Subcellular Distribution and Analysis of Technetium-99m-MIBI in Isolated Perfused Rat Hearts

Paulo A. Carvalho, Mary L. Chiu, James F. Kronauge, Mieko Kawamura, Alun G. Jones, B. Leonard Holman, and David Piwnica-Worms

Department of Radiology, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts

To address the apparent discrepancy between cultured cells and whole heart preparations, Langendorff-perfused rat hearts loaded with hexakis (2-methoxyisobutyl isonitrile) technetium (I) (^{99m}Tc-MIBI) were fractionated by a standard differential centrifugation method and fractional contents of ^{99m}Tc-MIBI were correlated with the mitochondrial marker, malate dehydrogenase (MDH), and mitochondrial substrates. The "cytosolic" fraction nominally contained 89% ± 3% of total ^{99m}Tc-MIBI, but also contained 91% ± 1% of total MDH activity by this method. Chromatographic analysis of activity in the "cytosolic" fraction demonstrated >95% of the agent was present as the original free cationic complex; binding to a small molecular weight cytosolic protein was not involved in localization. Addition of the mitochondrial uncoupler CCCP (5 μM) to both "mitochondrial" and "cell fragment" pellets released up to 84% ± 8% of ^{99m}Tc-MIBI content and addition of the mitochondrial substrate succinate (10 μM) in the presence of rotenone (1 μM) enhanced ^{99m}Tc-MIBI content by up to 139% ± 52% over the control. These correlative data from rat hearts indicate that approximately 90% of ^{99m}Tc-MIBI activity in vivo is associated with mitochondria in an energy-dependent manner as a free cationic complex, but migrates during fractionation/centrifugation.

1992; 33:1516–1521

Hexakis (2-methoxyisobutyl isonitrile) technetium (I) (^{99m}Tc-MIBI) is a myocardial perfusion imaging agent recently approved by the Food and Drug Administration that exploits the favorable scintigraphic properties of ^{99m}Tc (*1,2*). During initial investigations of the myocardial uptake mechanisms and subcellular distribution of ^{99m}Tc-MIBI in guinea pig heart slices, data suggested that this modestly lipophilic cationic agent passively diffused across the myocellular membrane and localized in the cytosolic fraction (*3,4*). Of the total recoverable activity using standard differential centrifugation techniques, 84% was associated with the cytosolic fraction (*4*). By use of similar techniques applied to newborn chick hearts, technetium complexes of the 2-carbomethoxy isopropyl isonitrile ligand (^{99m}Tc-CPI), another modestly lipophilic cationic agent, were found predominantly in the cell membrane fraction (61%) and cytosolic fraction (25%) (*5*). As expected, other highly lipophilic cationic alkylisonitrile ^{99m}Tc complexes were reported to be predominately associated with the membrane fraction in cultured rat heart cells or red blood cells (*6*). These previous studies were, however, limited by the lack of correlative functional or enzymatic markers to characterize the tissue fractions for cross contamination of cellular constituents or impurities.

Recently, studies using cultured chick heart cells (*7*), mouse fibroblasts (*8*) and tumor cell lines in vitro (*9*) have indicated that the fundamental cellular mechanism of uptake and retention of ^{99m}Tc-MIBI involves nonmediated diffusion across both plasma and mitochondrial membranes in response to large negative transmembrane potentials, thereby favoring mitochondrial localization of this agent at steady-state loading levels (*7,8*). Concurrently, little activity was found to be nonspecifically associated with the cell membrane based on pharmacological interventions that depolarize mitochondrial and plasma-membrane potentials (*7,8*). The nonflow-dependent mechanism of cellular distribution of ^{99m}Tc-MIBI would therefore require a three-component compartmental model (extracellular space, cytosol, mitochondrial inner matrix) exchanging in series in the myocytes, as opposed to a two-component model implied in the earlier reports with guinea pig hearts (*3,4*).

The purpose of this study was to reinvestigate the subcellular distribution and chemical integrity of ^{99m}Tc-MIBI in a whole heart preparation, thereby testing the models developed with cultured avian heart cells in a mammalian system. A mitochondrial inner matrix enzyme, malate dehydrogenase (*10*), and mitochondrial substrates were correlated with ^{99m}Tc-MIBI content in subcellular fractions to correct for any cross-contamination inherent in the methodology. Chromatographic and ultrafiltration tech-

Received Dec. 16, 1991; revision accepted Mar. 25, 1992.

For reprints contact: David Piwnica-Worms, MD, PhD, Department of Radiology, Brigham and Women's Hospital, 75 Francis St., Boston, MA 02115.

niques were also employed to test for possible binding to a soluble protein or metabolism of the parent compound.

METHODS

Experimental Solutions

Isolated hearts were perfused with modified Krebs-Henseleit buffer containing the following (m*M*): 118 NaCl; 5.4 KCl; 0.8 $MgSO_4$; 0.8 NaH_2PO_4; 1.2 $CaCl_2$; 26 $NaHCO_3$; 5.6 dextrose; 4.0 HEPES. This buffer was continuously bubbled with 95% O_2/5% CO_2 to achieve pH 7.4 ± 0.05 and the temperature maintained at 37°C in a water bath. Radionuclide infusion was performed by adding 1.5–3.3 mCi ^{99m}Tc-MIBI to 250 ml of buffer for prolonged net uptake protocols or by injecting a bolus of 0.8–3.0 mCi ^{99m}Tc-MIBI into a port in the perfusion apparatus placed immediately before the aortic cannula.

