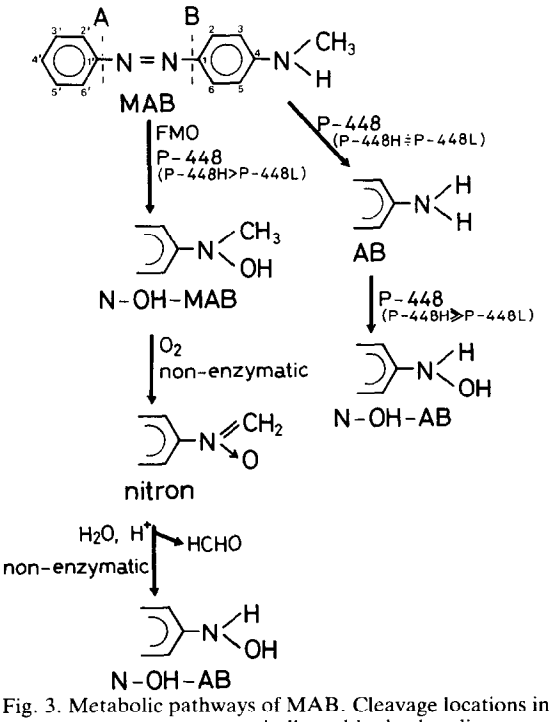

Fig. 3. Metabolic pathways of MAB. Cleavage locations in mass spectrometry are indicated by broken lines.

of cyt. P-448 are given in Table 1. The formation of N-OH-MAB and the increased formation of nitron, a degradation product of N-OH-MAB, were remarkable in the case of P-448-H compared with that of P-448-L, reflecting their N-hydroxylating activities. In the MC microsomes, at the protein level $P\text{-}450_d$ (the same as P-448-H) was only one-third of $P\text{-}450_c$ (the same as P-448-L) [6], whereas N-hydroxylating activity with P-448-H was several times as large as that with P-448-L. Thus, it can be concluded that in the N-hydroxylation of MAB the contribution of P-448-H is larger than that of P-448-L. N-OH-AB was detected specifically with P-448-H, while the rate of formation of AB from MAB was similar with the two forms of cyt. P-448. The ring hydroxylations, i.e. the formations of 4′-OH-MAB, 4′-OH-AB and 3-OH-AB, were 1.7 to 3 times higher with P-448-L than with P-448-H.

In summary, the contribution of P-448-H was large in the N-hydroxylation of MAB in contrast to the ring hydroxylation which was more efficiently catalyzed by P-448-L. These results suggest the important role of P-448-H in the metabolic activation of MAB in MC microsomes, although MC treatment *in vivo* not only enhances N-hydroxylation to initiate carcinogenesis but also accelerates other detoxification processes to inhibit carcinogenesis.

Table 1. Rate of formation of the metabolites of MAB with P-448-H and P-448-L

	Metabolites formed (nmoles/nmole cyt. P-448/min)	
	P-448-H	P-448-L
4′-OH-AB	0.201 ± 0.21	0.337 ± 0.025
3-OH-AB	0.641 ± 0.066	2.075 ± 0.109
4′-OH-MAB	0.735 ± 0.033	1.267 ± 0.061
N-OH-AB	7.065 ± 1.148	< 0.020
AB	17.532 ± 0.801	18.053 ± 0.444
Nitron	6.702 ± 0.433	1.059 ± 0.063
N-OH-MAB	0.830 ± 0.180	0.382 ± 0.039

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Transport of $^{42}K^+$, $^{201}Tl^+$ and $[^{99m}Tc(dmpe)_2 \cdot Cl_2]^+$ by neonatal rat myocyte cultures

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Interest in the mechanism of transport of monovalent cations has increased due to the widespread use of $^{201}Tl^+$ for the detection of myocardial infarction and ischemia by myocardial perfusion imaging [1]. Utilizing human erythrocytes, Skulskii *et al.* [2] indicated that the (Na^+, K^+)ATPase (EC 3.6.1.3) recognizes both K^+ and Tl^+ and that ion transport differed only in a greater affinity for Tl^+ than K^+. It has also been shown that Tl^+ is interchangeable with K^+ in stimulating Na^+ efflux from erythrocytes [2]. Based on these types of results, it is generally accepted that Tl^+ acts physiologically as a K^+ analog [3]. The utility of $^{201}Tl^+$ for myocardial imaging is limited by its high cost,

low energy emissions (70 keV), and long half-life (73 hr). The radionuclide preferred for use as a nuclear medicine diagnostic agent is ^{99m}Tc. This is due to its lower cost, nearly ideal gamma energy (140 keV), and short half-life (6 hr). The replacement of $^{201}Tl^+$ by a ^{99m}Tc-labeled monovalent cationic radiopharmaceutical for myocardial imaging has been suggested [4]. Several ^{99m}Tc-labeled cationic complexes have been synthesized, and their potential as myocardial imaging agents tested. One of these, $[^{99m}Tc\text{-}(dmpe)_2\cdot Cl_2]^+$ [dmpe = 1,2-bis(dimethylphosphino)-ethane], has been used successfully for imaging the heart of dogs and other species [5].

