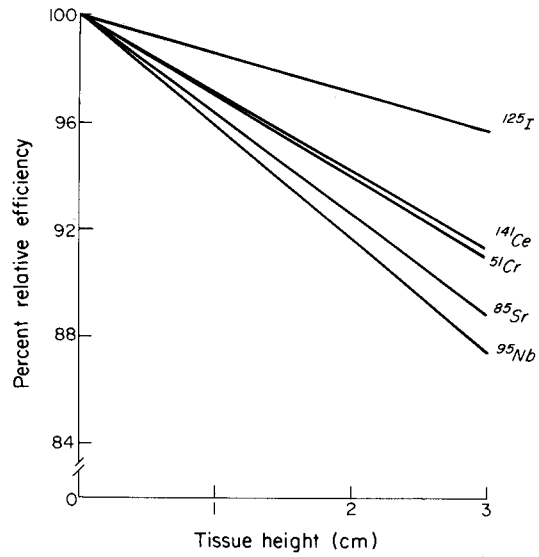

Fig. 6. Counting efficiency in a 3-inch well of each of the five nuclides distributed in carbonized tissue in the counting vial at different heights up to 3 cm (standard height currently used).

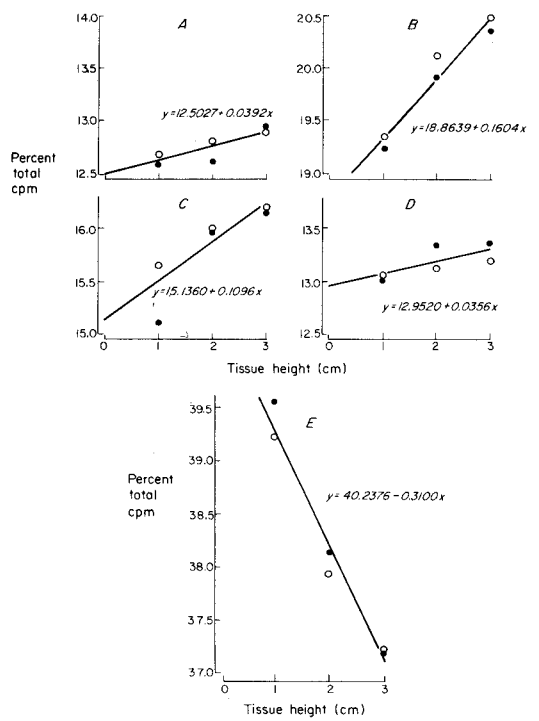

Fig. 7. Changes of ^{95}Nb spectral distribution percentages within each of the five regions of interest A, B, C, D and E (see appendix, Fig. 5) at different tissue heights in the counting vial.

both spectral distribution percentages (Table 2, Fig. 5) and efficiency percentages for each nuclide (Fig. 6) are needed. Efficiency values at a sample height of 3 cm only are calculated and related to the settled microsphere sample values because we have elected to maintain this constant height of carbon samples. Wet organ tissue samples, on the other hand, require a complex set of equations combining both the varying spectral distribution percentages (Fig. 7) and efficiency values (Fig. 6). Varying heights of tissue samples have to be used because it is hard to standardize and evenly pack various sized pieces of tissue from various sized organs. Changes of the spectral distribution of a particular nuclide within the various regions of interest are linearly related to height (weight) of the sample in our present instrument. In a previous instrument, some were linear and some were exponential, demonstrating that the response matrix of each instrument is different and has to be carefully evaluated. A linear relationship to height (weight) exists with respect to changes in efficiency as well. A set of equations for each nuclide, therefore, is needed to relate the wet tissues of varying heights to the settled samples. To calculate heterogenous samples requires complex information about the response matrix of the particular instrument.

Determining the response matrix for the individual gamma counter is critical to the success of the use of the method. Without accurate knowledge of the instrument's response to the specific counting situations in question, accurate calculation of the raw multichannel pulse-height data is impossible. When four or five different nuclides are used, the true counts for each nuclide in each vial must be determined from the complex raw data. When dealing with samples of differing heights and/or different sample types, a large programmable calculator or computer is necessary to carry out the involved calculations.