Fractionation Buffer I contained the following (m*M*): 230 mannitol; 70 sucrose; 3 MOPS; 2 EGTA; 0.2% bovine serum albumin (BSA) (w/v); pH 7.2 titrated with KOH. Fractionation Buffer II contained (m*M*): 110 KCl; 20 MOPS; 46 mannitol; 14 sucrose; 2 EGTA; pH 7.5 titrated with KOH.

Synthesis of radiolabeled ^{99m}Tc-MIBI was performed using a one-step kit formulation (provided by E.I. Dupont, Medical Products Division, North Billerica, MA) containing stannous chloride (0.075 mg) as a reducing agent for the technetium and MIBI as the $Cu(MIBI)_4BF_4$ salt. Kits were reconstituted, purified and tested for radiochemical purity by previously described methods (*7*).

Isolated Perfused Heart Preparation

Male or female Sprague-Dawley rats were anesthetized in a diethyl ether chamber. After deep anesthesia was achieved, the heart was removed through a rapid parasternal thoracotomy and immediately placed in ice cold (4°C) Krebs-Henseleit buffer containing heparin (~100 units). The heart was rapidly cleaned of connective tissue, and the aorta was identified and connected to the water-jacketed perfusion apparatus by a cannula for retrograde perfusion. All hearts were initially perfused for 10 min with Krebs-Henseleit buffer (37°C) to achieve mechanical stabilization. Buffer was infused at either constant flow (Master flex pump, Cole-Palmer Instrument Co., Chicago, IL) or constant pressure (~80 cm water) as identified in the Results. Two experimental perfusion protocols were employed: either perfusion for 17 min prior to a bolus injection of 0.8–3.0 mCi ^{99m}Tc-MIBI followed by an additional 3 min of perfusion or perfusion for 20 min with buffer containing 6–14 μCi/ml ^{99m}Tc-MIBI at a rate of 2.5–7.0 ml/min. All experiments were performed with approval of the Harvard Medical Area Standing Committee on Animals in compliance with the position of the American Heart Association on research animal use.

Tissue Fractionation

After perfusion, the hearts were placed in Buffer I at 4°C, further trimmed of connective tissue, minced into 1–2-mm pieces and homogenized with three to four strokes using a Potter-Elvehjem tissue grinder before centrifugation for 10 min at 500× g and 4°C. The supernatant was discarded and the pellet resuspended in 5 ml of Buffer II (4°C) for further homogenization in the presence of 0.5 mg/ml of protease (nagarse, Sigma Chemical Co., St. Louis, Mo). After 3 min of digestion, the protease reaction was inhibited using 10 ml of Buffer II containing 0.2% BSA on ice. Large tissue fragments were filtered and the filtrate was centrifuged for 5 min at 5000× g (4°C), yielding "supernatant component I" (Fig. 1). The pellet was resuspended in 10 ml of Buffer II with BSA on ice and centrifuged for 10 min at 500× g and then 3000× g (4°C) to obtain the "membrane fragment component" and "mitochondrial component," respectively, with the resulting supernatant termed "supernatant component II" (Fig. 1).

To determine the effect of centrifugation speed on separation of fractions, ^{99m}Tc-MIBI was added to the post-protease fraction obtained by the technique described above, except that the hearts were perfused with isotope-free Krebs-Henseleit and the post-protease fraction was centrifuged for 5 min at 4°C at either 15,000× g or 500× g.

Assays, Markers and Inhibitors

Malate dehydrogenase, a soluble inner matrix mitochondrial enzyme, was used as a marker to determine the distribution and leakage of mitochondrial contents into various fractions. A standard enzymatic fluorometric assay of the oxidation of NADH by oxaloacetate (*10*) was utilized to measure malate dehydrogenase activity (Kontron Instruments SFM 25, Zurich, Switzerland; excitation 340 nm, emission 460 nm).

To correlate net accumulation of ^{99m}Tc-MIBI in various fractions to the metabolic status of intact mitochondria, substrates specific for mitochondrial respiration were employed. Following protease inhibition and filtration, filtrate was divided to yield

