# Hexakis (2-Methoxy Isobutylisonitrile) Technetium-99m and Thallium-201 Chloride: Uptake and Release in Cultured Myocardial Cells

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Hexakis (2-methoxy isobutylisonitrile) technetium-99m ([$^{99m}$Tc]MIBI), a new tracer of myocardial blood flow, was compared with $^{201}$Tl Cl in cultures of myocardial cells of newborn rats. The kinetics of uptake and release of both tracers were assessed in basal conditions and in the presence of 5 m*M* cyanide, an inhibitor of the respiratory chain, 0.1 m*M* iodoacetate, an inhibitor of glycolysis, 10 $\mu M$ ouabain, an inhibitor of the Na-K ATPase, or with various pH values. The amplitude and frequency of contractions of the cells were also monitored in the same conditions. Results show that the washin and washout kinetics of [$^{99m}$Tc]MIBI are slower than $^{201}$Tl ($T\frac{1}{2}$) of the washout curves were, respectively, of 28 min and 6 min). The kinetics of release of both tracers were not influenced by any of the inhibitors. There was a strong effect of the pH on the $^{201}$Tl uptake only. Moreover $^{201}$Tl uptake was decreased by 34% in the presence of cyanide plus iodoacetate. Otherwise the uptakes of $^{201}$Tl and [$^{99m}$Tc]MIBI were not decreased by any of the drugs. The cellular contractility was significantly diminished by cyanide and it was abolished by cyanide plus iodoacetate. It is concluded that (a) impaired contractility can be associated with normal $^{201}$Tl and [$^{99m}$Tc]MIBI kinetics in myocardial cells in culture, (b) that $^{201}$Tl uptake may depend on the level of ATP devoted to the maintenance of membrane integrity, (c) that [$^{99m}$Tc]MIBI shows slower kinetics but is less sensitive to metabolic inhibitors than $^{201}$Tl.

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Tetrakis (2-methoxy isobutylisonitrile) $^{99m}$Tc (I)*([$^{99m}$Tc]MIBI) is a new tracer of myocardial blood flow that is presently under investigation for clinical use (*1*). Until more experience is gained about [$^{99m}$Tc] MIBI, thallium-201 ($^{201}$Tl) remains the agent routinely used for the scintigraphic assessment of coronary artery disease and myocardial infarction. While there is no report yet on the mechanism of uptake and metabolic behavior of [$^{99m}$Tc]MIBI, these properties remain incompletely understood or even controversial for $^{201}$Tl (*2–5*).

These experiments were designed to assess the uptake and efflux of [$^{99m}$Tc]MIBI and $^{201}$Tl in myocardial cells with impaired metabolism. Using a model of beating myocardial cells in cultures allowed us to work in well controlled conditions and at constant tracer concentrations.

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## MATERIALS AND METHODS

### Cultures of Myocardial Cells

The cultures were prepared in accordance with the technique described by Harary (*6*) with few modifications. Cells were released from the ventricles of 2- to 4-day-old Sprague-Dawley rats by repeated cycles of trypsinization (trypsin at 0.2% concentration) and centrifuged at 900 *g* during 5 min. The pellets were resuspended in growth medium (Ham F-10) complemented with 14 m*M* Na HCO3, 1 m*M* CaCl2, 100 U/ml of penicillin, 100 $\mu$g/ml of streptomycin and 10% horse serum plus 10% fetal calf serum. The cultures were incubated at 37°C in a water-saturated atmosphere (5% CO2 – 95% air). To reduce the number of contaminating nonmuscular cells, the differential attachment technique was used (*7*). Cells attached during the first 3-hr period of incubation were discarded. A new plating of the supernatant was done in 2 ml of nutrient medium per 35 mm diameter petri dishes (65,000

cells/cm$^2$). By 3 days, confluent monolayers with at least 80% of synchronously beating ventricular cells were obtained and used for the experiments. For the contractility studies, plastic microspheres 2–3 $\mu$m in diameter (3M Co) were added to the cultures on day two. They attached to cell surfaces and were moved by contraction of individual cells in the layer. This provided an improved image for contraction measurement and recording (*8*).

