Differential Registration of Two Types of Radionuclides on Macroautoradiograms for Studying Coronary Circulation: Concise Communication

Hitonobu Tomoike, Ikuo Ogata, Yuji Maruoka, Kikuo Sakai, Takeshi Kurozumi, and Motoomi Nakamura

Research Institute of Angiocardiology and Cardiovascular Clinic, Faculty of Medicine, Kyushu University, Fukuoka 812, Japan

Double-radionuclide autoradiography proved to be feasible using combinations of Tc-99m and I-125, or Tc-99m and C-14. Because of the short half-life of Tc-99m (6 hr), we first registered Tc-99m on x-ray film. Given an adequate Tc-99m:I-125 activity ratio of 20:1, the exposure duration for Tc-99m was still too short for I-125 to blacken the x-ray film. The pure emission from C-14 is completely absorbed by a thin aluminum sheet—hence no problem there. After the decay of Tc-99m, therefore, it was entirely feasible to continue autoradiography with I-125 ($T_{1/2}$ = 60.2 days) or C-14 ($T_{1/2}$ = 5730 yr). Based on these conditions, we applied (a) tracer microspheres labeled with I-125 and Tc-99m to define the respective perfusion areas of the left anterior descending, septal, and left circumflex coronary arteries of the beating heart, and (b) Tc-99m pyrophosphate and C-14 antipyrine to demarcate respectively the localization of the infarct-avid substance and the regional blood flow. We verified the first procedure with postmortem angiography and the second with histochemistry.

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A graphic record has the advantage of increasing the topographical sensitivity of an experiment, and leads the way to a better understanding of physiological and/or metabolic evidence in relation to the anatomical findings (*1–4*). Moreover, autoradiography is one technique for determining the localization of radioactive tracers.

We have determined the area at risk in coronary perfusion before occlusion using I-125 microspheres and compared the physiological perfused area with the area of induced myocardial infarction on the same section of the heart (*5*). We have also used autoradiography with Tc-99m pyrophosphate to demarcate the distribution of the tracer in macro and microautoradiograms in case of acute myocardial infarction (*6*). These techniques allow for a high regional resolution of the tracer distributions, but autoradiograms with a single radionuclide limit further analysis of the tracer kinetics. For example, autoradiographical visualization of multiple radionuclides without overlap may not only permit serial or spatial documentation of coronary blood flow, but also provide a technique for analyzing the relationship between coronary blood flow and specific distributions of tracers such as Tc-99m pyrophosphate.

Reported techniques with the double-tracer autoradiogram were based on the physical characteristics of the nuclides, namely, differences in half-life, specific energy, and decay type (*7–10*). However, these techniques have not been applied to the study of coronary circulation. Recently, we were able to differentiate two kinds of radionuclides, making use of differences in emission

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For reprints contact: Hitonobu Tomoike, MD, Research Institute of Angiocardiology and Cardiovascular Clinic, Faculty of Medicine, Kyushu University, Fukuoka 812, Japan.

energies as well as half-life. One pair was I-125 and Tc-99m, and the other was C-14 and Tc-99m. We now describe in detail our current techniques for double-tracer autoradiograms and describe applications to pathophysiological studies of the coronary circulation.

METHODS

I. Principles of the differential registration of two types of radionuclides. Iodine-125 emits Te x-rays of ~27 keV and gamma photons at 35 keV, while Tc-99m emits higher-energy photons at 140 keV. Although both radiations are easily registered on a conventional x-ray film, exposure time adequate for differential autoradiography between Tc-99m and I-125 depends on the radioactivity at the time of measurement. In addition, the half-lives of Tc-99m and I-125 are 6 hr and 60.2 days. Accordingly, the differential registration of Tc-99m and I-125 on x-ray film should be feasible by using an appropriate ratio of the radioactivity between them and changing both the exposure time and the time lag for the exposure. To test this hypothesis, we explored the effects of activity concentration of both nuclides on the grain density of the x-ray film.