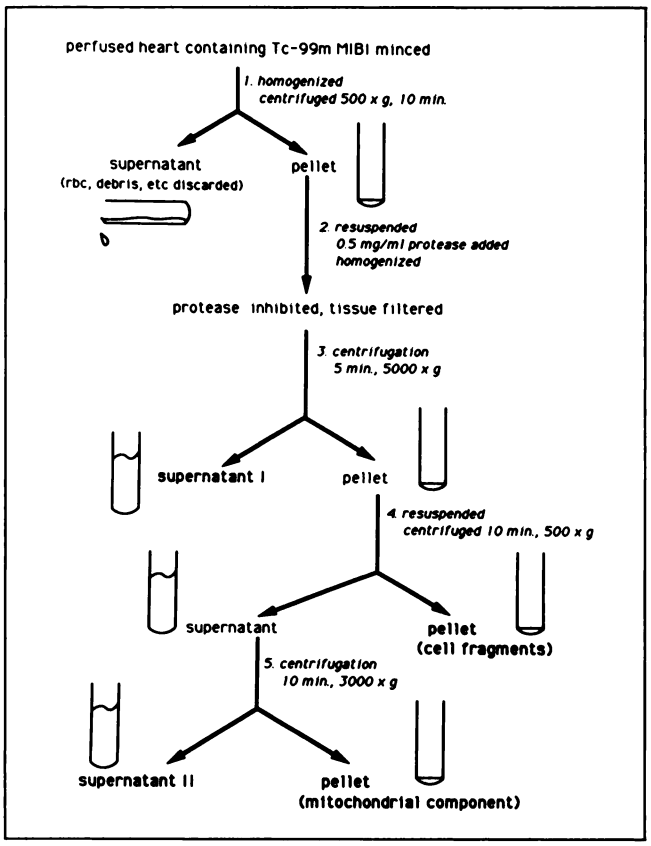

FIGURE 1. Schematic diagram of the differential centrifugation procedure applied to Langendorff-perfused rat hearts containing ^{99m}Tc-MIBI.

"control" or "treatment" samples. To the treatment sample was added 10 m*M* succinate, a substrate specific for Site II of the electron transfer chain, and 1 μM rotenone, an inhibitor of Site I (*7*). Samples were incubated at room temperature for 10 min followed by centrifugation for 5 min at 5000× g (4°C). The effect of mitochondrial substrate addition to fractions in which ^{99m}Tc-MIBI was added in vitro was also studied: here, hearts were perfused with isotope-free Krebs-Henseleit buffer for 20 min and then fractionated as described, but with the radiolabel being added after the protease inhibition and filtration step. Samples were again divided into control and treatment groups, incubated 10 min at room temperature with or without substrate and then centrifuged for 5 min at 5000× g (4°C). To define intact mitochondria that could be depolarized, 5 μM carbonylcyanide m-chlorophenylhydrazone (CCCP), an uncoupler of oxidative phosphorylation (*7*), was added to fractions as indicated. All reagents were purchased from Sigma Chemical Co. and dissolved in dimethylsulfoxide (DMSO) prior to addition to buffers. Final DMSO concentration was typically <0.25%, a level that has been shown to have no effect on cellular kinetics of ^{99m}Tc-MIBI (*7*).

Fractions were weighed in tared centrifuge tubes when required and then all samples were assayed for ^{99m}Tc activity in a well-type gamma detector (Omega 1, Canberra, Meriden, CT) and corrected for geometric differences in sample volumes. The protein content of pellets was determined by a biuret assay (*11*).

For analysis of relative ^{99m}Tc-MIBI activity in each tissue fraction, residual supernatant counts contaminating each respective pellet were subtracted. This correction assumed 0.65 g cell protein/g cell dry weight and 0.8 g mitochondrial protein/g mitochondrial dry weight (*12*) combined with the experimentally determined values of 21 ± 2 (n = 8) g wet weight/g cell protein and 26 ± 3 (n = 8) g wet weight/g mitochondrial protein measured on pellets from the standard protocols illustrated in Figure 1. The correction factors for the variable centrifugation protocol were 29 ± 8 (n = 4) and 16 ± 3 (n = 4) g wet weight/g pellet protein for 500× g and 15,000× g centrifugations, respectively. Results were then normalized to total recoverable ^{99m}Tc activity and expressed as percent of total fractional activity.

Analysis of In-Vivo Administered ^{99m}Tc-MIBI

Male Sprague-Dawley rats were anesthetized with diethyl ether and injected via an exposed jugular vein with ^{99m}Tc-MIBI (15–20 mCi, 5.5–7.4 GBq/0.5 ml). At 15 min postinjection, the animals were killed by ether asphyxiation, and the hearts were removed and washed free of blood in cold phosphate-buffered saline (0.15 *M*; pH 7.4, 4°C). The heart was minced with scissors into 1–2-mm pieces, diluted 2:1 by weight with buffered saline and homogenized in a Potter-Elvehjem teflon and glass tissue grinder. The homogeneous mixture was centrifuged at 3000× g for 20 min at 4°C, then triplicate 500-μl aliquots of the supernatant containing primarily "cytosolic components" were applied to a Centricon 30 Kdalton cutoff microconcentrator (Amicon, Beverly, MA) and centrifuged in a 30° fixed-angle rotor at 3000× g for 45 min. The filtrate was applied to a 10 Kdalton cutoff microconcentrator and centrifuged as above followed by similar processing through a 3 Kdalton cutoff microconcentrator. After each initial pass through the filter, an additional 2 × 250 μl of buffer was added and centrifugation repeated to wash through activity retained in the concentrate volume (~50 μl) that was not protein bound. The concentrated fraction of activity was collected by inverting the microconcentrator and centrifuging, thereby differentiating between counts associated with a large molecular weight protein and nonspecific binding to the membrane. Control studies were performed with the preinjected ^{99m}Tc-MIBI agent in buffered saline to monitor for binding to the filter plastic.

The "cytosolic" component from the homogenized heart was also tested for protein binding of any technetium containing species by chromatography using two different HPLC systems. The first was a silica-based gel permeation chromatography system (GPC) using an ^{125}I protein column as the stationary phase (1 cm × 25 cm) (Waters Assoc., Milford, MA) eluted with an aqueous buffer mobile phase (0.05 *M*, Na_2HPO_4/NaH_2PO_4, pH 7.0) at a flow of 1 ml/min. The second system was an anion exchange chromatography system (AEC) consisting of an Accell anion exchange column (4.6 mm × 250 mm) (Waters Assoc., Milford, MA) eluted with a linear gradient of phosphate-buffered (0.05 *M*, pH 7.0) saline (0.15 *M* to 1.5 *M*) over 10 min at 1 ml per min. On both of these systems, the parent lipophilic cation is retained indefinitely until a methanol gradient to at least 80% is performed.

