

Mechanism of Localization of ^{99m}Tc -Labeled Pyrophosphate and Tetracycline in Infarcted Myocardium

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The gross and subcellular localizations of ^{99m}Tc -labeled pyrophosphate and tetracycline in myocardial infarcts were studied in a rabbit model. Experiments utilizing double-nuclide labeling were carried out using a useful mapping technique. Concentration of the various chelates decreases in an expected manner from the center of the infarcted area toward its periphery, but it is higher near the epicardial surface than toward the endocardium. Technetium-99m-pyrophosphate is concentrated in the same infarcted areas as ^{45}Ca ion or ^{32}P -pyrophosphate, but to a much greater degree. The uptake is dependent on both the degree of necrosis and residual blood flow. Gel filtration experiments with rabbit serum indicate that ^{99m}Tc -tagged pyrophosphate, tetracycline, and diphosphonate are mainly protein-bound, whereas ^{32}P -pyrophosphate is not. Subcellular localization studies show that ^{99m}Tc -tetracycline and ^{99m}Tc -pyrophosphate are bound primarily to soluble protein, and only a small fraction is associated with nuclei, mitochondria, and microsomes. The uptake of technetium chelates in myocardial infarcts may be due to the formation of polynuclear complexes with denatured macromolecules rather than to the deposition of calcium in mitochondria.

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Although several chelates of ^{99m}Tc have been used for imaging acute myocardial infarcts (1-3), the mechanism of localization of these chelates is poorly understood. The electron-microscopic work of Shen and Jennings (4) and others (5) suggested the formation of calcium phosphate complexes, possibly at the hydroxyapatite in mitochondria of infarcted myocardial cells. Thus, the bone-scanning agents were thought to localize by surface adsorption to calcium phosphate deposited in the mitochondria of dead cells.

Utilizing a rabbit model of myocardial infarction, we first localized the area with maximum ^{99m}Tc concentration by means of a mapping technique. Then, using double labeling, we investigated the mechanism of localization in subcellular fractions. We observed nonspecific localization in nuclei, mitochondria, and cytoplasm. This study confirms our previous observation with a tissue-culture model of cellular ne-

crosis (6,7) and recent results with other radiopharmaceuticals (8,9).

MATERIALS AND METHODS

Agents used in this investigation were stannous pyrophosphate (Mallinckrodt/Nuclear), Sn-HEDP (Proctor & Gamble), ^{45}Ca , $\text{Na}_4^{32}\text{P}_2\text{O}_7$, and ^3H -tetracycline (New England Nuclear). Fifteen rabbits (New Zealand albinos weighing 2-3 kg) were anesthetized with sodium pentobarbital. The heart was exposed through a left thoracic incision and the left anterior descending coronary artery was ligated either proximally or distally, depending upon the infarct size desired. The incision was immediately closed