**Uptake and Efflux Measurements in Basal Conditions**

Thirty minutes before the experiment, incubation medium was replaced by serum-free medium. The pH was set at 7.4. The cells were incubated with 37 kBq of [$^{201}$Tl]chloride or [$^{99m}$Tc]MIBI which was obtained from a frozen formulation containing tetrakis (2-methoxy isobutylisonitrile) copper (I) tetrafluoroborate and formamidine sulfinic acid in a phosphate buffer and mannitol matrix. When radiolabeled with sodium pertechnetate $^{99m}$Tc injection, a hexakis cationic complex was formed.

For the uptake kinetics measurements, incubation times of 2, 5, 10, 20, 30, 45, 60, 120, and 180 min were used (n = 31 dishes for $^{201}$Tl and n = 44 dishes for [$^{99m}$Tc]MIBI). At the end of the incubation, the dishes were rapidly washed three times with cold saline. Then the cells were dissolved in 1% SDS and 10 m*M* sodium borate and transferred into a test tube for activity counting in a gamma well-counter. The amount of proteins was measured in each culture dish by the method of Lowry (range 200–300 $\mu$g proteins). The ratio between the intra and extracellular concentrations of the tracer (Ci/Ce) was then calculated by r × intracellular activity/(total injected activity-intracellular activity) where r represents the ratio of the extracellular to the intracellular volumes, i.e., $10^6$/ amount of proteins in $\mu$g (*9*).

For the efflux kinetics measurements, the incubation time was 20 min with both tracers. Different loads of $^{201}$Tl were used (Table 1). Then the medium was rapidly removed and replaced by a nonradioactive one. Samples of the medium were sequentially taken at 2, 5, 10, 20, 30, 45, 60, and 120 min and counted for their tracer activity. Finally the cells were washed, dissolved and also counted. From these values the time evolution of $^{201}$Tl and [$^{99m}$Tc]MIBI intracellular activities were calculated.

**TABLE 1**
**Experimental Conditions of Efflux Measurements After Incubation with 37 KBq of Either $^{201}$Tl or [$^{99m}$Tc]MIBI***

| Duration of incubation with the tracer (min) | Number of samples ($^{201}$Tl/[$^{99m}$Tc]MIBI) | Drug in the medium |
|---|---|---|
| 20 | 20/9 | None |
| 2 | 6/0 | None |
| 5 | 9/0 | None |
| 20 | 6*/0 | None |
| 20 | 5/6 | 5 m*M* cyanide |
| 20 | 5/6 | 5 m*M* cyanide plus 0.1 m*M* iodoacetate |
| 20 | 5/6 | 0.1 m*M* iodoacetate |
| 20 | 5/0 | 10 $\mu$*M* ouabain |

* 370 KBq of $^{201}$Tl.

**Effect of Metabolic Inhibitors and of the pH of the Medium**

The effect of 5 m*M* sodium cyanide (CN), an inhibitor of the respiratory chain, 0.1 m*M* sodium iodoacetate (IAA), an inhibitor of glycolysis, or 10 $\mu$*M* ouabain, an inhibitor of Na-K ATPase, were evaluated.

For the uptake measurements, the cells had been pretreated during 15 min with the inhibitor before the 37 kBq dose of $^{201}$Tl or [$^{99m}$Tc]MIBI was added. Incubation time was 20 min.

For the efflux kinetics measurements the cells were prepared like in the basal conditions, i.e., incubated for 20 min with the tracer. Then the radioactive medium was rapidly removed and replaced by the same amount of nonradioactive medium containing the inhibitor (Table 1). Sampling was done as already described.