Five hundred μCi/ml of pertechnetate (Tc-99m) was diluted with distilled water and concentration ratios of 1:1, :2, :4, :8, :16, and :32 dilution were prepared. A 0.1-ml sample from each of these specific activities was spotted on chromatography paper. Similarly, 72 μCi/ml of I- 125 was diluted in distilled water and the same six specific activities were prepared. Six 0.1-ml aliquots were similarly spotted on other chromatography paper. These spiked papers were dried at room temperature, coated with a thin polyethylene sheet (1.27 μm in thickness) and placed in contact with x-ray film. Starting immediately, some films were left in contact for 2 hr, then removed; others were left for eight days, then removed. After a further lapse of 14 days, contact was resumed with both groups and was maintained for eight more days. The other pair of tracers was C-14 and Tc-99m. The former is a pure beta emitter with a mean energy of 55 keV and a half-life of 5730 yr. Its emitted electons are readily absorbed by a sheet of 17-μm aluminum foil. The 140-keV photons of Tc-99m pass through this foil easily, but the half-life is short (6 hr).

II. Application of double-tracer autoradiography. *I-125 and Tc-99m.* carbonized-plastic tracer microspheres (diameter 15 μm, labeled with I-125) were suspended in 20% dextran with a few drops of Tween 80 and were sonicated with an ultrasonic probe for 5 min before being mixed with arterial blood (*11*). Human serum albumin microspheres (HSAM, diameter 7-25 μm) were labeled with Tc-99m and then shaken mechanically to obtain homogeneous dispersion. Ten milliliters of arterial blood, containing 20 μCi of I-125 microspheres and 20 mCi of Tc-99m HSAM, were then injected over 5 min by an autoperfusion system from the carotid arteries to the left circumflex coronary artery and to both the left anterior descending and septal arteries of anesthetized open-chest dogs to demarcate the respective perfusion areas. The number of both tracer microspheres was 2 million, which resulted in ~40,000 microspheres per gram tissue. After intravenous administration of saturated KCl, the heart was immediately excised, embedded in 10% carboxymethyl cellulose, and frozen in an acetone dry-ice bath (−75°C) for 30 min. The frozen heart was mounted on a stage and sliced with a cryotome to give transverse sections 50 μm thick, as shown in Fig. 1.

The 50-μm samples were then taken up on adhesive tape. For Tc-99m autoradiography the slices were immediately coated with Saran-Wrap, firmly pressed into contact with x-ray film in the cassette, and the contact maintained for 2 hr in a deep-freezer. After 14 days for Tc-99m decay, I-125 autoradiography was similarly carried out, except that 20 days of exposure time were used (Fig. 1).

To determine whether the distribution of tracer microspheres actually represented the perfused areas of respective coronary arteries, in five dogs the autoradiograms were compared with the angiograms of the adjacent ventricular slices. To do this, after the heart was excised, a barium-gelatin mixture was perfused simultaneously into the two main coronary arteries with 100 mm Hg pressure in the autoperfusion system. After the heart was frozen in an acetone/dry-ice bath as described above, the left ventricle was sectioned into three transmural slices of approximately equal thickness (1.0-1.5 cm) parallel to the atrioventricular groove. In each ventricular slice, transverse sections 50 μm thick were obtained with a cryotome, and the remaining slice

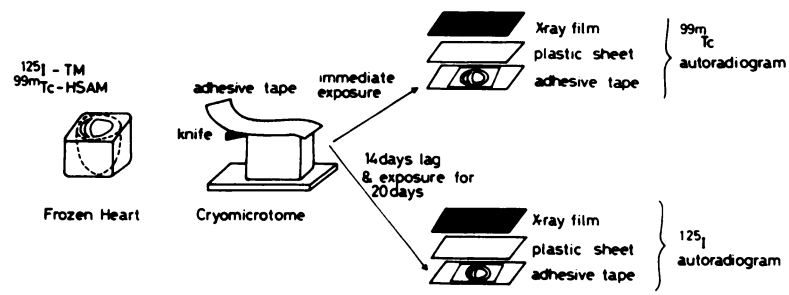

FIG. 1. Procedure for Tc-99m and I-125 autoradiograms. To register Tc-99m, one must store cassette containing sample and x-ray film in deep freezer, since sliced sample disintegrates quickly at room temperature. Plastic sheet used to prevent sample from adhering to x-ray film. Freezer is unnecessary for I-125 autoradiography, since there is considerable dessication during freezing time.