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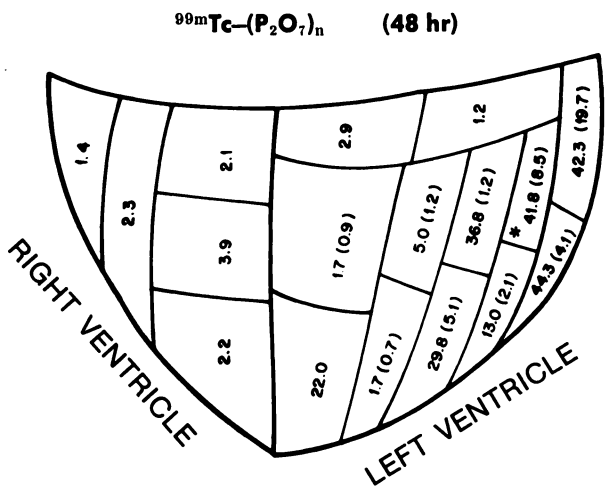
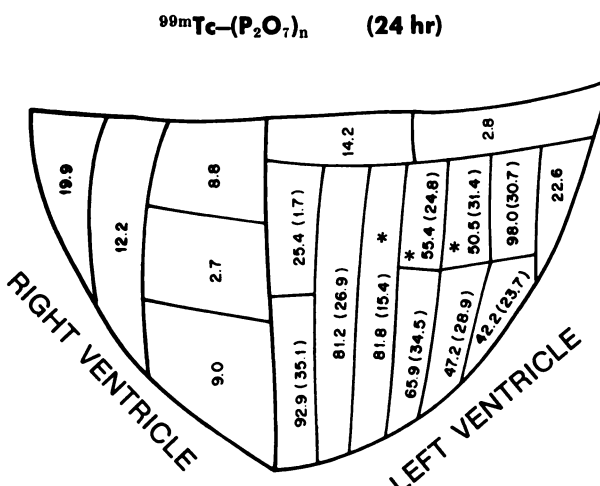
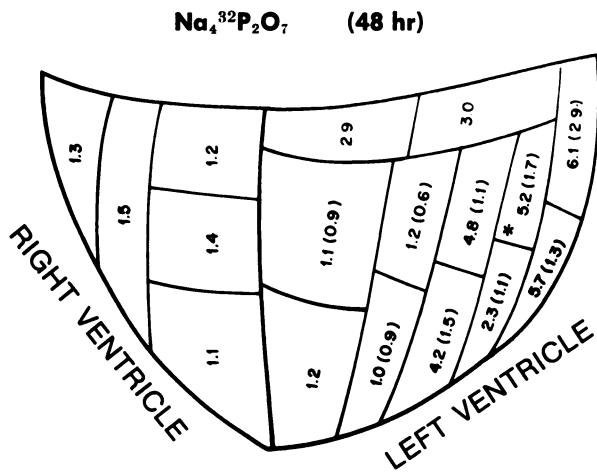
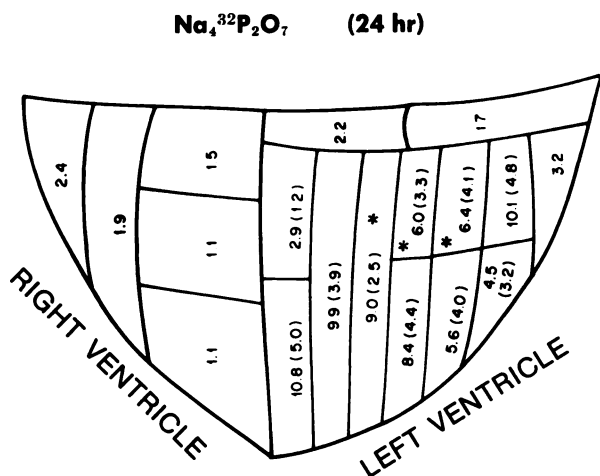


FIG. 1. Relative activity distribution of ^{99m}Tc -pyrophosphate (bottom) and ^{32}P -pyrophosphate (top) in ventricular segments with respect to central septal activity (24 hr after infarct and 3 hr after injection).

FIG. 2. Relative activity distribution of ^{99m}Tc -pyrophosphate (bottom) and ^{32}P -pyrophosphate (top) in ventricular segments with respect to central septal activity (48 hr after infarct and 3 hr after injection).

and the animal was allowed to recover. At time intervals varying from 3 hr to 14 days after ligation, the animals were injected with various combinations of ^{45}Ca , ^{32}P -pyrophosphate, ^{99m}Tc -pyrophosphate, ^{99m}Tc -tetracycline, and ^3H -tetracycline. Usually one gamma- and one beta-emitter were selected for simultaneous injection. For mapping studies, the animals were killed at 3–4 hr after injection, and the heart was immediately transferred to cold isotonic saline solution, kept on ice bath, and washed free of blood and clots. The heart was mapped, and the samples were cut out, numbered, and weighed. Samples of normal myocardium, lung, liver, kidney, bone, cartilage, diaphragm, abdominal and leg muscle, blood, and fat were also obtained. The 140-keV gamma photons of ^{99m}Tc and the 512-keV gamma photons of ^{85}Sr were measured in a gamma well detector. Beta activities from tritium, ^{32}P , and ^{45}Ca

were determined in a liquid-scintillation counter (Packard), the samples being dissolved in ten times their weight of Protosol (New England Nuclear) by overnight incubation at 50°C and then diluted with 10 ml of liquid-scintillation mixture (Aquasol, New England Nuclear). The activity per milligram of tissue was determined after dark adaptation, and the ratios of ventricular segments with respect to average activity in the septum was determined for beta and gamma photons. Similar myocardial mappings were performed in three sham-operated animals.

For subcellular distribution studies, the rabbits were killed 18 hr after injection. Normal and infarcted tissue were carefully separated, and each was homogenized with a mixture of 0.25 M sucrose and 0.25 M KCl solution in a Potter–Elvehjem homogenizer. The nuclear, mitochondrial, microsomal, and soluble-protein fractions were separated by differ-

ential centrifugation in a Beckman ultracentrifuge, and the distributions of gamma and beta activities were determined as above.

To study the interaction of ^{99m}Tc -chelates with myocardial tissue, fresh noninfarcted papillary muscle of rabbit was incubated in Ringer's bicarbonate buffer solution, with the chelates added, for various periods at 37°C and then aerated with 95% oxygen and 5% carbon dioxide in a metabolic shaker. The tissues were removed from the medium, washed free of supernatant activity with fresh buffer solution, and the activities per milligram of wet and dry tissue were determined.