The effect of various pH values ranging between 6 and 9 were studied in cells preincubated during 30 min in Hepes buffered serum-free growth medium (Ham F-10 complemented with 14 m*M* NaCl, 1 m*M* $CaCl_2$, antibiotics and 10 m*M* Hepes; pH was adjusted with 1*M* NaOH). The cells were then incubated during 20 min with $^{201}$Tl or 60 min with [$^{99m}$Tc]MIBI.

**Contractility Measurements**

Dishes containing cultures were placed in a thermostated chamber gassed with a 95% air – 5% $CO_2$ mixture, provided with inlet and exit ports connected to polyethylene tubing permitting medium change without variations of environment, on the stage of an inverted phase contrast microscope (Olympus). After 15 min of equilibration the medium was removed and replaced by a medium containing CN (5 m*M*) alone or with IAA (0.1 m*M*). The cells were magnified with a 40× objective. Their image was monitored by a low light level television camera. A video motion detector monitored a selected raster line segment. It provided every 16 ms the position of the border of a selected microsphere moving along that raster line. These movements were recorded during 10 min to 3 hr for measurements of frequency and amplitude of the cell contractions. Results were expressed in percent of the control values.

All the results were statistically analysed using Student's t-test.

## RESULTS

**Basal Conditions**

The kinetics of uptake in basal conditions are shown in Figure 1. Since no major change in the $^{201}$Tl Ci/Ce values occurred after 20 min, this was the duration of incubation retained for the other experiments with $^{201}$Tl. With [$^{99m}$Tc]MIBI an almost linearly increasing uptake could be observed during the first 45 min of incubation. No significant change was found between the second and third hours.

The washout curves are shown on Figure 2. T½ was 6 min for $^{201}$Tl and 28 min for [$^{99m}$Tc]MIBI. The $^{201}$Tl curves were identical with any $^{201}$Tl load.

**Effect of Metabolic Inhibitors and of the pH of the Medium**

Table 2 shows that the addition of CN plus IAA produced a significant decrease of $^{201}$Tl uptake ($p <$

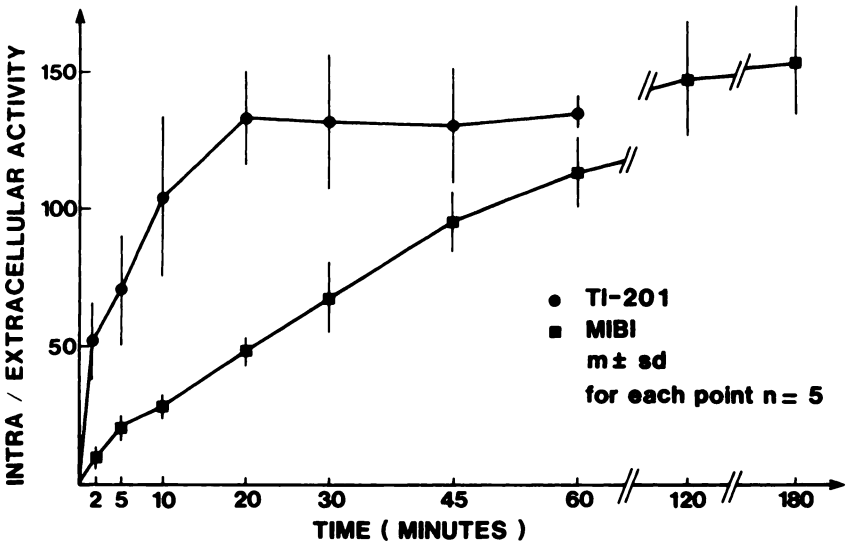


**FIGURE 1**
Kinetics of $^{201}$Tl and [$^{99m}$Tc]MIBI uptake in basal conditions.

0.001). A slight, but not significant increase was observed with IAA alone. CN alone had no effect. With [$^{99m}$Tc]MIBI there was a moderate but significant increase in the presence of CN plus IAA.

The washout curves of $^{201}$Tl and [$^{99m}$Tc]MIBI were unchanged when the cells were incubated with CN and/or IAA (Table 1).