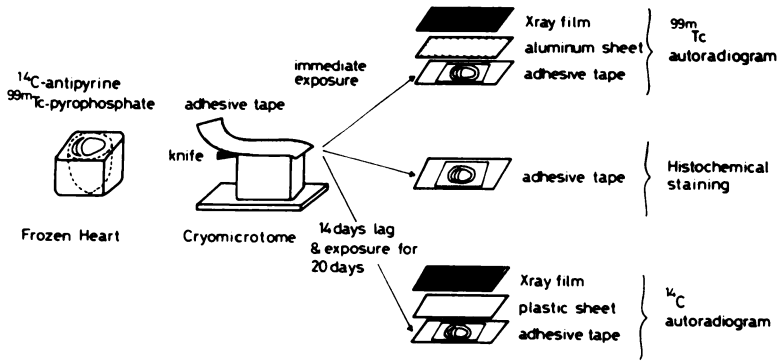

FIG. 2. Procedure used for Tc-99m and C-14 autoradiograms. Details like those in Fig. 1. Specimen should be dried and stored cold for histochemical staining.

(about 1.0 cm in thickness) was used for stereoscopic angiograms, without magnification.

C-14 and Tc-99m. As previously described (*6*), experimental acute myocardial infarction was induced in a canine model by ligating the left anterior descending coronary artery. Forty-eight hours (five dogs) and seven days (two dogs) after the ligation, 20 mCi of Tc-99m tagged to pyrophosphate (following the manufacturer's instruction) was administrated intravenously, and 2 hr later, 500 μCi of C-14 antipyrine was injected into the left atrium. Although the antipyrine technique has been widely applied in the quantitative autoradiographic investigation of regional changes in flow, (*12*), in the present study we limited this technique to determine the area of flow distribution. Ninety seconds after the antipyrine the dog was given an intravenous dose of saturated KCl, the heart was immediately excised, coated with 10% carboxymethyl cellulose, and immersed in the acetone/dry-ice bath (Fig. 2). The embedded heart, which was usually frozen completely within 30 min, was set on the stage and sliced transversely using a cryotome (Fig. 2).

For the autoradiographic registration of Tc-99m pyrophosphate, a sliced specimen was immediately covered with an aluminum sheet and pressed onto the x-ray film. This closely adhering preparation in the x-ray cassette was stored for 2 hr in a deep-freezer to document the localization of Tc-99m. Fourteen days later when Tc-99m decay was complete, a 20-day autoradiogram was made with the C-14 (Fig. 2).

Exposed films were developed, fixed, and rinsed in an automatic film processor. The grain densities of the developed films were measured with a densitometer. Histochemical demonstration of myocardial necrosis was