To determine the response matrix of our instrument as well as the efficiency values, suitable models of the various sample types were developed. For standardizing the wet tissue heights and determining the efficiencies for each nuclide at different tissue sample heights, a 20% gelatin solution was used to approximate the composition of the wet tissue sample. Different tissue heights and count rates could be easily produced and reproduced for each nuclide. The use of the gel also provided a simple way of determining the efficiency relationship between microspheres dispersed and then allowed to settle to the bottom of the vial. The 20% gelatin solution was made using a sterilizing cyanide solution (to eliminate bacterial and fungal growth) as the diluent. After heating to 70°C for thorough solution, the gelatin was cooled to 35°C in preparation for pouring into the counting vials. Microspheres labeled with each of the five nuclides were placed into separate vials and the count rates adjusted to the appropriate levels. Each height and count rate was performed in duplicate to be certain of the thoroughness of the mixing of the microspheres in the gelatin and the reproducibility of the system. When satisfied with the count rates for each sample, gelatin (at 35°C) was added briskly from a syringe to produce the required height in the vial. Small wooden dowels were used to mix the liquid gelatin thoroughly for about a minute, and the vials were then placed in an ice bath to produce rapid gelling, thereby trapping the beads in an evenly dispersed position. Variability in the spectral distribution percentages for a particular sample height indicated poor mixing.

After the initial counts were obtained with the microspheres evenly dispersed, the gels were warmed to 50°C and then centrifuged to pull the microspheres to the bottom of the vial. This duplicates the settled or blood sample configuration and gives a direct comparison between the microspheres in tissue and in blood samples. From this comparison, changes in spectral distribution and efficiency for the two situations can be determined.

All sample counts were done as accurately as possible. Generally, 10^6 total counts per sample was used to achieve a counting error of 0.2% or less. After completing the counting, the data were analyzed for discrepancies. Poor mixing of the microspheres was associated with an unusual spectral distribution for that sample height. When this occurred, samples were rewarmed, remixed, and counted again. Generally, when the initial mixing was thorough, no problems with poor dispersion occurred.

Carbon powder standards were prepared by injecting microspheres labeled with one of each of the five different nuclides used into each of five small animals. After carbonizing as described above, the animals were ground up separately in a blender producing fine granular powder with one

of each of the five nuclides thoroughly mixed with the carbon. Nonradioactive (cold) granular carbon was produced or purchased to be used as powder diluent.

To prepare the carbon standard samples, a small amount of each of the five types of radioactive carbon granules was put into vials with the count rate adjusted by the addition or subtraction of granules until the appropriate level was achieved. The amount used just covered the bottom of the vial and was therefore equivalent to the settled microsphere situation. Nonradioactive carbon was then added to bring the total carbon level up to 3 cm. The vials were shaken well to mix the radioactive and nonradioactive carbon thoroughly. A cotton pledget was used to wipe the top and sides of the vial down to the 3-cm level and then was placed on top of the 3 cm of carbon. All count rates were obtained in duplicate and ranged from 10^3 to 10^6 counts per minute for all samples. This was done to determine what, if any, effect different count rates had on the spectral distribution in the range of counts that we normally encounter. The efficiency of counting carbonized tissue at a height of 3 cm could then be calculated.

From all this data, the following equations are calculated. For each nuclide, geometric configuration and count rate spectral distributions for the five regions of interest are calculated as a percentage of the total counts. Duplicates are averaged and all of the data compiled and analyzed. Plots and regression analyses of the relationship between tissue height and spectral distribution for each nuclide in each region of interest are made for the wet tissue samples. The spectral distribution for the blood (settled) samples and the carbon samples is compiled, each in their own separate table (Tables 1 and 2). Since each of these sample configurations is constant (the former settled at the bottom of the vial, the latter at a height of 3 cm), only one set of percentages is required. Next the totals for each vial counted while dispersed (wet tissue and carbon sample) are averaged and compared to the total counts of the microspheres on the bottom (settled blood sample configuration). Multiplied by 100, this ratio gives the efficiency difference between the microspheres dispersed at various heights and the microspheres on the bottom. This efficiency is linear with respect to height, so for wet tissues, equations are used to make this adjustment. For carbon samples, the 3-cm height value only is used for the efficiency adjustment.

In summary, the spectral distribution percentages as a function of the total are calculated for each type of sample. For blood and carbon samples, since only one sample configuration is used for each, the spectral distribution percents are compiled in a table and entered into the computer in that way (Tables 1 and 2). For blood, the efficiency difference is zero, since this is used as the calculation norm. For carbon, the efficiency difference at 3-cm height is all that is required. For wet tissue samples, since both the spectral distribution and the efficiency difference percentages vary with sample height (weight), a series of equations is used to describe the response of the instrument. All of this information is necessary to convert the raw count data to usable count data for the calculation of blood flows, cardiac outputs, etc.