The effect of the pH of the medium is shown on Figure 3. It had a strong linear positive correlation with $^{201}$Tl Ci/Ce (n = 83 for three different experiments, r = 0.93) and a weak one with [$^{99m}$Tc]MIBI Ci/Ce (n = 57 for two different experiments, r = 0.56).

### Contractility

Results from preliminary experiments have demonstrated that 3-day-old cells in cultures are spontaneously beating at a stable frequency (range 90 to 260 bpm). Although there were variations from one batch to another, the cells belonging to the same batch were beating at the same frequency. When the temperature was kept constant, the beating frequency of cells would remain stable for hours. Renewing the medium did not affect the contraction parameters.

When the medium containing CN was added, a significant decrease of the frequency of contractions was observed (Fig. 4). However the cells never stopped beating, even after 3 hr. All cells treated with CN plus IAA stopped beating after 5 to 7 min of incubation (Fig. 4). Similar negative findings were observed concerning the amplitude of contractions.

## DISCUSSION

### Mechanism of $^{201}$Tl Uptake

Our results demonstrate that in this model of cultured myocardial cells, normal $^{201}$Tl kinetics can be associated with severely impaired contractility.

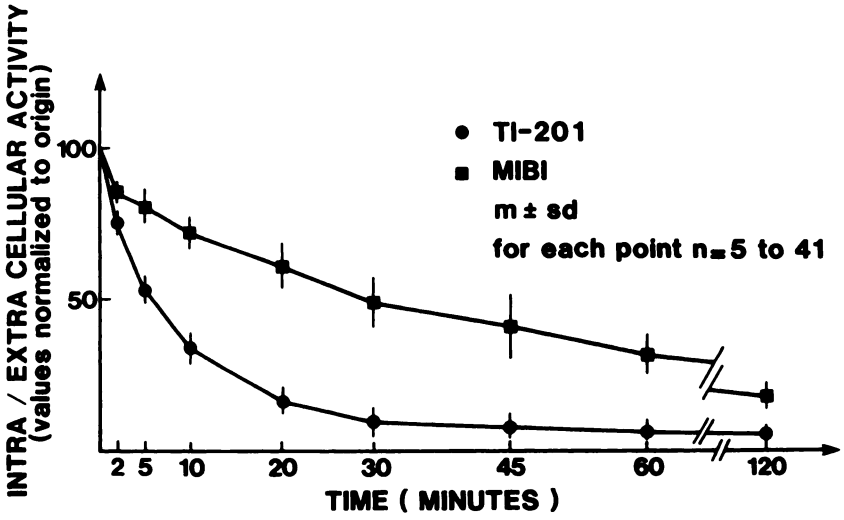


**FIGURE 2**
Kinetics of $^{201}$Tl and [$^{99m}$Tc]MIBI efflux in basal conditions.

**TABLE 2**
Variations in the Intra/Extracellular Concentrations (Ci/Ce) of $^{201}$Tl and [$^{99m}$Tc]MIBI After 20 min of Incubation in the Cultured Myocardial Cells Treated with Inhibitors

| | $^{201}$Tl | | [$^{99m}$Tc]MIBI | |
|---|---|---|---|---|
| Incubation with | n | % of Ci/Ce* control value | n | % of Ci/Ce* control value |
| 5 m*M* NaCN | 21 | 97 ± 10 | 10 | 112 ± 17 |
| NaCN 5 m*M* + IAA 0.1 m*M* | 21 | 66 ± 13‡ | 10 | 114 ± 10† |
| IAA 0.1 m*M* | 15 | 119 ± 26 | 10 | 98 ± 13 |
| Ouabain 0.01 m*M* | 10 | 106 ± 26 | 6 | 125 ± 22 |

*m ± sd.
†$p < 0.01$.
‡$p < 0.001$.