The dialyzing tubes (cellulose, average flat width 0.39 in.) were washed with distilled water and heated for 30 min at 75°C to remove impurities in the membranes. The tubes were then filled to a constant volume of 6 ml with a mixture of 100 μCi of the radiopharmaceutical in saline and 250 mg of human serum albumin in saline. They were then dialyzed for 36 hr against 5 liters of isotonic saline in a cold room with an Oxford dialyzer. After dialysis, the radioactivities of the contents, cellulose, and dialyzing media were determined and the percentage of material retained was calculated from the standard activity.

For gel filtration with Sephadex (medium) G-50, the column was carefully washed and loaded with a known amount of rabbit serum containing the tracers. The column was washed with isotonic saline solution and the activities in different fractions and protein contents were determined with a gamma well, a beta counter, or an ultraviolet spectrometer.

RESULTS

A good correlation was obtained between the site of the infarct and the uptake of the various radiopharmaceuticals. In sham-operated animals, no differences in ventricular concentrations of radioactivity were found, although there was increased concentration in the surgical wound. When the heart was needled without arterial ligation, a localized rise of calcium, ^{32}P -pyrophosphate, and ^{99m}Tc activity could be produced. After ligation of the anterior descending coronary artery, the region of increased radioisotope concentration could be related to the level of ligation, with the largest infarcts produced by proximal ligation. The widest areas of infarction were produced by serial ligation of several coronary arteries (Fig. 1: the asterisks indicate sites of coronary artery ligation). Of greatest importance was the difference in ratios between the outer and inner layers of myocardium in regions of infarction. The outer layer had as much as a tenfold increase in radioactivity relative to the inner layer. In most

previous studies, a lower ratio of infarcted to normal myocardium was obtained, since the sample contained normal as well as infarcted tissue. In our study we have carved out the hemorrhagic or pale outer layer of the infarct from the normal tissue near the endocardium. In this way a more meaningful myocardial mapping was obtained. The numbers in Figs. 1–4 indicate the ratio of activity in each sample block with respect to the activity in the normal septal tissue. The numbers without parentheses show the infarcted-to-normal-myocardium ratio in the outer layer, and those within parentheses show the ratio for the inner layer.

The myocardial mappings of ^{99m}Tc -labeled pyrophosphate and tetracycline, along with ^{32}P -pyrophosphate, ^{45}Ca , and ^3H -tetracycline, were made 3 hr to 10 days after ligation and 3–4 hr after intravenous injection. The results are shown in Figs. 1–4, with the time after ligation indicated. The histograms in

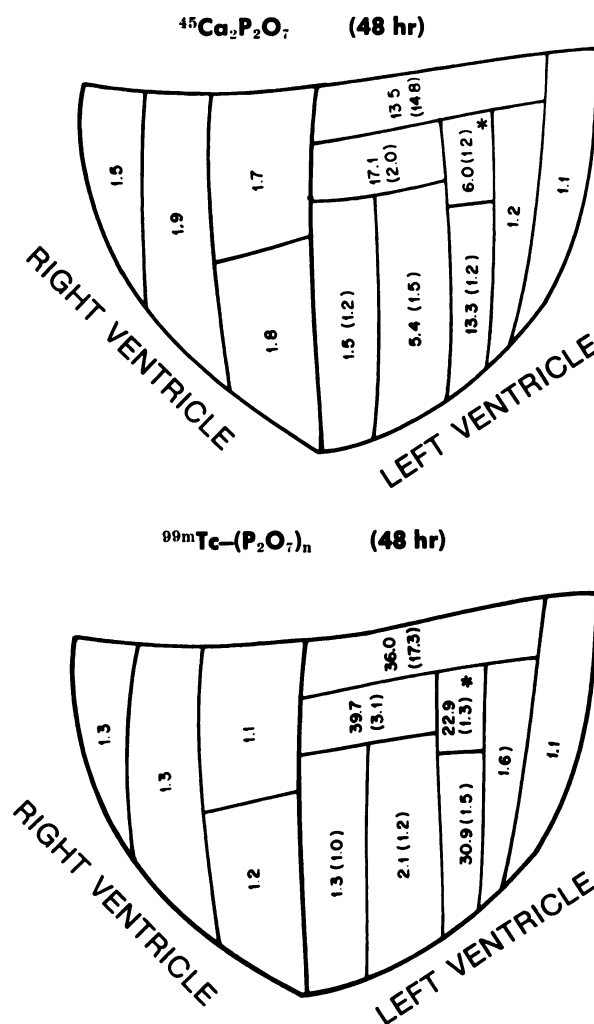


FIG. 3. Relative activity distribution of ^{99m}Tc -pyrophosphate (bottom) and ^{45}Ca (top) in ventricular segments with respect to central septal activity (48 hr after infarct and 3 hr after injection).