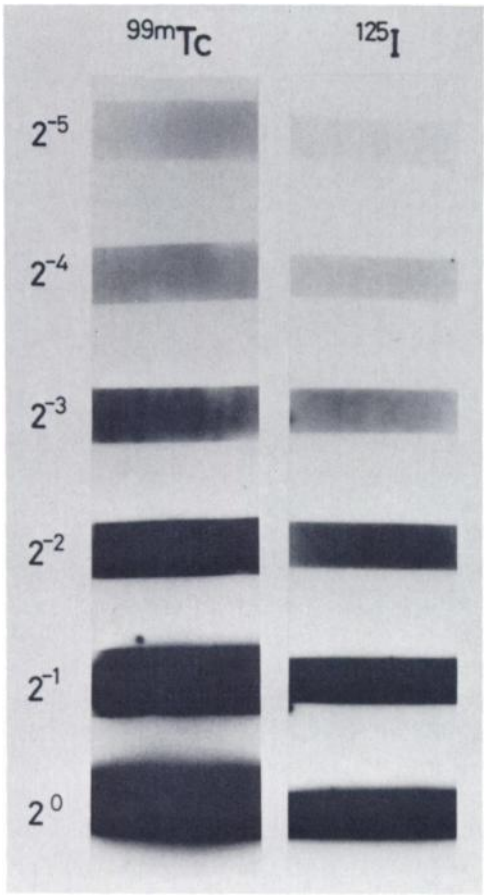

FIG. 3. Activity gradients and difference of exposure time. 2^0–2^{-5} activity concentrations for Tc-99m (15.6–500 μCi/ml) and I-125 (0.45–14.4 μCi/ml) were made on chromatography paper. Left: immediate 2-hr exposure of x-ray film to Tc-99m. Right: eight day exposure to I-125. Note that there is "bloom" in higher-activity strips with Tc-99m (2^{-1} and 2^0). Inhomogeneity seen in Tc-99m strips at 2^{-3} and 2^{-4} may be due to inconsistencies in spacing between specimen and film.

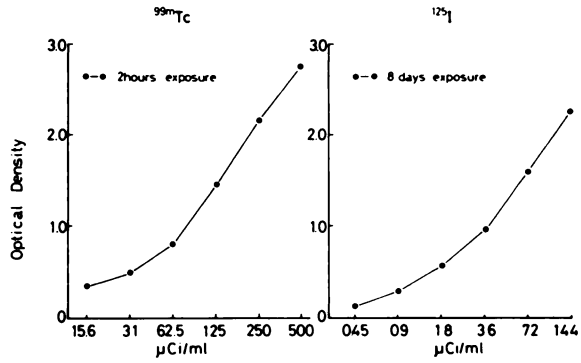

FIG. 4. Optical density (by densitometer) against activity concentration. Left: average optical density with Tc-99m, exposed for first 2 hr. Right: average optical density at eight days of I-125 exposure. Note exponential relation between radioactivity and optical density.

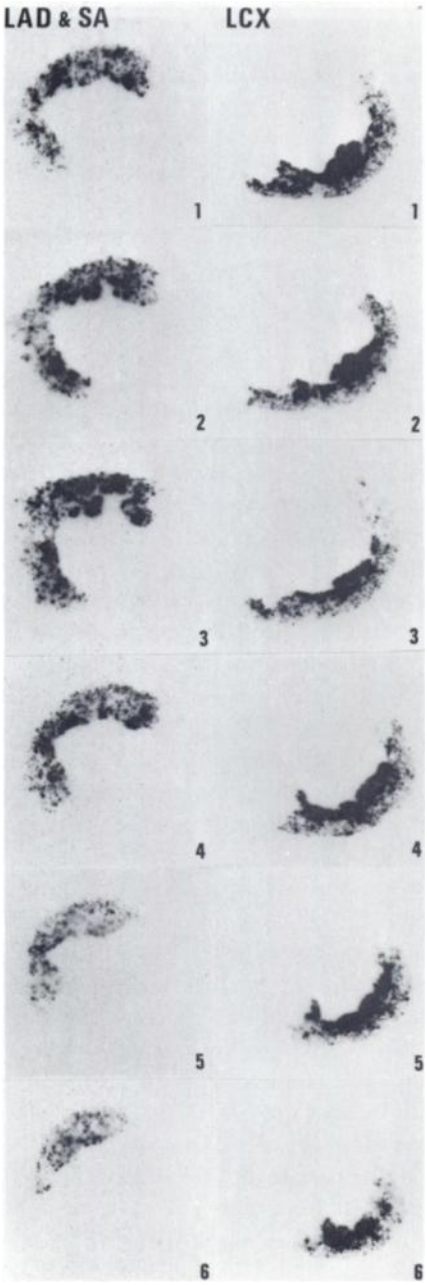

FIG. 5. Autoradiograms showing differential registration of blood supply through LCX as well as LAD + SA from base (1) to apex (6). LCX = left circumflex coronary artery, LAD = left anterior descending coronary artery, SA = septal artery. Each spot probably represents individual microsphere.

performed with nitroblue tetrazolium (NBT) staining as described previously (*5,6*).