We have recently compared the mechanism of transport of K^+, Tl^+ and $[^{99m}Tc(dmpe)_2\cdot Cl_2]^+$ by human erythrocytes and concluded that the influx of $[^{99m}Tc(dmpe)_2\cdot Cl_2]^+$ is by a mechanism different from that of either K^+ or Tl^+ [6]. In this report, we have extended these studies to the beating neonatal rat myocyte system [7]. The data indicate that in the myocyte the characteristics of the uptake of $^{201}Tl^+$ are very similar to those of $^{42}K^+$ although a portion of the $^{201}Tl^+$ influx appears to be independent of the (Na^+,K^+)ATPase. In contrast, the uptake of the $[^{99m}Tc(dmpe)_2\cdot Cl_2]^+$ complex is completely independent of the (Na^+,K^+)ATPase.

The procedures described by Lampidis *et al.* were modified for the preparation of cultures of beating neonatal rat myocytes [7]. The ventricles from twenty 24- to 48-hr-old neonatal rats were rinsed and minced in cold, sterile Hanks' Ca^{2+} and Mg^{2+}-free balanced salt solution (HBSS). The minced tissue was trypsinized (0.05% trypsin in HBSS at 37°) in a 25-ml stir-flask for 10-min intervals. The supernatant fraction from each trypsinization was decanted off and fresh medium was added. The supernatant fraction from the sixth through ninth trypsinizations were retained, and these trypsin solutions were promptly inactivated by adding equal volumes of sterile Eagle's minimal essential medium (MEM-E) containing 10% fetal calf serum (FCS) and 1% antibiotic. The cell suspensions were combined and centrifuged at 200 *g* for 10 min. The pellet containing the cells was resuspended in the 10% FCS-MEM-E medium. The cell count was adjusted to 2.5×10^5 cells/ml.

The myocytes (approximately 2.5×10^4 cells/well) were seeded into 12 well plates. The plates were incubated at 37°, 5% CO_2 for 18 hr to allow for attachment of the cells. At 18 hr after seeding, the cells were incubated at 37°, 5% CO_2, and treated for 3 hr with medium containing 10 mM hydroxyurea to minimize fibroblast growth. The plates were then rinsed three times with medium and thereafter maintained for 2–3 weeks with medium containing 1.5% bovine colostrum, 0.05% FCS (to inhibit growth of fibroblasts), and 1% antibiotic. The bovine colostrum was received frozen. After thawing, aliquots were centrifuged several times at approximately 40,000 *g* for 30–60 min. The middle layer from the centrifuged material was pipetted off and spun again at approximately 40,000 *g* for 10 min. The supernatant fractions were then filtered sequentially through 0.8, 0.45, and 0.2 μm sterilization filter units. The sterile solutions were dispensed into 50–100 ml bottles and frozen until needed. The cells on the plates, which were used for uptake experiments 2–3 weeks after seeding, were approximately 90% myocytes. For the influx studies, the plates of myocytes were thoroughly rinsed with K^+-free Ringer's sulfate buffer (80 mM Na_2SO_4, 1 mM $MgSO_4$, 0.4 mM $CaSO_4\cdot 2H_2O$, 20 mM $NaHCO_3$, 14 mM dextrose, pH 7.4). The cells were allowed to equilibrate in the Ringer's buffer with or without 0.15 mM ouabain for 10–25 min, after which the labeled monovalent cation ($^{42}K^+$, $^{201}Tl^+$, or $[^{99m}Tc(dmpe)_2\cdot Cl_2]^+$) was added. The competing unlabeled cation such as K^+, Tl^+, or $[^{99}Tc(dmpe)_2\cdot Cl_2]^+$ was added at the same time as the labeled cation. The plates were incubated at 37° for a timed interval. At the end of the incubation period, the plates of myocytes were rinsed with ice-cold phosphate-buffered saline (PBS; 0.1 mM phosphate, 150 mM NaCl, pH 7.2), and treated with 7% trichloroacetic acid (TCA). The precipitated cellular material was scraped from the surface of each well with a rubber policeman and pipetted into counting tubes with the TCA. Each well was rinsed twice with PBS. These rinses were also drawn off and retained for counting. The amounts of radioactivity remaining in the combined TCA precipitated cells and the PBS rinses were expressed as the percent of radioactivity added to each well. Data at each time point are reported as the mean plus or minus standard error of the mean. In Figs. 1–3, the number of experiments is indicated as "n". Statistical analysis of the data was done using the double-tailed Student's *t*-test, and P values of <0.05 were taken as being significant. $^{42}K^+$ (0.013 mCi/mg), $^{201}Tl^+$ (carrier-free), $[^{99}Tc(dmpe)_2\cdot Cl_2]^+$ and $[^{99m}Tc(dmpe)_2\cdot Cl_2]^+$ (carrier-free) were obtained from DuPont/NEN Products (North Billerica, MA). Ouabain was obtained from the Sigma Chemical Co. (St. Louis, MO). Bovine colostrum was obtained from Rossbach Farms (West Townsend, MA) and Homestead Farm (Boxborough, MA). Neonatal rats were obtained from the Charles River Breeding Laboratories, Inc. (Wilmington, MA).