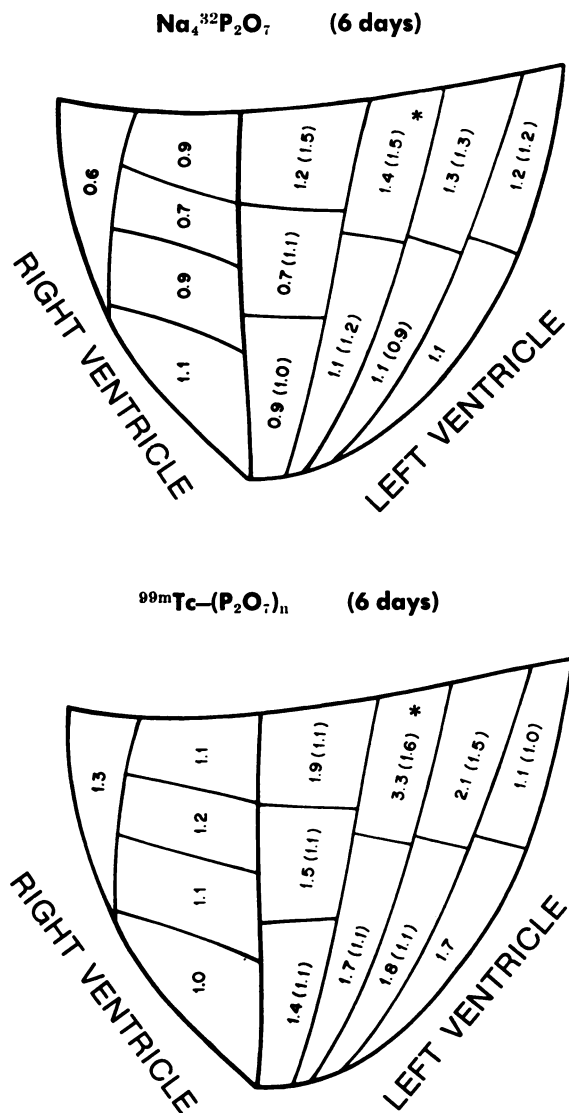


FIG. 4. Relative activity distribution of ^{99m}Tc-pyrophosphate (bottom) and ³²P-pyrophosphate (top) in ventricular segments with respect to central septal activity (6 days after infarct and 3 hr after injection).

Figs. 5 and 6 indicate the infarct-to-septum ratios for the above-mentioned tracers at 24, 48, 72, 144, and 168 hr after ligation. The left-hand column indicates the ratio in the outer layer, and the right-hand column the ratio for the inner layer. The ratio for ^{99m}Tc is 3–4 times that for ⁴⁵Ca, and the ratio for ⁴⁵Ca is 2–3 times that for ³²P-pyrophosphate. The corresponding ⁴⁵Ca and ³²P activities were normalized with respect to ^{99m}Tc activity. The localizations of ^{99m}Tc chelates, ⁴⁵Ca, and ³²P-pyrophosphate reach maximum values at 48 and 72 hr and continue until the necrotic cells are replaced by fibroblasts; a ratio close to unity is restored for all the tracers after 7 days. Similar results were obtained with ^{99m}Tc-tetracycline, ⁴⁵Ca, and ³H-tetracycline except that the ra-

tio of ^{99m}Tc to ³H was 2–3, rather than the value of 6 obtained for ^{99m}Tc-pyrophosphate and ³²P-pyrophosphate.

Tables 1 and 2 show the subcellular distribution of ³²P, ⁴⁵Ca, and ^{99m}Tc-pyrophosphate in normal and infarcted myocardium. Note that only a small fraction of radioactivity is associated with nuclei, mitochondria, and microsomes, whereas a large fraction is associated with soluble protein for both the normal and infarcted tissue. In each fraction of infarcted tissue the amount of activity per fraction is higher than that in the normal tissue. Similar values (Table 3) were obtained for ^{99m}Tc- and ³H-tetracycline.