The identity of the technetium containing complexes in the rat heart was ascertained by analysis of activity in the heart homogenate "cytosolic" component on the same reversed-phase, high-performance liquid chromatography system (RP-HPLC) used to validate the purity of the ^{99m}Tc-MIBI kit. Sample preparation for this technique involved diluting the cytosolic fraction with ice cold ethanol (1:1, v:v) to denature and precipitate proteins and centrifuging at 3000× g for 5 min at 4°C. A determination of counts/volume indicated no significant binding of ^{99m}Tc-MIBI to the precipitated proteins. Samples of the clear supernatant were analyzed on a RP-HPLC system consisting of a C-8 bonded stationary phase (5 μm sperical particles, 10 cm × 0.46 cm) (Brownlee, OS-MP cartridge, Rainin Instruments, Woburn, MA) and a linear gradient mobile phase of 100% aqueous buffer ($(NH_4)_2SO_4$, 0.05 *M*) to 95% methanol in 5 min. Serial radiometric and UV detection were used to monitor chromatographic eluations.

Statistical Analysis

Values are mean ± s.e.m. unless indicated in the text [(n) is the number of observations]. Statistical significance was determined by one-way analysis of variance or the two-tailed Student's t-test (paired or unpaired) as indicated (*13*).

RESULTS

Subcellular Distribution

To evaluate potential sources of error in assigning activity to specific subcellular compartments, the influence of various centrifugation rates on ^{99m}Tc activity in the post-protease pellet from perfused rat hearts were tested. For centrifugation rates of 500× g, 5000× g and 15,000× g, for 5 min each, the pellet-associated activity was 36% ± 5% (n = 4), 32% ± 4% (n = 8) and 50% ± 6% (n = 4) of total activity, respectively. The 500× g and 5000× g separations were not significantly different, but that at 15,000× g increased pellet-associated activity by 56% ($p < 0.05$). Because the purpose of this series of experiments was to correlate fractional distribution of ^{99m}Tc-MIBI activity with a mitochondrial marker while approximating

previously published methods, the 5000× g protocol was further analyzed.

In five perfused rat hearts injected with a bolus of ^{99m}Tc-MIBI, the centrifugation protocol shown in Figure 1 resulted in fractions containing ^{99m}Tc-MIBI activity distributed as shown in Figure 2 (% of total): supernatant I, 64% ± 5.3%; supernatant II, 23% ± 2.4%; cell fragments, 11% ± 2.6%; and mitochondrial component, 1.5% ± 0.6%. Although there was no statistically significant difference in fractional distribution between bolus and infusion (Fig. 2) or between constant pressure and constant flow protocols (p = ns), there was a trend with the infusion protocols toward enhanced mitochondrial-associated activity (40% increase) and decreased cell fragment-associated activity (11% decrease). However, note that 65% ± 7% of the ^{99m}Tc-MIBI activity that could be pelleted on the post-protease centrifugation was releasable into supernatant II by further manipulations and centrifugations with this protocol. In fact, control experiments demonstrated that three successive resuspensions of the post-protease pellet in Buffer II (without mitochondrial substrate), each followed by centrifugation (5000× g; 5 min), resulted in only 1% of total ^{99m}Tc-MIBI associated with the final pellet versus 39% without these manipulations.

Correlative evaluation of supernatants and pellets for the presence of malate dehydrogenase and ^{99m}Tc-MIBI activity is shown in Figure 3. Pooling the activity associated with both supernatants, 91% ± 1% (n = 4) of total malate dehydrogenase and 89% ± 3% of total ^{99m}Tc-MIBI contents were found in these "cytosolic" fractions. These techniques could not discern any significant differences in fractional activity of enzyme or agent with bolus or constant infusion protocols (p = ns) when normalized to total activity recovered by each method.

To further assay pellet fractions for the presence of viable mitochondria, substrate-specific mitochondrial membrane polarization was induced by addition of succinate (final concentration: 10 m*M*) and rotenone (final concentration: 1 μM) to filtrates prior to centrifugation (Fig. 4). Two protocols were followed:

1. For ^{99m}Tc-MIBI activity loaded into perfused heart preparations by the infusion protocol, the addition of succinate and rotenone increased pellet-associated tracer by 19% ± 3.6% (n = 5) over control values ($p < 0.01$). There did not appear to be any significant difference whether substrate was added to filtrate immediately after the protease treatment or to the partially purified supernatants of the next two centrifugation steps.
2. For ^{99m}Tc-MIBI activity added in vitro to the filtrate immediately after protease treatment of isotope-free perfused hearts, addition of succinate and rotenone increased pellet-associated tracer by 182% ± 66% (n = 8) over control ($p < 0.05$).