These findings could be consistent with the predominance of a passive, coronary blood flow dependent uptake instead of a metabolically dependent uptake of $^{201}$Tl. Using different models other authors have drawn similar conclusions. Leppo et al. utilized isolated contracting rabbit hearts and did not observe any effect of hypoxia on $^{201}$Tl uptake and clearance (*4*). A predominantly passive process was observed after coronary reperfusion in intact dogs by Okada et al. (*10*) and Forman et al. (*2*). Weich et al. noticed that with acidosis the extraction fraction significantly diminished while after alkalosis it did not change (*11*). They also observed that it reversibly decreased under hypoxia. Melin et al. noticed that changes of extraction fraction do not parallel those of the myocardial blood flow (*5*). However these experiments on extraction fraction cannot be compared with those on Ci/Ce because extraction fraction was measured from the passage of a bolus of activity of $^{201}$Tl in the coronary arterial circulation while our Ci/Ce was obtained with an extracellular $^{201}$Tl concentration kept constant in the medium during the whole experiment. Concerning the $^{201}$Tl release, the fact that in our study it was not different between the controls and the cells treated with cyanide plus IAA also supports the existence of a passive phenomenon.

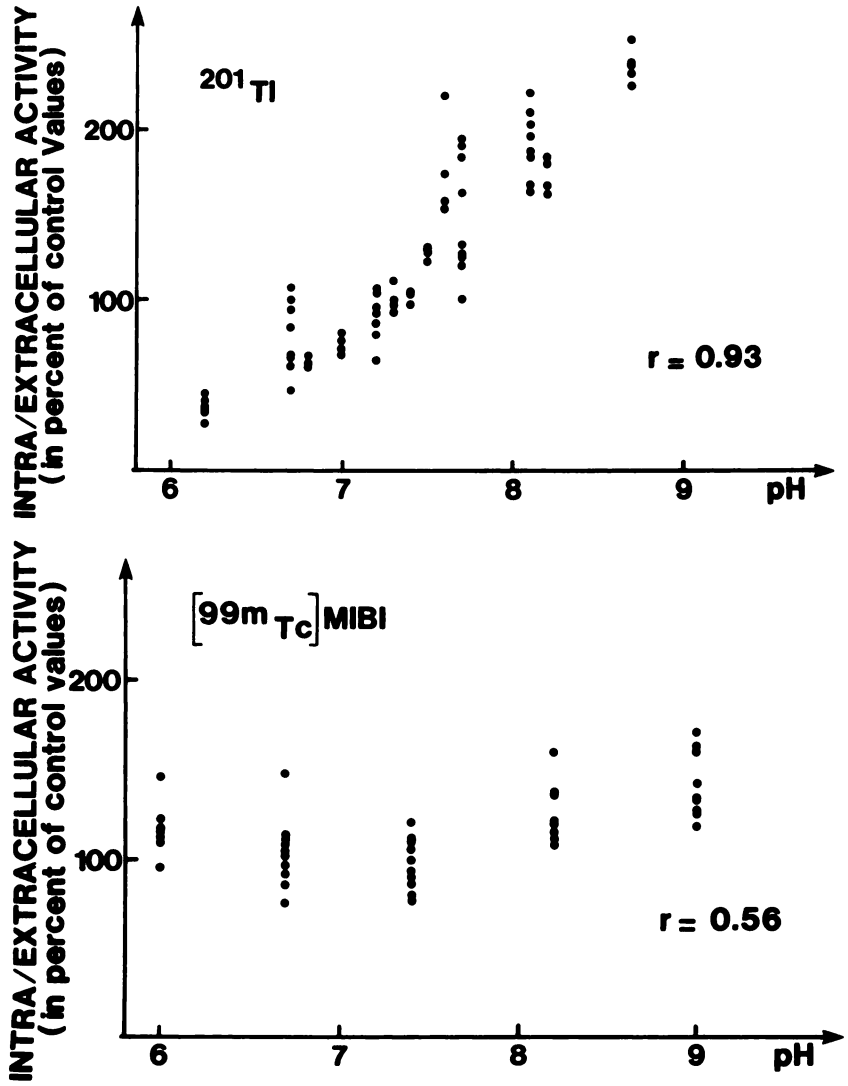


**FIGURE 3**
Relationship between the pH of the medium and the uptake of $^{201}$Tl (top) and [$^{99m}$Tc]MIBI (bottom).

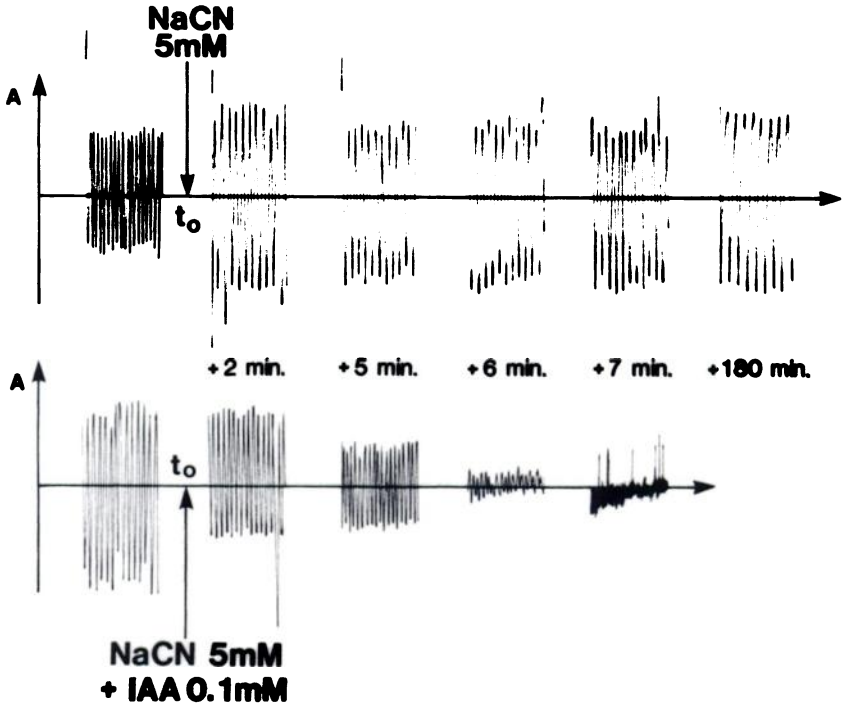


**FIGURE 4**
Effect of cyanide with or without iodoacetate on the contractions of two cardiac cells in culture. With cyanide alone (top), the frequency was decreased but the cell was still beating after 3 hr in incubation. With cyanide plus iodoacetate (bottom) contractions stopped after 7 min.