The data in Fig. 1 demonstrate that the beating myocytes have a cationic transport system and that the influx of $^{42}K^+$ into the cells is time dependent. This transport of $^{42}K^+$ was inhibited by 0.15 mM ouabain, indicating that it was mediated by the (Na^+,K^+)ATPase. In addition, $^{42}K^+$ influx was inhibited by unlabeled K^+ (10 mM) or Tl^+ (10 mM). The competition by Tl^+ supports the concept that Tl^+ is recognized as a K^+ analog by the (Na^+,K^+)ATPase system.

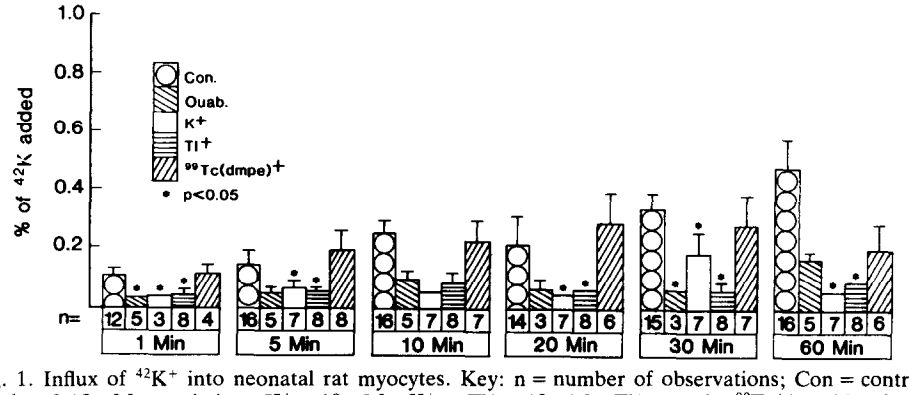

Fig. 1. Influx of $^{42}K^+$ into neonatal rat myocytes. Key: n = number of observations; Con = control; Ouab = 0.15 mM ouabain; K^+ = 10 mM K^+; Tl^+ = 10 mM Tl^+; and $^{99}Tc(dmpe)^+$ = 5 mM $[^{99}Tc(dmpe)_2\cdot Cl_2]^+$. Error bars represent S.E.M. An asterisk (*) = $P < 0.05$.

In contrast, $[^{99}Tc(dmpe)_2 \cdot Cl_2]^+$ at 5 mM had no significant effect on the $^{42}K^+$ influx, suggesting that the uptake of $^{42}K^+$ and $[^{99m}Tc(dmpe)_2 \cdot Cl_2]^+$ is mediated by non-competing mechanisms. Although ^{99}Tc is a low specific activity beta emitter, in these studies the ^{99}Tc label functioned as a non-radioactive analog of the $[^{99m}Tc(dmpe)_2 \cdot Cl_2]^+$ complex.

As shown in Fig. 2, the rate and magnitude of $^{201}Tl^+$ influx in the myocytes were much greater than for $^{42}K^+$. The $^{201}Tl^+$ influx was inhibited by either 0.15 mM ouabain or 10 mM Tl^+. Although 10 mM K^+ diminished $^{201}Tl^+$ influx, the effect was not statistically significant. This was apparently due to the fact that $^{201}Tl^+$ uptake is governed by active and diffusional processes [2]. As in the $^{42}K^+$ influx studies, $[^{99}Tc(dmpe)_2 \cdot Cl_2]^+$ at 5 mM had no effect on $^{201}Tl^+$ influx.