Sample Counting and Processing

All samples should be counted on the same day or, if not, count loss due to spontaneous decay compensated for. Since the half-life, and therefore the daily proportion of count loss, of all nuclides is known, this is a simple calculation to make. In practice, for the nuclides we use, a 2–3 day difference is insignificant.

The number and spectral distribution of counts in each sample is obtained and stored by whatever process is used. The total number of counts of each nuclide in each sample is then calculated and from these counts the number of microspheres of each nuclide label present in the sample calculated from the specific activity/half-life plot. With this information the reliability of measurements is checked from the number of microspheres present in any single sample or group of samples under investigation.

TECHNIQUE OF INJECTION

The number of microspheres desired for an experiment is determined and the appropriate number of counts per microsphere for that day is obtained from the count per microsphere chart. The multiple dose vial is then well shaken and, if necessary, may be placed in an ultrasonicator for 1 min. With sterile precautions, an appropriate amount is withdrawn into a disposable tuberculin syringe and placed directly into a specially designed

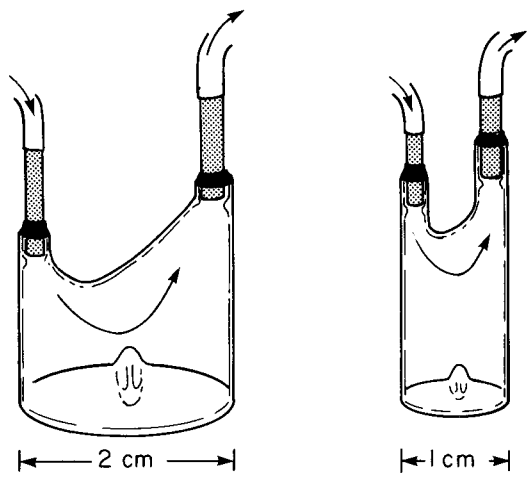

Fig. 8. Diagrammatic illustrations of the glass vials used to inject the microspheres. The size used depends on animal size and hence the number of microspheres to be injected and the flushing volume.

injector vial (Fig. 8). Alternatively, the microspheres are drawn up into a disposable tuberculin syringe attached to a disposable 3-way stopcock. A second syringe is then attached to the third outlet of the stopcock, the stopcock closed to the needle, and the microsphere solution flushed back and forth 10-15 times between the two syringes. In our experience, this is the simplest and most effective method for breaking up aggregations. The microsphere solution is then returned to one of the syringes, the stopcock opened to the needle, and the microspheres placed in the injector vial. We have used this particular type of injector vial in preference to a syringe since, with a syringe, wastage of the rather expensive microspheres is increased and occasionally a glass-barrelled syringe jams during injection. In addition, the injector vials we have designed fit into the counting vials. The geometry of the microspheres counted in the injector vial before and after injection into the circulation is thus as close as possible to the geometry of subsequent blood samples. This obviates a major problem in relating counts in different sample configurations and is generally more accurate than counting the microspheres in a syringe. The injector vial is counted briefly and microspheres added or removed in order to obtain the desired number according to the prior determination. The vial is then filled with 0.1% Tween 80 in saline, leaving only a small air bubble, and the final count obtained after allowing the microspheres to settle. After this count, in preparation for injecting the microspheres into the animal, the vial is placed in a vortex mixer in order to assure that we break up any aggregations. The injector vial is then attached to the relevant catheter and a syringe containing saline attached to the other blunt needle. The vial is inverted so that the flat surface is uppermost, and the baffle in the flat surface then serves to mix the microspheres during injection. The vial is agitated continuously to maintain mixing of the microspheres, which are then injected over 15-20 sec with 5-20 ml saline depending on the size of the animal. A further advantage of this injector vial over a syringe is that a large bolus of microspheres is not delivered rapidly into the intravascular space; rather, a more gradual well mixed solution containing the microsphere is infused. After completing the injection, the vial is detached and the catheter flushed with 2-10 ml heparinized saline. No microspheres have remained in the catheter after this procedure.[17] The residual microspheres are allowed to settle, and the injector vial is counted again in order to calculate the number of counts and thereby the number of microspheres injected.

If calculations are to be made based on the total number of microspheres injected (vial counted before - vial counted after injection), extreme care must be taken that there is no loss of the microsphere solution in attaching to and detaching from the catheter and during counting. In addition, the time difference between counting the injector vial and subsequent counting of tissues should be considered and, if necessary, corrected for.