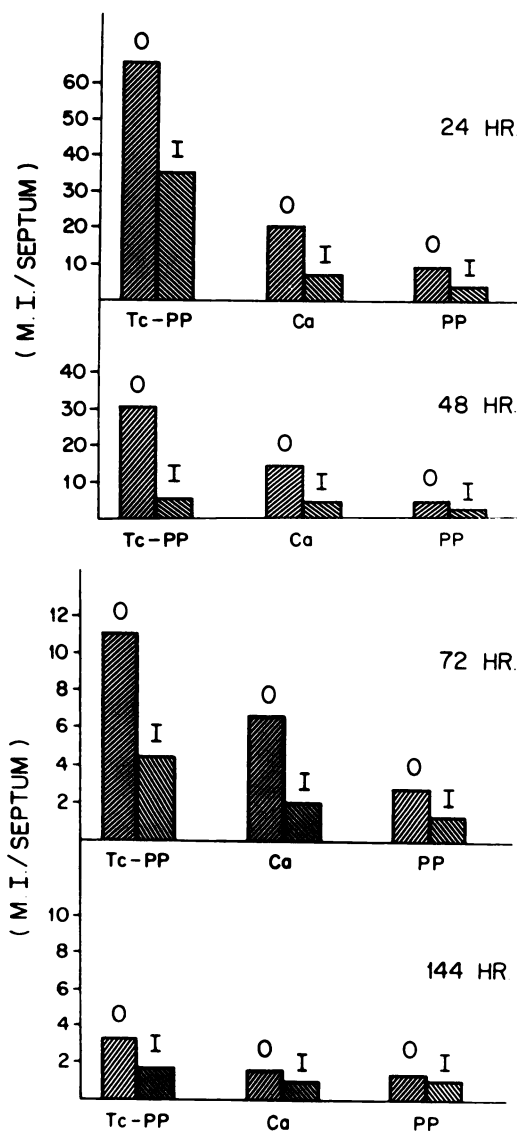


FIG. 5. Uptake ratios between damaged ventricular wall and normal interventricular septum, for both inner (I) and outer (O) portions of wall, in neighborhood of coronary artery ligation. Times after ligation are indicated. Tracers are ^{99m}Tc-pyrophosphate (Tc-PP), ⁴⁵CaP₂O₇ (Ca), and Na₄³²P₂O₇ (PP).

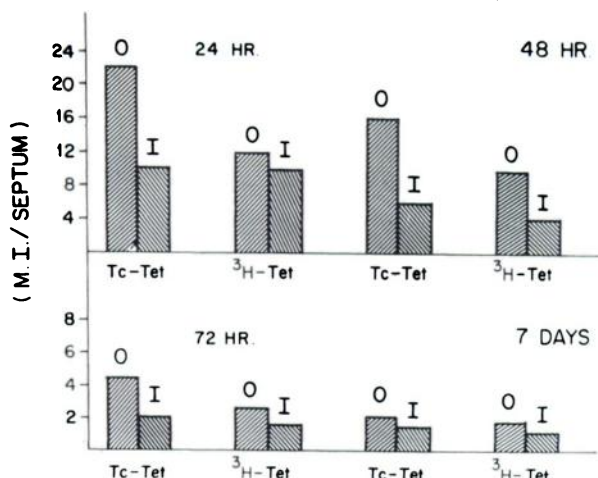


FIG. 6. Infarct-to-normal uptake ratios as in Fig. 5, but with tetracycline tracers labeled with technetium (Tc-Tet) and tritium (³H-Tet). Inner and outer portions of ventricular wall are indicated, as are times since ligation of coronary artery.

The localization of ^{99m}Tc-chelate in normal papillary muscle is dependent on concentration and time. The uptake reaches a maximum value after 1 hr of incubation in the metabolic shaker (Fig. 7). Similar results were obtained when live cells were incubated with ^{99m}Tc chelates in Eagle's minimum essential medium (6,7). Thus, localization of ^{99m}Tc chelates in living tissue must be a diffusion-dependent phenomenon. On the other hand, the localization in infarcted tissue depends both on blood flow and irreversible binding with damaged tissue components.

To understand the binding of ^{99m}Tc chelates with serum proteins, we have performed gel filtration and dialysis experiments. The elution patterns of ^{99m}Tc-pyrophosphate, compared with ⁴⁵Ca and ³²P-pyrophosphate, are shown in Figs. 8 and 9, respectively. The ^{99m}Tc activity curve could be superimposed on the curve for serum protein. Here also we observe that ^{99m}Tc chelation enhances protein-binding affin-