The combined enhancement over control by substrate addition to all "fragment" pellets and "mitochondrial"

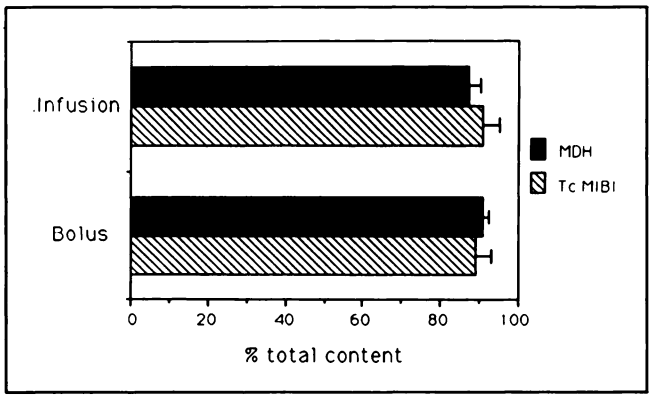

FIGURE 3. Correlative ^{99m}Tc-MIBI and malate dehydrogenase (MDH) content in the "cytosolic" fractions obtained from perfused rat hearts. Results are expressed as percent of total recoverable activity of radiolabel and enzyme from all fractions using either bolus (n = 4) or infusion (n = 4) loading protocols.

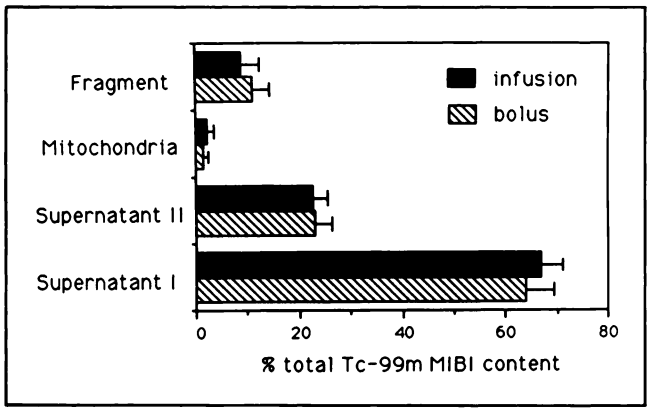

FIGURE 2. Nominal distribution of ^{99m}Tc-MIBI in fractions obtained from perfused rat hearts. Fractional tracer content is expressed as percent of total recoverable ^{99m}Tc-MIBI from either bolus (n = 5) or infusion (n = 6) loading protocols (see Methods).

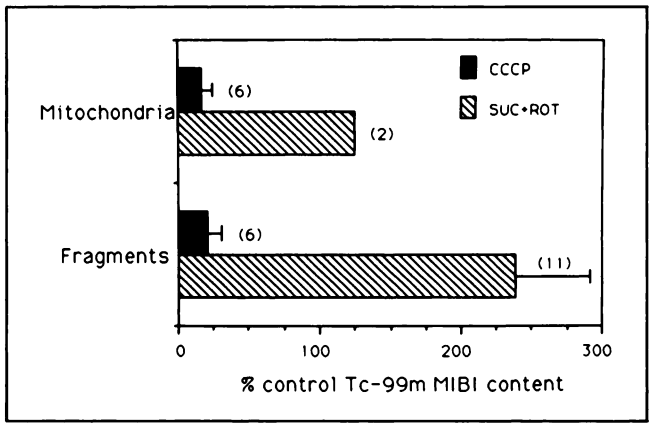

FIGURE 4. Effects of succinate (10 m*M*) plus rotenone (1 μM) or CCCP (5 μM) on ^{99m}Tc-MIBI content in the "cell fragment" and "mitochondrial" pellets. Results are expressed as percent of a paired untreated sample. Numbers in parentheses are the number of observations.

pellets was 139% ± 52% (n = 11) and 24% (n = 2), respectively (Fig. 4). The combined increase in pellet-associated activity in ^{99m}Tc-MIBI above control by addition of mitochondrial substrate for all in vivo and in vitro protocols was 119% ± 46% (n = 13; $p < 0.025$).

Conversely, for perfused hearts loaded with tracer, addition of the mitochondrial uncoupler CCCP (final concentration: 5 μM) to supernatants 3 min before centrifugation of the "cell fragment" pellet or "mitochondrial" pellet reduced pellet-associated ^{99m}Tc-MIBI activity to 20% ± 9.7% and 16% ± 8.2% of control (n = 6; $p < 0.001$), respectively (Fig. 4). There was no significant difference between continuous infusion and bolus loading protocols. These data indicated that CCCP-releasible (mitochondrial) ^{99m}Tc-MIBI activity was present in both pellets using this differential centrifugation technique.

Analysis of Possible Protein Binding in Cytosol

Control experiments on ultrafiltration of ^{99m}Tc-MIBI through microconcentrators demonstrated that the majority of the activity passed through all three filters. A substantial amount of activity, however, was also observed bound to the filter material for the 30 and 10 Kdalton cutoff microconcentrators. When a comparison was made between control ^{99m}Tc-MIBI from kits and ^{99m}Tc-MIBI recovered from the "cytosolic" component of the rat heart homogenate, no statistically significant differences were observed in this molecular weight range (Table 1). Indeed, only 7.6% ± 2.7% of the "cytosolic" activity was found associated with the fraction characterized as protein or biomolecules greater than 3 Kdalton in molecular weight compared with 5.0 ± 1.2 percent in this fraction for the ^{99m}Tc-MIBI agent alone.