If the microspheres are injected directly into a cardiac chamber, particularly the left ventricle, it is important to warm the injecting solution, since a sudden cold flush may produce ectopic ventricular beats. It is important to monitor either an electrocardiogram or an arterial pressure pulse during injection to check for the presence of normal sinus rhythm throughout the injection. Ectopic ventricular beats during the microsphere injection may significantly alter the distribution pattern, particularly if coronary blood flow is measured by this technique.

QUANTITATION OF BLOOD FLOW

The proportion of systemic output distributed to any organ can be calculated without actually measuring flow itself by relating the number of

microspheres in that organ to the total number injected. However, in most instances, an estimate of actual flow rates to organs is desired when using this technique. Calculation of flows can be performed in two ways. In the first, cardiac output can be measured by an independent means and an individual organ flow derived from the proportion of microspheres found in that organ related either to the total number of microspheres injected[19,22,54] or the total number of microspheres present in the whole animal.[24] In large animals, or in circumstances where the whole animal is not counted, the total number of microspheres injected is obtained by careful counting of the injector vial before and after injection of the microspheres into the animal. If a syringe is used for the microsphere injection, this too can be counted before and after the injection or a similar volume as was injected into the animal can be counted subsequently as a standard.[25] These latter two techniques are less reliable than the one we use.

$$\text{Organ flow (ml/min)} = \frac{\text{Cardiac output (ml/min)} \times \text{No. of microspheres in organ}}{\text{Total no. of microspheres injected}} \quad (1)$$

The second method requires the measurement of flow to one organ by an independent means.[28] Since the number of microspheres in that organ as well as any other organ are known, flow to any organ can be calculated from the formula:

$$\text{Unknown organ flow (ml/min)} = \frac{\text{Known organ flow (ml/min)} \times \text{No. of microspheres in organ with unknown flow}}{\text{No. of microspheres in organ with known flow}} \quad (2)$$

Cardiac output or systemic blood flow can be calculated either by adding all organ flows together or by using the total number of microspheres injected as the number of microspheres in the organ with unknown flow, thereby calculating the cardiac output as the unknown flow. Several techniques have been used to measure cardiac output independently. The least reliable, the indicator dilution technique using Indocyanine green, was used in many of the earlier studies in monkeys[27,31] and rabbits.[24] Indicator dilution curves have also been used to quantitate the distribution of pulmonary arterial blood flow in dogs.[33] More commonly used to quantitate flow are techniques to measure blood flow to an individual organ by an independent means. In the early studies on the circulation of fetal lambs, blood flow to the placenta was measured by the steady state diffusion method infusing antipyrine into the fetus.[28,62,63] Flow to other organs may be measured by independent techniques; these include the direct collection of venous effluent[33,37] or the use of an electromagnetic flow transducer either around or inserted into a vessel. Currently the most common method used is that of a surrogate organ flow known as the reference sample technique. Before, during, and after injection of the microspheres, blood is collected from an artery at a constant rate. Several different techniques have been described. We use one of two simple methods. In the first the arterial catheter is attached, via a blunt needle, onto a previously weighed heparinized 20-ml glass syringe. The syringe is placed in a Harvard withdrawal pump set generally at a withdrawal rate of between 7 and 10 ml/min. The withdrawal is commenced and after about 5 sec, when it is apparent that blood is being withdrawn without difficulty into the syringe, the microspheres are injected over about a 20-sec period (see above). The withdrawal is continued for a further period and, generally, the total withdrawal time is either 1 or $1\frac{1}{4}$ min. The syringe is then detached and reweighed in order to calculate the volume of blood withdrawn (blood weight/specific gravity of blood). The blood is then transferred carefully into counting vials and the syringe flushed repeatedly with distilled water that both washes out any residual blood and microspheres and also hemolyzes the blood. Three counting vials generally suffice, and after washing we have not detected any residual counts in the syringe, indicating that all microspheres have been washed into the counting vials. The second technique uses a small Holter (model 913) roller pump that withdraws the blood and ejects it directly into counting vials. The flow rate in this technique is between 8 and 12 ml/min, and the blood is collected in 4 vials over a period of 2 min. With both techniques we have counted all tubing and found that at the flow rates used no