Each perfused area was traced manually by three independent observers: the increased focus of activity of Tc-99m pyrophosphate, the decreased focus of activity of C-14 antipyrine, and the infarcted area documented by NBT staining. The sizes of these areas were then measured with a planimeter. The average area for the three observers was used as representative of the respective samples. Least-squares techniques were applied

FIG. 6. (a,b) Autoradiograms of coronary perfusion from left circumflex coronary artery (a) and both left anterior descending and septal coronaries (b). In a, microspheres labeled with I-125 were injected into left circumflex coronary artery; in b, Tc-99m labeled HSAM was injected into left anterior descending and septal coronary arteries. (c) Angiogram of ventricular slice adjacent to sections in (a) and (b). Border areas were dotted in by observing x-ray films in a stereoscope. LV = left ventricle.

to calculate the interrelationship between areas subjected to different techniques.

RESULTS

The developed films of the activity gradients are shown in Fig. 3. Figure 4 plots average optimal density against radioactive concentration based on the data of Fig. 3. When the concentrations were 500 μCi/ml for Tc-99m and 72 mCi/ml for I-125, an adequate exposure was obtained in the first 2 hr with Tc-99m, and in eight days with I-125. No autoradiographic blackening was detectable after 2 hr with I-125, nor even with eight days of exposure to Tc-99m after a time lag of 14 days. The minimum dose of I-125 to give grains on the x-ray film was 0.045 μCi with eight days exposure time. There was an exponential relation between dose and grain density from 62.5 to 500 μC/ml with Tc-99m and from 0.45 to 14.4 μCi/ml with I-125 (Fig. 4). The Tc-99m to I-125 activity ratio for comparable blackening was about 20:1 (Fig. 4). There is considerable "bloom" in strips with higher Tc-99m radioactivity (Fig. 3). With C-14 and Tc-99m the separation of the effects is easy because of the extreme difference in the half-lives and the fact that the beta particles from C-14 are easily stopped by thin aluminum foil, whereas the Tc-99m gammas are not.

Figure 5 shows the distribution of the left circumflex

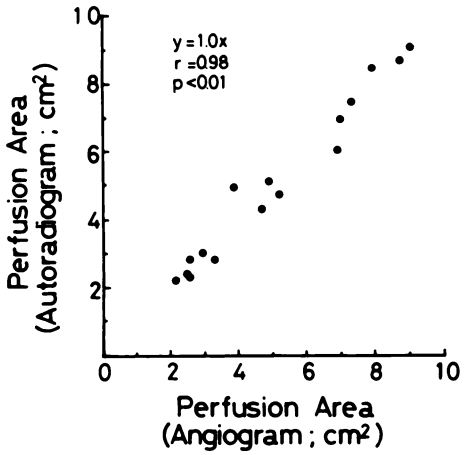

FIG. 7. Perfusion areas of 16 sample sets from five dogs, determined by angiogram (abscissa) and autoradiogram (ordinate). Note consistent linear correlation in normally perfused heart.

coronary artery with I-125-labeled plastic carbonized microspheres and that of the left anterior descending and septal arteries with Tc-99m-labeled HSAM. The numbering 1 to 6 is from base to apex. The boundary between the perfusion areas was sharply demarcated and the doubly perfused zone was all but negligible.