However, the effects of variations of the pH value show that the ionic environment plays a significant role too. It must also be emphasized that we did not calculate the actual inward and outward transmembrane flux of $^{201}$Tl through the membrane but rather the time evolution of the intra and extracellular concentrations after loading of the intra or extracellular compartments. Moreover the uptake and washout experiments are completely parallel since during the uptake experiments the cells concentrate $^{201}$Tl while the net result of the washout phase is a release of intracellular $^{201}$Tl. Some investigators have concluded that an active process can be at least partly responsible for $^{201}$Tl uptake (*3–5*). In fact, although we found that a decreased mechanical function does not necessarily imply an altered $^{201}$Tl uptake, this is not definite proof that $^{201}$Tl uptake is totally independent of ATP concentration. In effect it has already been suggested that ATP could be functionally compartmentalized for maintenance of essential membrane integrity (*12*). Therefore it remains possible that under cyanide induced hypoxia and its concomitant decrease in overall ATP and creatine phosphate concentrations, the cell reacts by decreasing its mechanical function but keeps the remaining ATP to preserve its membrane integrity and function. The suggestion by Higgins et al. (*12*) that the glycolytically derived ATP is used to maintain the membrane integrity and function would then fit with the fact that in our experiments, when IAA was added to cyanide, the $^{201}$Tl intracellular concentration started to decline. This is also consistent with the results of Doorey et al. (*13*) who noticed that CN plus IAA impaired contractility more severely than CN alone, suggesting that glycolytically derived energy can support some level of mechanical work. Therefore it can be hypothesized that $^{201}$Tl uptake depends at least partly on the glycolytically derived ATP devoted to the maintenance of membrane integrity.