microspheres have adhered to the tubing. This may not be true at slower flow rates. We have not found it necessary to employ one of the more cumbersome techniques, such as withdrawing blood under mineral oil directly into a counting vial, as with the two methods used we have not lost any microspheres. The volume of blood withdrawn is not critical as long as more than 400 microspheres are present in the sample.[17] The duration of withdrawal is important, since the microspheres are injected over a 20-sec period and the circulation time may not be known. Using the technique with a roller-pump withdrawal, we have checked 15-30 sec periods for 1-5 min after microsphere injection in order to define the period, invariably no more than 60 sec, after which no microspheres are circulating.[19,37] This procedure should be performed in all studies, particularly after any intervention, in order to be sure that all microspheres have been cleared from the circulation during the period the reference sample is obtained. Flow to any and all organs can now be calculated with ease using the relationship described above (2) where the "known" organ is the reference sample. In order for the reference sample technique to be accurate, the number of microspheres present needs to satisfy the statistics outlined above; furthermore, the concentration of microspheres in the reference sample has to be representative of that perfusing all organs, i.e., mixing has to be adequate. If during preliminary studies it appears that the concentrations are not similar in different arteries, such as in the sheep where the brachiocephalic trunk arises just above the aortic valve and incomplete mixing or streaming occurs in this region, several reference samples can be obtained in order to relate different organs to reference samples with microsphere concentrations closer to those organs. For example, in the sheep, reference samples should be obtained from both an upper and lower artery. In certain circumstances, in studies of the fetal circulation, three reference samples have been obtained.[64] The only limitation to multiple-reference samples is the volume of blood withdrawn, and if this is not large, any number may be used. In animals with a small blood volume, we have made a point of replacing the volume withdrawn with donor blood during the latter half of the withdrawal once the microspheres have been injected. The systemic arterial reference sample technique has been used in many circumstances and many species, including fetal[23,45,53,64] and neonatal[19] lambs, sheep,[17,22,38,52] dogs,[17,19,37,38] monkeys,[26] and newborn[65] and adult[25,46] rabbits, and provides a simple and accurate method of quantitating arterial flow during the injection of microspheres. Hales[21,22] has also used this technique on the venous side to measure cardiac output in adult sheep. The microspheres were injected into a peripheral vein and the reference sample withdrawn from the pulmonary artery.

VALIDATION AND COMPARISON WITH OTHER METHODS OF FLOW MEASUREMENT

Distribution Patterns

Microsphere distribution patterns have been compared directly with those obtained by several other different techniques. In one study, a physical model consisting of four branching tubes was perfused with a rotary pump. Labeled microspheres were injected into the system and the effluent collected from each tube at varying flow rates. The concentration of microspheres in each was very similar, indicating that the distribution pattern of microspheres closely correlated with the actual distribution of flow.[28] In fetal lambs, the proportional distribution of blood flow to the placenta through each umbilical artery was checked by comparing the flows measured by electromagnetic flow transducers with the calculated flows based on microsphere distribution. The correlation between the ratios of flow to each umbilical artery by the two methods was extremely high.[28] In the newborn lamb, renal and femoral venous return were measured by electromagnetic flow transducers and the ratio of femoral to renal flow compared to that measured by the microsphere technique.[24] The ratios obtained by the two methods were similar.

The accuracy of the microsphere method for measuring the distribution of cardiac output, individual organ flows, or the distribution of flow within organs has been tested less directly by comparing diffusable indicators with microspheres. In dogs, Delaney and Grim[6] compared gastric blood flow and its distribution measured by the clearance of radioactive potassium ^{42}K with that measured by injection of ^{24}Na-labeled 16 or 20μ diameter glass microspheres. They first tested the validity of the clearance technique by simultaneous determination of total gastric flow by collecting

venous effluent and injection of ^{42}K. The average difference between the two techniques was 5.4%. The distribution of microspheres was also in close agreement with the partition of ^{42}K. Comparisons of cardiac output distribution, as measured with microspheres and radioactive iodoantipyrine or ^{86}Rb, have shown a high correlation in some tissues but differences in others.[21,47] Since distribution patterns as measured with microspheres are extremely close to those measured more directly with electromagnetic flow transducers or by collection of venous effluent, it is likely that the differences recorded when using diffusable indicators and microspheres are due to inaccuracies in the indicator techniques.

Measurement of Cardiac Output

Measurements of cardiac output calculated from the injection of microspheres have been compared with cardiac output measured by indicator dilution techniques as well as cardiac output maintained artificially with some form of cardiopulmonary bypass using a roller pump. The most accurate comparison can be made using roller-pump outputs, since these are calibrated by timed collections. Measurements of total systemic blood flow during total cardiopulmonary bypass were made with microsphere injection and a reference sample and compared with simultaneous measurements obtained from the calibrated pump on 30 occasions in 15 monkeys.[26] Twenty-six of 30 measurements made with the microspheres were within 15% of the outputs of the calibrated pump (Fig. 9A). Archie et al.[19] compared cardiac output determined by microsphere injections and reference samples with cardiac output provided by a calibrated pump during right heart bypass. Repeated studies were performed in two dogs and one lamb (Fig. 9B). The extremely high correlation between the two techniques is shown, and all determinations by microspheres were within 15% of known flow; 80% of microsphere determinations were within 10% of known flow.