Figure 3 shows that the rate and magnitude of the uptake of $[^{99m}Tc(dmpe)_2 \cdot Cl_2]^+$ by the myocyte cultures were similar to $^{201}Tl^+$ and greater than that of $^{42}K^+$. In contrast to the $^{42}K^+$ and $^{201}Tl^+$ uptake, however, the influx of the $[^{99m}Tc(dmpe)_2 \cdot Cl_2]^+$ complex was not inhibited by 0.15 mM ouabain, 10 mM K^+ or 10 mM Tl^+ but was inhibited significantly by excess $[^{99}Tc(dmpe)_2 \cdot Cl_2]^+$.

When the washed myocytes loaded with $^{201}Tl^+$, $^{42}K^+$, or $[^{99m}Tc(dmpe)_2 \cdot Cl_2]^+$ were lysed by TCA and the counts associated with the cell debris separated from the lysate, essentially none of these cations was associated with the cellular debris (data not shown). This suggests that these monovalent cations were actually transported into the cells and were not just bound to the outside membrane.

For several years, the radionuclide $^{201}Tl^+$ has been the agent-of-choice for nuclear medicine myocardial perfusion imaging [1]. Because of its monocationic characteristics,

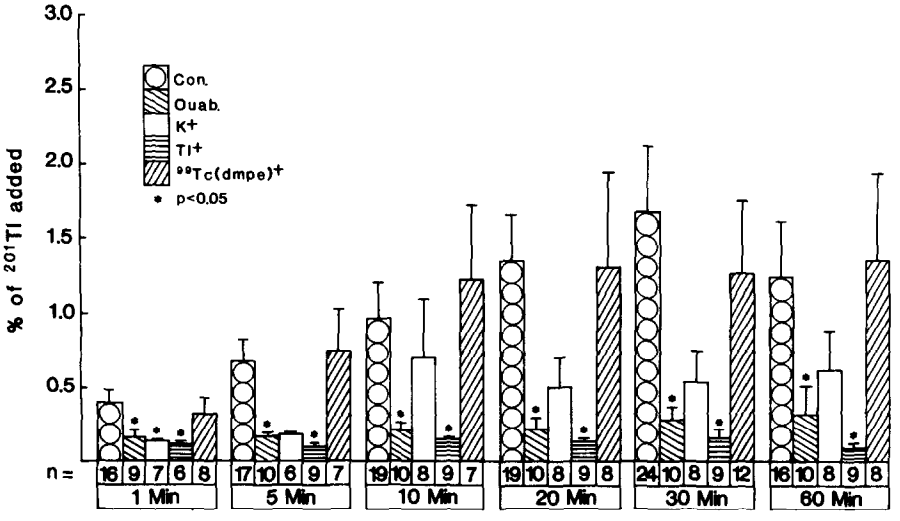

Fig. 2. Influx of $^{201}Tl^+$ into neonatal rat myocytes. Key: n = number of observations; Con = control; Ouab = 0.15 mM ouabain; K^+ = 10 mM K^+; Tl^+ = 10 mM Tl^+; and $^{99}Tc(dmpe)^+$ = 5 mM $[^{99}Tc(dmpe)_2 \cdot Cl_2]^+$. Error bars represent S.E.M. An asterisk (*) = $P < 0.05$.