TABLE 1. SUBCELLULAR DISTRIBUTIONS OF ³²P-PYROPHOSPHATE AND ^{99m}Tc-PYROPHOSPHATE IN NORMAL AND INFARCTED MYOCARDIUM

	Normal myocardium		Infarcted myocardium	
	³² P (%)	^{99m} Tc (%)	³² P (%)	^{99m} Tc (%)
Nuclei	5.6 ± 0.6	14.4 ± 1.4	6.5 ± 0.7	19.5 ± 2.0
Mitochondria	6.9 ± 0.7	16.5 ± 1.7	5.5 ± 0.6	21.4 ± 2.1
Microsome	2.9 ± 0.3	5.2 ± 0.5	2.8 ± 0.3	5.3 ± 0.5
Soluble proteins	84.5 ± 8.5	63.9 ± 6.4	85.2 ± 8.5	53.9 ± 5.4

TABLE 2. SUBCELLULAR DISTRIBUTIONS OF ⁴⁵Ca₂P₂O₇ AND ^{99m}Tc-(P₂O₇)_n IN NORMAL AND INFARCTED MYOCARDIUM

	Normal myocardium		Infarcted myocardium	
	⁴⁵ Ca (%)	^{99m} Tc (%)	⁴⁵ Ca (%)	^{99m} Tc (%)
Nuclei	11.20 ± 1.1	15.0 ± 1.5	10.5 ± 1.2	15.8 ± 1.6
Mitochondria	18.3 ± 1.8	17.8 ± 1.8	18.2 ± 1.7	16.5 ± 1.7
Microsome	7.0 ± 0.7	8.0 ± 0.8	10.3 ± 1.0	6.0 ± 0.6
Soluble proteins	63.5 ± 6.4	59.2 ± 5.9	61.0 ± 6.1	62.6 ± 6.2

TABLE 3. SUBCELLULAR DISTRIBUTIONS OF ³H-TETRACYCLINE AND ^{99m}Tc-(TETRACYCLINE)_n IN NORMAL AND INFARCTED MYOCARDIUM

	Normal myocardium		Infarcted myocardium	
	^{99m} Tc (%)	³ H (%)	^{99m} Tc (%)	³ H (%)
Nuclei	25.0 ± 2.5	23.1 ± 2.3	24.6 ± 2.5	14.6 ± 1.5
Mitochondria	21.8 ± 2.2	26.7 ± 2.7	24.1 ± 2.4	18.8 ± 1.9
Microsome	5.9 ± 0.6	8.2 ± 0.8	8.3 ± 0.8	12.9 ± 1.3
Soluble proteins	47.3 ± 4.7	43.0 ± 0.4	43.0 ± 4.3	53.7 ± 5.4

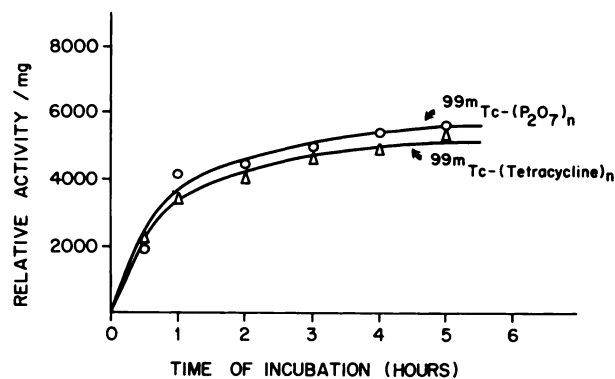


FIG. 7. Time dependence of uptake of ^{99m}Tc -pyrophosphate and ^{99m}Tc -tetracycline in fresh papillary muscle of rabbit.

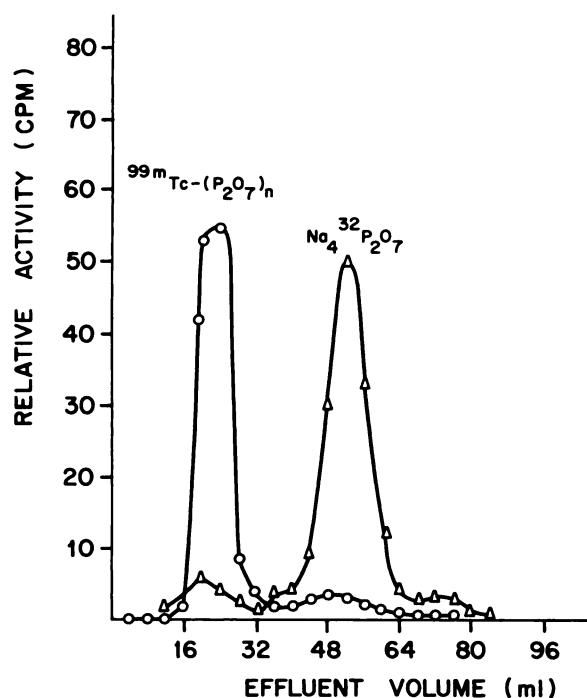


FIG. 8. Sephadex gel filtration of serum of rabbit injected 3 hr earlier with ^{99m}Tc -pyrophosphate and ^{32}P -pyrophosphate. Column (G-50, 1.6×24 cm) is washed with isotonic saline solution.

ity. Only a small fraction of the ^{99m}Tc activity is not bound to serum protein. On the other hand, most of the ^{32}P -pyrophosphate appears to be free in serum. Similar types of elution curves were obtained for ^{99m}Tc -pyrophosphate and ^{45}Ca ion when most of the calcium ion was free in the serum.