Cardiac output measured with indocyanine green dye has also been compared with that measured by microspheres and a reference sample. The correlation of these two methods is not as high as is that comparing microspheres and roller-pump outputs, since the accuracy of cardiac output measurement by the indicator dilution technique is considerably less. Nevertheless, the comparisons are still quite

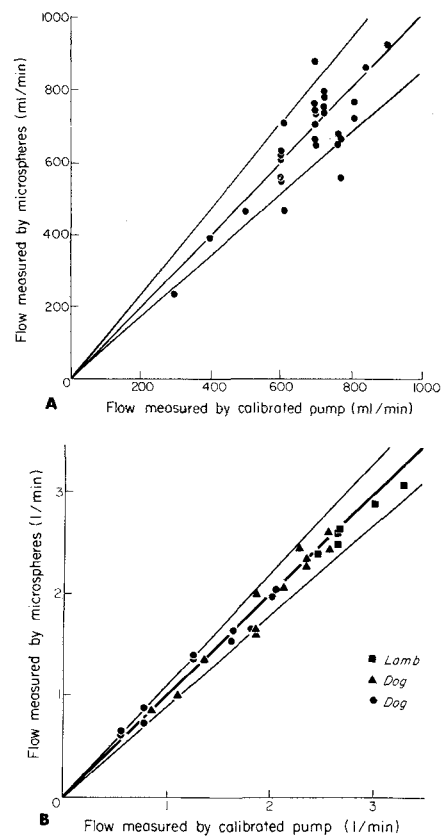

Fig. 9. (A) Comparison of total systemic blood flow measured by the microsphere/reference sample technique with known roller-pump flows during total cardiopulmonary bypass in rhesus monkeys.[26] Line of identity ± 15% is shown. $\hat{y} = 0.987X - 5.42$; $r = 0.845$; $Sy \cdot x = 14.43$. (B) Comparison of cardiac output measured by the microsphere/reference sample technique with known roller-pump flows during right heart bypass.[19] Each symbol represents measurements in one animal. Line of identity ± 10% is shown. $\hat{y} = 0.95X + 0.10$; $r = 0.944$; $Sy \cdot x = 0.11$.

good. In one study in monkeys,[26] 65% of 30 measurements of cardiac output by microspheres were within 20% of those measured by indicator dilution. In a second study,[27] a fairly high correlation was also found. In lambs, cardiac output was determined with microspheres and several minutes later with indocyanine green.[19] Cardiac output was varied in each animal by intravenous infusion of either isoproterenol or dextran. Sixty-two percent of the observations were within 20% of each other. Similar studies have been performed in rabbits in whom cardiac output was altered either by phlebotomy or dextran infusion. Ninety-eight paired observations were made and showed an extremely high correlation.[25]

Cardiac output determined by microspheres has also been compared with that measured using the Fick principle in newborn rabbits. The cardiac output using microspheres was 222 ml/kg/min compared with 230 or 260 ml/kg/min measured in the same laboratory albeit in different studies.[65]

Comparisons of Organ Blood Flows

Electromagnetic flow transducers. Cerebral blood flow was measured in a fetal lamb by implanting cannulating electromagnetic flow transducers in both external jugular veins. The flow calculated from the microsphere injection was 139 ml/min as compared to the measured venous flow of 126 ml/min.[28] In dogs, the right renal artery was cannulated and perfused with an electromagnetic flow transducer in the perfusion line. Twelve comparisons of renal blood flow measurements with the electromagnetic flow transducer and those calculated by the microsphere technique agreed within 6%.[17] In fetal lambs, pulmonary blood flow was measured by an electromagnetic flow transducer around the main pulmonary artery and calculated from microsphere injections. In two instances, pulmonary blood flow was increased significantly above resting level by continuous infusion of acetylcholine into the fetus. As seen in Fig. 10, the differences in flow measured by the two techniques are small.[64] Umbilical placental blood flows measured by an electromagnetic flow transducer around the common umbilical artery and by microspheres also have shown an extremely high correlation.[66]