Analysis of the "cytosolic" fraction by HPLC using both gel permeation (GP-HPLC) and anion exchange (AE-HPLC) under the nondenaturing elution conditions of the protein separation systems demonstrated only unreacted TcO_4^- or hydrophilic impurities eluting. Quantitative analysis confirmed >95% retention of the "cytosolic" activity on the columns for >90 min. Retention of activity on the column under this condition could have indicated: (1) a ^{99m}Tc-MIBI-protein conjugate that was extremely lipophilic, or (2) a labile conjugate such that the ^{99m}Tc-MIBI dissociated during elution, or (3) a ^{99m}Tc-MIBI conjugate that had decomposed to a lipophilic product, or (4) ^{99m}Tc-MIBI was simply present as the free cation that itself would not elute under these conditions.

However, the control experiment of reversed phase chromatographic analysis of "cytosolic" activity from rat heart labeled in vivo and extracted in protein denaturing conditions demonstrated 94% of the eluted activity with a retention time identical to free ^{99m}Tc-MIBI (data not shown). This result confirmed that the core technetium complex was retained in the heart as the intact cation and not metabolized (*14*).

DISCUSSION

Understanding the biological behavior of functional tracers such as the myocardial perfusion imaging agent ^{99m}Tc-MIBI can guide both appropriate clinical uses and further development of these diagnostic radiopharmaceuticals. Cultured chick heart cells have proven to be an efficacious and predictive cellular model for evaluating a wide number of candidates as myocardial imaging agents from this class of compounds (*15*). Cellular physiological studies indicate the ^{99m}Tc-MIBI, a modestly lipophilic cation, is sequestered within mitochondria of living cells thermodynamically driven by the large negative transmembrane potentials in series across the sarcolemmal and mitochondrial inner membranes (*7,8*). Early data from whole heart preparations, on the other hand, largely derived from differential centrifugation techniques, indicated the vast majority of ^{99m}Tc-MIBI to be associated with the "cytosolic" fraction (*3–5*).

Mitochondrial Association of ^{99m}Tc-MIBI in Mammalian Heart

Data from the current study corroborated the apparent cytosolic localization of ^{99m}Tc-MIBI in whole heart preparations, but found this to be an artifact of the tissue homogenization and fractionation techniques, particularly during the centrifugation steps. Several lines of evidence indicated that mitochondrial-associated ^{99m}Tc-MIBI in the mammalian model was lost during the centrifugation procedure. First, approximately 90% of malate dehydrogenase activity (a soluble mitochondrial matrix marker; *16*) was found in the "cytosolic" fractions in direct correlation with the ^{99m}Tc-MIBI activity. This indicated that the technique had damaged mitochondria sufficiently to allow leakage of inner matrix enzyme contents. Second, crude as well as

TABLE 1

Ultra-centrifugation of ^{99m}Tc-MIBI In-vivo Labeled Rat Heart: Cytosolic Fraction Compared with Free ^{99m}Tc-MIBI Complex

	>30K	Filter	30K–10K	Filter	10K–3K	Filter	<3K
				(% total activity)			
Rat heart	1.6 ± .26	13.4 ± 1.6	1.7 ± .95	18.1 ± 2.5	4.3 ± 1.5	0.7 ± .06	60.2 ± 5.0
^{99m}Tc-MIBI	0.9 ± .18	18.9 ± 3.0	1.1 ± .23	17.3 ± 1.8	3.0 ± .78	0.6 ± .14	58.2 ± 4.5

Measurements are presented as percent of total activity loaded onto the largest molecular weight cutoff microconcentrator. Each subsequent filtrate was transferred to the next smaller pore filter. Values are for mean ± s.d. (n ≥ 3).

partially purified pellets showed significant increases in ^{99m}Tc-MIBI activity when substrate specific for mitochondrial respiration (succinate; *16*) was supplied in the fractionation buffer. This showed that using a suboptimal protocol produced nominal "cell fragment" pellets as well as "mitochondrial" pellets containing viable mitochondria. The latter were able to repolarize their inner membrane potential and thereby increase potential-dependent accumulation of the agent. These data also indicated that another mechanism of loss in the "cytosol" fraction (independent of irreversible membrane damage) was a transient reduction in membrane potential due to lack of substrate. Cold temperatures during isolation would also contribute to loss of membrane potential. Third, the impurity of the cell membrane pellets was further demonstrated by the significant decrease in pellet-associated counts following addition of the mitochondrial uncoupler CCCP (*16*). Last, increasing centrifugation speed to 15,000× g significantly augmented pellet-associated ^{99m}Tc-MIBI activity consistent with improved efficiency of mitochondrial recovery.

If the fractionation data from this mammalian model are correlated with markers of mitochondrial integrity, then the activity of ^{99m}Tc-MIBI attributed to various fractions can be corrected and reassigned as shown in Figure 5. This analysis would indicate that approximately 90% of the ^{99m}Tc-MIBI activity is localized to the mitochondria in this mammalian model, consistent with previous observations from cultured avian heart cells and other in vitro cell models.

The alternative explanation for localization of ^{99m}Tc-MIBI based on irreversible binding to a soluble protein or biomolecule cannot be confirmed by the present experiments. Although some retention of ^{99m}Tc-MIBI activity from heart homogenate was observed with the Centricon ultrafiltration devices, the results could be explained by hydrophobic adsorption to the membrane filter of the microconcentrators. Indeed, any small differences in retention between heart "cytosolic" and kit ^{99m}Tc-MIBI ultrafiltration may be due to differences in filtration rate or competitive binding of biomolecules with the membrane. Additionally, HPLC analysis gave indications that no more activity eluted from the column than could be accounted for as chemical impurities (i.e., <5%). These chromatographic results do imply, however, that if a ^{99m}Tc-MIBI-biomolecule conjugate exists in vivo it is either exceptionally lipophilic, which would be inconsistent with a soluble conjugate localized to the cytosol, or an exceptionally labile interaction that would be expected to dissociate and produce rapid washout from the heart, which is contrary to in vivo data. We conclude that irreversible binding to a cytosolic protein is not a significant mechanism of localization for ^{99m}Tc-MIBI.