A similar protein-binding tendency was observed in the dialysis experiment, where ^{99m}Tc -pyrophosphate and ^{99m}Tc -DTPA were mixed with pure human serum albumin and were dialyzed against isotonic saline in cellophane tubes in the cold room. Table 4 indicates that 50–60% of ^{99m}Tc -pyrophosphate was retained inside the cellulose tube containing human

serum albumin after 24 hr of dialysis, where only 3–7% of ^{99m}Tc -DTPA and ^{99m}Tc -pyrophosphate in the control were retained in the absence of human serum albumin. Since ^{99m}Tc -DTPA does not bind intensely with serum protein, it was used as a standard. The rest of the activity was in the dialyzing medium. In the absence of albumin, the cellulose membrane showed some tendency to bind a small fraction of ^{99m}Tc chelates.

DISCUSSION

These studies confirm the intense concentration of ^{99m}Tc -pyrophosphate in experimental myocardial infarcts. The rabbit model appears to be suitable for studying the problem, and the mapping technique shows the expected gradients of activity. Other studies have shown that both infarction and residual blood flow are needed in order to obtain the maximum concentration of the various scanning agents. While this study was not specifically designed to evaluate this problem, it was noted that the maximum concentration of radionuclide activity was not at the center of the infarct, where blood supply is presumed to be markedly limited. Nonetheless, in this particular model, even at the center of the infarct a very useful infarct-to-normal-muscle activity ratio was achieved. The differences between the activity in the inner and outer layers of myocardium are striking, probably because of the lesser degrees

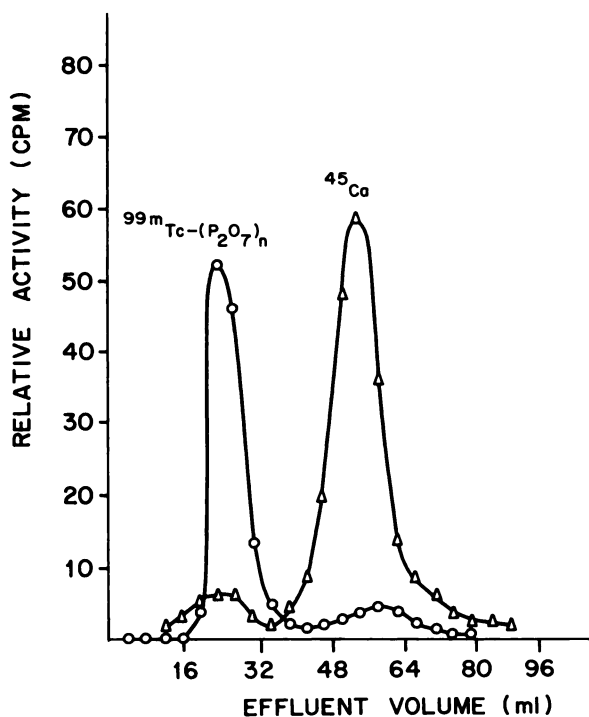


FIG. 9. Sephadex gel filtration of serum of rabbit injected 3 hr earlier with ^{99m}Tc -pyrophosphate and ^{45}Ca . Column (G-50, 1.6×24 cm) is washed with isotonic saline solution.

TABLE 4. DIALYSIS OF ^{99m}Tc -DTPA AND ^{99m}Tc -(PYROPHOSPHATE) $_n$ IN PRESENCE OF HUMAN SERUM ALBUMIN

	^{99m}Tc -DTPA (%)	^{99m}Tc -pyrophosphate (%)
Control:		
Retentate	2.7 ± 0.1	3.2 ± 0.1
Container	5.7 ± 0.9	7.1 ± 0.85
Albumin:		
Retentate	21.7 ± 1.1	55.7 ± 5.4
Container	2.8 ± 0.1	6.9 ± 1.0

of infarction produced in the inner layers of the heart due to better blood flow. The need for mapping gradients in this direction as well as along the surface of the heart is well demonstrated by these experiments.