Figure 6 shows the representative distribution of two kinds of radiotracers on the 50-μm sections (a + b), and an angiogram (c) of the adjacent ventricular slice. Areas determined in the in-situ beating heart by an autoradiographic technique correlated closely with those measured at autopsy by angiography (Fig. 7).

Figure 8 is a graphic demonstration of C-14-antipyrine, NBT staining, and the localization of Tc-99m pyrophosphate at 48 hr after coronary ligation. The perfused area documented by C-14 antipyrine coincided well with the negatively stained area seen with histochemistry. Technetium-99m pyrophosphate localized preferentially around the periphery of the infarcted area and the distribution was rather clustered. In Fig. 8(c), 7 days after coronary occlusion, there are distinct spots of Tc-99m pyrophosphate darkening. Both the underperfused area seen with C-14 antipyrine and the increased perfused area of Tc-99m pyrophosphate correlated closely with the infarct size as determined by NBT staining (Fig. 9).

DISCUSSION

Double-tracer autoradiography is feasible utilizing the physical differences between two radionuclides. Autoradiographic double-tracer techniques are now used to evaluate the interrelation among brain function, blood flow, and metabolism in the same brain section (*12,13*). Tracer hairs used in these studies were I-123 and C-14, or I-131 and C-14. Two autoradiograms were registered using the different half-lives of two tracers. The present

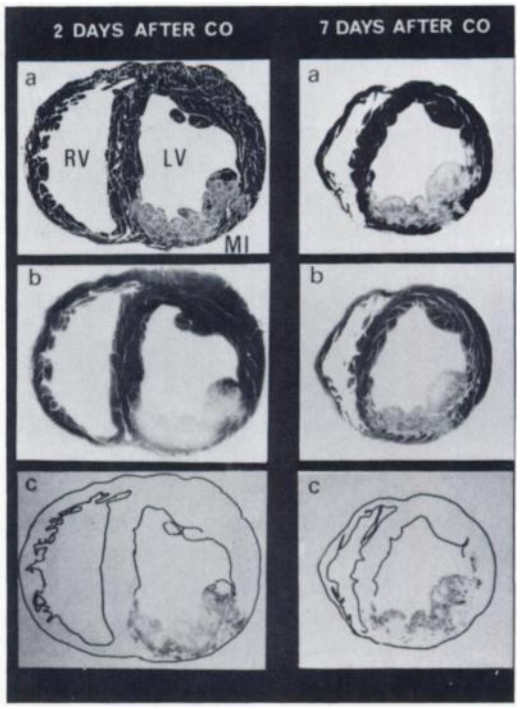

FIG. 8. Autoradiographic and histochemical findings at two and seven days after induced myocardial infarction in dog. From top, NBT staining (a), C-14 antipyrine distribution (b) and Tc-99m pyrophosphate localization (c). RV = right ventricle, LV = left ventricle, MI = myocardial infarction. Note tiny distinct spots of Tc-99m pyrophosphate in c at seven days after coronary occlusion. These spots might be due to microcalcification, since they coincide well with histochemical localization of calcium by von Kossa staining (unpublished observation).

method was based on the same principle, but the details of the technique were quite different.

In general, the lower the emission energy of the applied tracer, the greater is the blackening of the x-ray film. Therefore, to register Tc-99m radioactivity on film without contamination from I-125, a greater activity of Tc-99m is required. An adequate exposure time for the registration of Tc-99m is even then too short for a com-

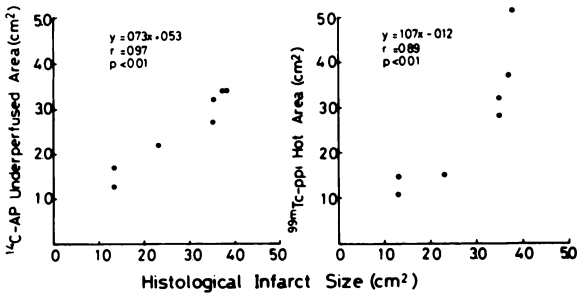

FIG. 9. Correlation between histological infarct size in seven dogs, determined by NBT staining and underperfused area (left panel) or Tc-99m PPi increased area of activity (right panel). Each point was obtained from representative section in each dog. AP = antipyrine, PPi = pyrophosphate.

parable image of the I-125 radioactivity. Although the calculated activity ratio of Tc-99m to I-125 for appropriate differentiation was about 20:1, we used 1000:1 activity to overcome the rapid loss of Tc-99m radioactivity during preparation of the slices.