### Mechanism of [$^{99m}$Tc]MIBI Uptake

Our results show that the uptake of [$^{99m}$Tc]MIBI was not altered by inhibitors of the respiratory chain, glycolysis or Na-K ATPase, demonstrating that its mechanism of uptake is less dependent on an active process than $^{201}$Tl. Sands et al. also observed that the uptake of two other [$^{99m}$Tc]isonitriles complexes by neonatal rat myocytes and human erythrocytes were unaffected by ouabain (*14*). However, they did not measure the effect of ouabain on the $^{201}$Tl uptake in their model. Whether or not the cellular uptake of [$^{99m}$Tc]MIBI is only due to the high lipophilicity of [$^{99m}$Tc]isonitrile complexes (*15*) remains to be demonstrated. However it cannot be concluded from our results that the process of uptake is totally passive.

### Advantages and Limitations of the In Vitro Model

Using in vitro cultures presents several advantages. It allows the elimination of all interactions due to neuronal and hormonal influences that cannot be avoided and are not always controlled in vivo. Local parameters such as pH and temperature can be easily monitored. Contractility can be assessed at the cellular

level. The extracellular concentration of the chemicals, drugs and tracers administered to the cultures can be precisely known and kept constant. Finally the washout of radiotracers can be easily followed from samples taken successively from the medium.

On the other side, conclusions drawn from an in vitro experiment with animal tissue cannot always be extended to in vivo conditions in humans. Moreover beating cultured cells are obtained from the myocardium of newborn rats which is more heavily reliant on carbohydrate as a substrate than the adult myocardium (*16*).

Although hypoxia was not real in the medium but was simulated by CN, our results remain valid since a dramatic effect was produced on the contractility. Cyanide is known to reversibly inhibit mitochondrial electron transport and as a consequence oxidative phosphorylation. Its mechanical effects in rat and canine myocardium are similar to those induced by hypoxia (*17*). Doorey et al. (*13*) noticed that the threshold concentration producing a decline in contractile amplitude of cultured chick heart cell is 0.01 m*M*. They found that at 0.13 m*M*, intracellular ATP declined by 67%. The reversibility of CN inhibition has also been observed in one of our flasks in which frequency of contractions decreased from 200 to 150 with CN and went back to 180 after CN had been washed out.

**Clinical Implications**

Our results cannot be interpreted like the demonstration that even irreversibly damaged tissue will show a normal uptake of $^{201}$Tl or [$^{99m}$Tc]MIBI if blood flow is normally delivered. Other experiments should be designed to address this particular issue. However, they strongly suggest that, if they can be extrapolated to human scintigraphy, a normal uptake of these tracers does not imply a normal mechanical function of the myocardial wall. It seems that as long as the myocardial cell can maintain some production of ATP through the glycolytic pathway, it will maintain a normal $^{201}$Tl and [$^{99m}$Tc]MIBI uptake. This is of interest since the development of non traumatic revascularisation techniques in cardiology, like intravenous fibrinolytic treatment and percutaneous coronary angioplasty. Scintigraphy provides an elegant method to assess the efficacy of revascularisation. According to our results it would seem that a normal uptake following such an intervention would mean that the circulation has been successfully reestablished, and that at least a minimal cellular metabolic activity is present, without predicting the eventual outcome, i.e., complete recovery or irreversible necrosis.

Concerning the comparison between $^{201}$Tl and [$^{99m}$Tc]MIBI, we can conclude that [$^{99m}$Tc]MIBI uptake is less sensitive to the metabolism of the cells than $^{201}$Tl. Therefore [$^{99m}$Tc]MIBI could be a more reliable tracer of myocardial blood flow than $^{201}$Tl.

## NOTES

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