Direct collection of venous effluent. Coronary blood flow measured by microspheres was compared with direct collection of coronary venous return during right heart bypass.[17,37,38] Blood from both venae cavae was drained into a reservoir and returned into the pulmonary artery by a roller pump. A catheter was introduced into the right ventricular cavity in order to drain coronary venous return from the right heart. Different levels of coronary blood flow were obtained by altering the pump output, by constricting the aorta, or by producing hypoxemia and hypercapnia. During the period of microsphere injection, while the reference sample was obtained, coronary venous return was collected directly into a graduated cylinder. Collected coronary venous return and calculated coronary blood flow, in 27 measurements in 7 dogs, were within 5% of each other in 12, 10% in 19, and 20% in 24.[37] Similar studies were performed in a second group of dogs and a group of sheep.[17] In the dogs, after injection of microspheres into the left atrium, the differences between calculated and measured flows averaged −3.6%. The slopes of the regression lines of each injection did not differ significantly. In the sheep, calculated coronary blood flow after left atrial in-

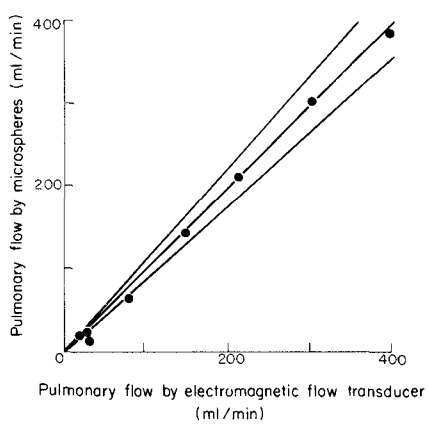

Fig. 10. Comparison of pulmonary blood flow measured by the microsphere/reference sample technique with that measured by a precalibrated electromagnetic flow transducer in fetal lambs. Line of identity ± 10% is shown.

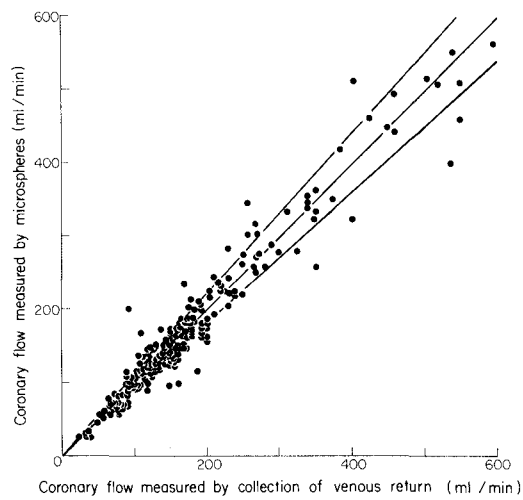

Fig. 11. Comparison of coronary blood flow measured by the microsphere/reference sample technique with that measured by collection of the coronary venous return in dogs and sheep.[38] Line of identity ± 10% is shown. $\hat{y}$ = 1.00446X; $Sy \cdot x$ = 13%.

jection averaged 1.4% less than measured flow. A third and significantly larger group of animals was also studied in this manner.[38] In these, total and regional myocardial blood flow were measured and altered by several different maneuvers. Two-hundred-fifty comparisons during right heart bypass were made (Fig. 11); these also included measurements from the previous two studies.[17,37] Sixty-six percent of the calculated flows were within 10% of measured flows and 90% within 20% of measured flows. In the same report, regional blood flow was evaluated by comparing microsphere distribution with that of antipyrine. When appropriate size microspheres were employed, there were no major differences between the two techniques of measurement of regional flow.

Left lower lobe pulmonary arterial blood flow was measured in two dogs by the microsphere technique and compared with pulmonary venous return from that lobe, collected directly into a graduated cylinder. Total pulmonary blood flow was calculated using indocyanine green dye, and the proportion of microspheres lodging in the left lower lobe related to the total number in the lung to calculate left lower lobe flow. The mean difference between the two measurements was 3%.[33]

Miscellaneous techniques for comparison. Umbilical blood flow calculated from the microsphere technique and measured by the steady-state diffusion method have been compared in fetal lambs. In eight of nine observations, the difference between the two methods was less than 10%.[23] Renal blood flow measured by the microsphere technique agreed closely with values obtained by PAH clearance.[21]

SUMMARY

When appropriately and correctly applied, the microsphere technique is relatively simple and extremely accurate. Distribution patterns, both of total systemic arterial blood flow or venous return as well as within specific organs, can be measured. Several techniques have been applied to quantitate flow using microspheres; the reference sample method is extremely simple and by far the most accurate of all. Collection of venous effluent is perhaps more accurate but requires extensive surgery and is almost certainly the least physiologic. Other methods used for quantitation, such as bolus injections of indocyanine green dye or infusions of diffusable indicators, are considerably less accurate and therefore significantly reduce the reliability of the microsphere technique.