Our studies with ^{99m}Tc chelates and the chelating molecules indicate that chelation of metal ions enhances the protein-binding affinity as well as the localization in an infarct. We also observed that certain metal ions (e.g., Ca^{+2} , Sr^{+2} , Hg^{+2} , and lanthanides) accumulate in the infarcted tissue; this is also true for pyrophosphate anion. It is still uncertain whether these cations or anions form metal chelates at once with the chelating molecules in the blood pool, and only later localize in the infarcted tissue. The binding of sodium diphosphonate, pyrophosphate, or polymethylenephosphonate with the calcium ion of hydroxyapatite has been investigated extensively (10–13). The binding of divalent metal ions Ca^{+2} , Mg^{+2} , etc., with serum protein (14) is also a well-known phenomenon. Recently Kaye et al (15) and Garcia (16) showed that ^{99m}Tc -labeled bone-scanning agents bind not only with hydroxyapatite, but also with organic matrix (collagen, etc.) in bone.

The subcellular distribution studies of the ^{99m}Tc chelates and other tracers show that most of the tracers are associated with serum protein. This agrees very well with the results of the gel filtration and dialysis experiments. Subcellular distribution studies with ^{99m}Tc -HEDP-labeled dead cells indicate that most of the ^{99m}Tc activity is associated with the soluble-protein fraction. The nonspecific binding of ^{99m}Tc -pyrophosphate with fibrinogen, albumin, and α , β , and γ globulin was observed by Krishnamurthy et al (17). Our gel filtration experiment indicates that ^{32}P -pyrophosphate does not bind with the serum protein; only when pyrophosphate is chelated with reduced technetium ion does protein binding occur. That most of the ^{99m}Tc -pyrophosphate activity is protein-bound is indicated by elution in the early fraction with serum albumin. Similar results were obtained from our dialysis experiments, in

which the cellulose membrane retains molecules larger than 12,000 daltons. In dialysis experiments, where ^{99m}Tc -pyrophosphate was dialyzed in the presence of human serum albumin, serum protein binding increased the molecular weight of the ^{99m}Tc -pyrophosphate-albumin complex, leading to its longer retention in the cellulose tube. Similar results were obtained with ^{99m}Tc -diphosphonate and ^{99m}Tc -tetracycline, but the protein binding of ^{99m}Tc -DTPA is lower than that of ^{99m}Tc -pyrophosphate. We thus observe a correlation between protein binding capacity and localization of ^{99m}Tc chelate in infarcted myocardium. The early removal of ^{99m}Tc -pyrophosphate from the blood pool (by skeletal uptake and renal excretion) makes it the best infarct-imaging agent, whereas other ^{99m}Tc chelates are removed only slowly by the kidney and liver.

Denatured macromolecules bind with many divalent metal ions and a host of metal chelates to form polynuclear complexes. We suggest this as a possible mechanism for the localization of metal chelates of ^{99m}Tc , ^{113m}In , etc., in dead cells in myocardial infarcts, facilitated by membrane damage. Figure 10 suggests how the strength of the bond between the ^{99m}Tc chelate and macromolecules depends on the number of receptor sites on the macromolecule, the orientation of the ^{99m}Tc chelate during attachment, and the number of available chelating atoms in the ^{99m}Tc chelate. The flexibility of denatured macromolecules helps them further in fitting complete octahedral ^{99m}Tc complexes.

Although the concept of hydroxyapatite formation in the mitochondria of dying myocardial cells provides an attractive theory for the concentration of bone-scanning agents, our subcellular distribution experiments did not substantiate this theory. They suggested, rather, that some other type of binding of technetium chelates to macromolecules might be the major factor in myocardial infarct imaging with bone-scanning agents. This would explain the relatively low mitochondrial concentration of technetium activity and the much lower concentrations of ^{32}P -pyrophosphate and ^{45}Ca ion in the infarcted myocardium. It would also explain why other technetium

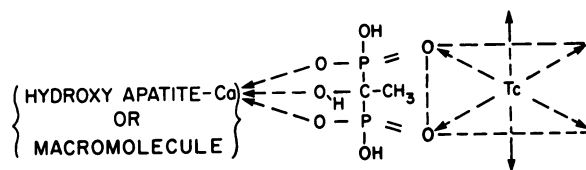


FIG. 10. Possible mode of binding of ^{99m}Tc -HEDP to calcium ion on hydroxyapatite crystal or macromolecule. Arrows indicate site of bond formation. Complete possible octahedral structure of ^{99m}Tc -HEDP is not shown.

chelates, such as ^{99m}Tc -labeled tetracycline and glucoheptonate (1,3), have some ability to concentrate in myocardial infarcts although they are in no way considered bone-scanning agents.

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