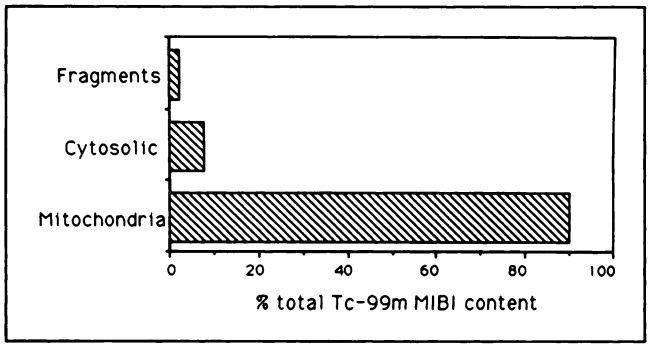

FIGURE 5. Corrected fractional distribution of ^{99m}Tc-MIBI expressed as percent of total activity obtained from perfused rat hearts. Technetium-99m-MIBI associated with malate dehydrogenase in supernatants I and II, or releasable by treatment of pellets with CCCP, was assigned to the mitochondrial fraction. CCCP-insensitive ^{99m}Tc-MIBI in the nonmitochondrial pellet was assigned to fragments and all residual activity was assigned to the cytosol.

Correlative Models and Clinical Implications

Data in other mammalian preparations are consistent with the model and conclusions of the present study. For example, in reperfused open-chest dogs, severe ischemic injury produced by LAD occlusion for 3 hr followed by 90 min of reperfusion results in the uncoupling of segmental reperfusion flow (measured with microspheres) and ^{99m}Tc-MIBI activity, such that accumulation of the agent is less than 30% of the corresponding reperfusion flow (*17*). This would be consistent with energy-dependent mitochondrial localization of ^{99m}Tc-MIBI. Similar results are reported in swine (*18*). In Langendorff-perfused rat hearts, inhibition of oxidative phosphorylation or lytic membrane disruption reduce net uptake and enhance clearance of ^{99m}Tc-MIBI (*19*), and in guinea pig heart slices incubated under hypoxic conditions, there is less accumulation of ^{99m}Tc-MIBI compared to control (*4*). Thus, in general, a significant proportion of tracer accumulation appears dependent upon metabolic status. Furthermore, by optimizing the differential centrifugation technique applied to guinea pig hearts loaded in vivo with ^{99m}Tc-MIBI, recent preliminary data indicate directly that >90% of the activity is found in the mitochondrial fraction in a substrate-dependent manner (*20*).

Under steady-state conditions in the presence of a constant extracellular concentration of ^{99m}Tc-MIBI, negative plasma-membrane potentials can pre-concentrate the agent into the cytosol approximately five-fold over extracellular space; mitochondria are then capable of further concentrating the agent up to 300-fold over the cytosol (*7, 16*). This is a result of intermediary metabolism of substrates (fatty acids, glucose, pyruvate), which delivers reducing equivalents to mitochondrial electron transfer cytochromes (*16*). Negative inner matrix membrane potentials generated by translocating protons out of the matrix during vectoral electron transfer (proton-motive hypothesis) provide the final bioenergetic sink for the lipophilic cationic ^{99m}Tc-MIBI.

Thus, in clinical use, these data imply that ^{99m}Tc-MIBI is both a perfusion and tissue viability agent. These prop-

erties might be best exploited during post-reperfusion evaluation when tracer accumulation would reflect myocardial viability under conditions where reestablishment of flow can be documented independently (perhaps angiographically). In the presence of severe myocellular post-ischemic injury, both mitochondrial and plasma-membrane potentials would decline (depolarize) from metabolic derangements and membrane disruption, thereby decreasing the driving force for ^{99m}Tc-MIBI accumulation. The level of myocardial accumulation of ^{99m}Tc-MIBI could serve as a noninvasive single-photon probe of tissue bioenergetics, specifically targeting the status of the membrane potentials. Alone or in combination with other tracers, ^{99m}Tc-MIBI could monitor the presence of severe, possibly irreversible, injury (*21*). Because the state of mitochondrial energetics is a fundamental aspect of a variety of tissue insults (toxic, chemotherapeutic, radiation) in addition to ischemia, there is every reason to believe that these principles should also be applicable to other tissues capable of accumulating ^{99m}Tc-MIBI, such as skeletal muscle, liver, kidney and a variety of tumors.

CONCLUSIONS

In summary, when fractionation of a marker of mitochondrial contents is correlated with the distribution of ^{99m}Tc-MIBI, the agent can be localized to mitochondrial compartments. No evidence for in-vivo binding to a cytosolic protein is found. This extends to intact mammalian myocardium results previously obtained with cultured cells in vitro. Caution is warranted when using tissue fractionation techniques to localize diffusible radiopharmaceuticals with energy-dependent sequestration mechanisms.