Selection of the appropriate size microspheres allows for definition of arteriovenous anastomoses as well as the measurement of organ blood flows and distribution of blood flow within those organs. In most instances, smaller microspheres (15μ diameter or 8-10μ diameter) have significant advantages over larger ones. They are distributed more like red cells, obstruct less of the vascular bed, are less variable in size, and can be given in significantly greater numbers. This latter point is important, since the statistical criteria need to be satisfied and the use of small spheres allows for the more reliable measurement of blood flow to small organs or to small regions of organs.

APPENDIX

The channel groups or regions of interest (ROI) (Fig. 5) used for the five nuclides ^{125}I, ^{141}Ce, ^{51}Cr, ^{85}Sr, and ^{95}Nb are:

ROI	Channels
A	1-56
B	57-143
C	144-231
D	232-363
E	364-511

Calculations of the total number of counts per minute (cpm) for each of the five nuclides when combined are based on the spectral distribution percentages for carbonized tissues (Table 2).

^{95}Nb–ROI E contains cpm derived only from ^{95}Nb and has fraction 0.3973 of the total ^{95}Nb cpm. Therefore, the cpm in ROI E (C_E) are related to the total ^{95}Nb cpm by

$$C_E = 0.3973\ Nb$$

$$\therefore (1)\quad Nb = 2.5170\ C_E$$

^{85}Sr–Both ^{85}Sr and ^{95}Nb contribute to the cpm in ROI D (C_D). From the fractions of the total cpm for each nuclide in this ROI

$$C_D = 0.1263\ Nb + 0.5294\ Sr$$

$$= (0.1263 \times 2.5170\ C_E) + 0.5294\ Sr$$

$$\therefore 0.5294\ Sr = C_D - 0.3179\ C_E$$

$$\therefore \quad (2)\quad Sr = 1.8889\ C_D - 0.6005\ C_E$$

^{51}Cr–The cpm in ROI C (C_C) are made up of contributions from ^{51}Cr, ^{85}Sr, and ^{95}Nb

$$\therefore \quad C_C = 0.1623\ Nb + 0.0951\ Sr + 0.7142\ Cr$$

$$= (0.1623 \times 2.5170\ C_E) + 0.0951$$

$$\times (1.8889\ C_D - 0.6005\ C_E) + 0.7142\ Cr$$

$$\therefore 0.7142\ Cr = C_C - 0.4085\ C_E - 0.1796\ C_D + 0.0571\ C_E$$

$$= C_C - 0.3514\ C_E - 0.1796\ C_D$$

$$\therefore \quad (3)\quad Cr = 1.4002\ C_C - 0.4920\ C_E - 0.2515\ C_D$$

Similarly for ^{141}Ce and ^{125}I

(4) $Ce = 1.2950\ C_B - 0.3819\ C_E - 0.5084\ C_D - 0.2887\ C_C$

(5) $I = C_A - 0.0427\ C_E - 0.1288\ C_D - 0.116\ C_C - 0.2950\ C_B$

In summary the equations used are:

(1) $Nb = 2.517\ C_E$

(2) $Sr = 1.8889\ C_D - 0.6005\ C_E$

(3) $Cr = 1.4002\ C_C - 0.2515\ C_D - 0.492\ C_E$

(4) $Ce = 1.295\ C_B - 0.2887\ C_C - 0.5084\ C_D - 0.3819\ C_E$

(5) $I = C_A - 0.295\ C_B - 0.1116\ C_C - 0.1288\ C_D - 0.0427\ C_E$

A different technique that can be applied is to calculate the total number of cpm of ^{95}Nb present (step 1) and then, based on the spectral distribution percentages (Table 2), subtract the proportion of that total number of cpm in each of the four lower ROI. This leaves each ROI without any cpm attributable to ^{95}Nb. ROI D now contains cpm of only ^{85}Sr. The total number of cpm of ^{85}Sr can now be calculated from the spectral distribution percentages (Table 2).

(6) Total ^{85}Sr cpm = 1.8889 (cpm ROI D)

The proportion of this total number of cpm of ^{85}Sr in each of the three lower ROI can then be subtracted leaving these three ROI free of both ^{95}Nb and ^{85}Sr counts.

(7) Total ^{51}Cr cpm = 1.4002 (cpm ROI C)

The process is then repeated for ^{141}Ce and ^{125}I.

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