In case of a paired administration of Tc-99m and C-14, the beta emission from the carbon was completely absorbed by thin aluminum foil. Accordingly, it is possible to register Tc-99m on an x-ray film without any interference from C-14. In both sets of tracers a 14-day waiting period is sufficient for the complete decay of the 6-hr Tc-99m. An I-125 or C-14 autoradiogram can thus be obtained readily with no need to protect the film from Tc-99m irradiation, which is the advantage of the present technique.

Resolution of the autoradiography depends on the energy level of the tracers as well as the thickness of the sample. We used transverse heart sections 50 μm thick, and the specimen and film were firmly pressed together, thus minimizing scattering effects and improving the spatial resolution. With I-125 and C-14 the resolution is considered suitable for microscopic analysis, but in the Tc-99m autoradiography the resolution is not sharp due to the higher energy with its increased scattering. In the present study there was no appreciable difference between Tc-99m and I-125 in the quality of the macroautoradiograms. In a previous report (*6*), we showed the microautoradiogram obtained with Tc-99m, and this also supports the highly regional resolution of Tc-99m on the autoradiogram.

Autoradiography can yield several different maps, depending on the tracers applied. Consequently, it permits area-to-area comparisons with anatomical and pathological findings. In the application of I-125 and Tc-99m, tracer microspheres were used, and because of the nondiffusible characteristics, once the tracer was trapped, the location of microspheres did not change appreciably (*11*). In addition, we verified the rationality of this autoradiographic method by comparing the in-vivo perfused area with a conventional postmortem angiogram. Therefore, repeated determinations of the perfused area by the tracer distribution may also be feasible.

In the case of C-14 and Tc-99m, we examined topographically the relationship among regional flow distribution, Tc-99m pyrophosphate uptake, and infarcted area at 48 hr and seven days after coronary ligation. Regional uptake of Tc-99m pyrophosphate corresponded with the distribution of blood flow and myocardial necrosis (Fig. 9). Thus, the usefulness of Tc-99m pyrophosphate in demarcating the infarcted area was confirmed graphically. Despite the presence of a rather homogeneous flow reduction and myocardial necrosis, Tc-99m pyrophosphate accumulated preferentially around the periphery of the necrosis. These findings demonstrate the active process of Tc-99m pyrophosphate accumulation coupled with the level of myocardial necrosis as well as the degree of myocardial blood flow.

Although we showed only two applications of double-tracer autoradiography, there is no doubt that this procedure has great potential for application, not only in studying the pathophysiology of the coronary circulation but also in providing a reference for current graphic recordings such as echocardiography, CT scanning, and imaging by nuclear magnetic resonance. Both microspheres and antipyrine proved useful in demarcating the area of tracer distribution, but a further refinement of the technique is necessary to quantitate the absolute amount of regional flow, as related to quantitative autoradiography.

This technique is also economical. An expensive gamma counter or a biochemical laboratory need not be set up. We obtained various maps concerning anatomy, physiology, metabolism, and pathology using multitracer autoradiography and histochemistry. For acquisition of quantitative information, the thickness of the specimen must be uniform and a high-precision cryotome must be used.

This technique was also applicable to microautoradiography using a cryomicrotome, as described previously (*6*). Technetium-99m, I-125, and C-14 are popular isotopes and can be used for tracer experiments, so the present technique may prove to have wide application, not only for studies of coronary circulation but for others as well.

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