ACKNOWLEDGMENTS

The authors thank Georgia Washington for her secretarial assistance. A preliminary report of this data was presented at the 37th Annual Meeting of the Society of Nuclear Medicine, Washington, DC (*J Nucl Med* 1990;31:832).

Supported by grants from the National Institutes of Health (HL42966 and CA34970) and Department of Energy (ER 60526). P.A.C. is a recipient of a scholarship from CAPES, MEC, Brazil. D.P.-W. is an Established Investigator of the American Heart Association.

REFERENCES

1. Jones AG, Abrams MJ, Davison A, et al. Biological studies of a new class of technetium complexes: the hexakis (alkylisonitrile) technetium (I) cations. *Int J Nucl Med Biol* 1984;11:225–234.
2. Wacker FJ Th, Berman DS, Maddahi J, et al. Technetium-99m-hexakis 2-methoxyisobutylisonitrile: human biodistribution, dosimetry, safety, and preliminary comparison to thallium-201 for myocardial perfusion imaging. *J Nucl Med* 1989;30:301–311.
3. Mousa SA, Williams SJ. Myocardial uptake and retention of Tc-99m hexakis-aliphatic isonitriles: evidence for specificity [Abstract]. *J Nucl Med* 1986;27:995.
4. Mousa SA, Williams SJ, Sands H. Characterization of in vivo chemistry of cations in the heart. *J Nucl Med* 1987;28:1351–1357.
5. Piwnica-Worms D, Kronauge JF, Holman BL, Lister-James J, Davison A, Jones AG. Hexakis (carbomethoxy isopropyl isonitrile) technetium (I), a new myocardial perfusion imaging agent: binding characteristics in cultured chick heart cells. *J Nucl Med* 1988;29:55–61.
6. Sands H, Delano ML, Gallagher BM. Uptake of hexakis (t-butylisonitrile) technetium(I) and hexakis-(isopropylisonitrile) technetium(I) by neonatal rat heart and human erythrocytes. *J Nucl Med* 1986;27:404–408.
7. Piwnica-Worms D, Kronauge JF, Chiu ML. Uptake and retention of hexakis (2-methoxyisobutylisonitrile) technetium (I) in cultured chick myocardial cells: mitochondrial and plasma membrane potential-dependence. *Circulation* 1990;82:1826–1838.
8. Chiu ML, Kronauge JF, Piwnica-Worms D. Effect of mitochondrial and plasma membrane potentials on accumulation of hexakis (2-methoxyisobutylisonitrile) technetium (I) in cultured mouse fibroblasts. *J Nucl Med* 1990;31:1646–1653.
9. Delmon-Moingeon LI, Piwnica-Worms D, Van den Abbeele AD, Holman BL, Davison A, Jones AG. Uptake of the cation hexakis (2-methoxyisobutylisonitrile)-technetium-99m by human carcinoma cell lines in vitro. *Cancer Res* 1990;50:2198–2202.
10. Ochoa S. Malic dehydrogenase from pig heart. *Methods Enzymol* 1955;1: 735–739.
11. Gornall AG, Bardawill CJ, David MM. Determination of serum proteins by means of the biuret reaction. *J Biol Chem* 1949;177:751–766.
12. LeFurgey A, Ingram P, Lieberman M. Quantitative microchemical imaging of calcium in Na-K pump inhibited heart cells. *Cell Calcium* 1988;9: 219–235.
13. Glantz AS. *Primer of biostatistics*, 2nd edition. McGraw-Hill, Inc. NY. 1987:379.
14. Kronauge JK, Kawamura M, Holman BL, et al. Metabolic studies of the myocardial perfusion agent Tc-MIBI. In: Mazzi U, ed. *Technetium and rhenium in chemistry and nuclear medicine 3*. New York: Cortiona International; 1990:677–681.
15. Piwnica-Worms D, Kronauge JF, Holman BL, Davison A, Jones AG. Comparative myocardial uptake characteristics of hexakis (alkylisonitrile) technetium (I) complexes: effect of lipophilicity. *Invest Radiol* 1989;24: 25–29.
16. Darley-Usmar VM, Rickwood D, Wilson MT, eds. *Mitochondria: a practical approach*. Oxford: IRL Press; 1987:321.
17. Sinusas AJ, Trautman KA, Bergin JD, et al. Quantification of area at risk during coronary occlusion and degree of myocardial salvage after reperfusion with technetium-99m methoxyisobutyl isonitrile. *Circulation* 1990; 82:1424–1437.
18. Freeman I, Grundwald AM, Hoory S, Bodenheimer MM. Effect of coronary occlusion and myocardial viability on myocardial activity of Tc-99m-sestamibi. *J Nucl Med* 1991;32:292–298.
19. Beanlands RSB, Dawood F, Wen W-H, et al. Are the kinetics of technetium-99m methoxyisobutylisonitrile affected by cell metabolism and viability? *Circulation* 1990;82:1802–1814.
20. Crane PD, Piwnica-Worms D, Kronauge JF, et al. Cardiac retention of Tc-99m-sestamibi is related to mitochondrial viability [Abstract]. *J Nucl Med* 1991;32:957.
21. Piwnica-Worms D, Chiu ML, Kronauge JF. Divergent kinetics of Tl-201 and Tc-99m sestamibi in cultured chick ventricular myocytes during adenosine triphosphate depletion. *Circulation* 1992;85:1531–1541.