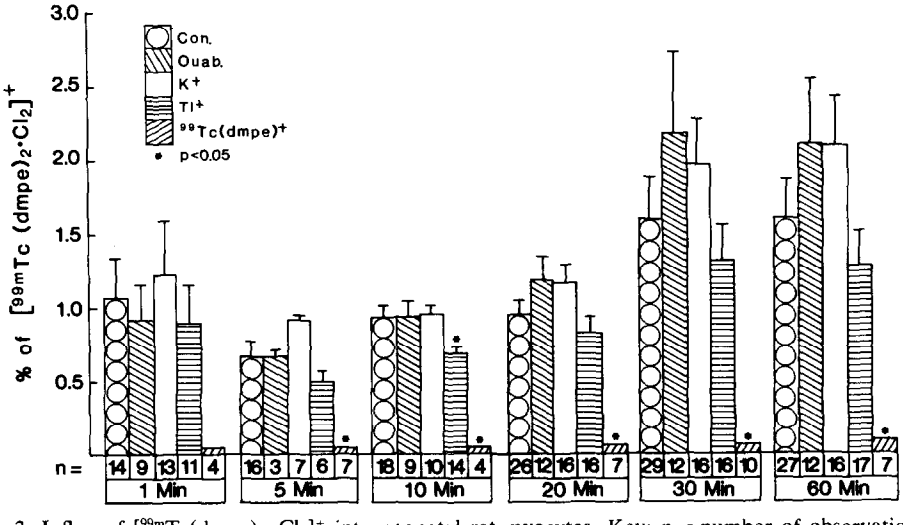

Fig. 3. Influx of $[^{99m}Tc(dmpe)_2 \cdot Cl_2]^+$ into neonatal rat myocytes. Key: n = number of observations; Con = control; Ouab = 0.15 mM ouabain; K^+ = 10 mM K^+, Tl^+ = 10 mM Tl^+, $^{99}Tc(dmpe)^+$ = 5 mM $[^{99}Tc(dmpe)_2 \cdot Cl_2]^+$. Error bars represent S.E.M. An asterisk (*) = $P < 0.05$.

$^{201}Tl^+$ is believed to be taken up by the (Na^+,K^+)ATPase as a K^+ analog. Studies by Skulskii *et al.* [2] and Britten and Blank [8] have demonstrated that Tl^+ acts as a K^+ analog and, in fact, has greater affinity for the (Na^+,K^+)ATPase. Furthermore, using cultured myocardial cells, McCall *et al.* [9] have reported that $^{201}Tl^+$ uptake is inhibited by either excess levels of non-radioactive Tl^+ or by the addition of either KCN or 2,4-DNP [9]. In related work, Zimmer *et al.* [10] reported that uptake of Tl^+ could be inhibited by 1 mM ouabain. The characteristics of the influx of $^{42}K^+$ and $^{201}Tl^+$ in the cultured neonatal rat myocytes demonstrated that the $^{201}Tl^+$ kinetics were similar to those of $^{42}K^+$ and that most of the influx was ouabain sensitive. The transport of $^{42}K^+$ and $^{201}Tl^+$ was reduced by the addition of unlabeled cation suggesting saturable transport systems. The transport of $[^{99m}Tc(dmpe)_2 \cdot Cl_2]^+$, whose radius is similar to the hydrated potassium ion, differed greatly from $^{42}K^+$ and $^{201}Tl^+$. The diameter of the potassium ion is approximately 5 Å [5], and the diameter of the $[^{99m}Tc(dmpe)_2 \cdot Cl_2]^+$ is approximately 8 Å [4]. Although efficacy as an imaging agent has been demonstrated in laboratory animals [4, 11], the data presented here suggest that $[^{99m}Tc(dmpe)_2 \cdot Cl_2]^+$ is taken up by a mechanism different from that of K^+ and Tl^+. The rate and magnitude of $[^{99m}Tc(dmpe)_2 \cdot Cl_2]^+$ uptake were greater than those of $^{42}K^+$ and were not inhibited by ouabain. Moreover, there was no competitive effect from either unlabeled K^+ or Tl^+.

A similar study using the (Na^+,K^+)ATPase of human erythrocytes confirmed the results obtained using the cultured neonatal rat myocardial cells [6]. The results reported here do not support the hypothesis that $[^{99m}Tc(dmpe)_2 \cdot Cl_2]^+$ represents a K^+ analogue which is taken up by the myocardium by a mechanism similar to that of either $^{201}Tl^+$ or $^{42}K^+$, and myocardial imaging efficiency shown in animals is apparently not reflective of (Na^+,K^+)ATPase activity. Thus, an alternative mechanism independent of the (Na^+,K^+)ATPase is apparently involved in the $[^{99m}Tc(dmpe)_2 \cdot Cl_2]^+$ transport into both human erythrocytes and neonatal rat myocytes.

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Effect of sulfotransferase inhibitors on the 2-acetylaminofluorene-mediated lowering of rat liver *N*-hydroxy-2-acetylaminofluorene sulfotransferase activity

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2-Acetylaminofluorene (AAF) induces liver cancer in rats when administered in various feeding protocols [1–3]. *In vivo* covalent binding of AAF to cellular nucleic acids and proteins also has been found in rat liver [4]. Metabolic conversion of AAF by liver enzymes to a more reactive form, an ultimate carcinogen, is required for AAF covalent binding to cellular molecules [5]. A principal metabolic pathway implicated in the formation of protein–AAF and DNA–AAF adducts involves conversion of AAF to *N*-hydroxy-AAF (*N*-OH-AAF) by the hepatic microsomal cytochrome P-450-dependent monooxygenases [6, 7], followed by conversion of *N*-OH-AAF to the *N*-*O*-sulfate ester of *N*-OH-AAF by an *N*-OH-AAF sulfotransferase [8, 9] believed to be aryl sulfotransferase IV [10–12]. Higher levels of hepatic *N*-OH-AAF sulfotransferase activity in male rats have been correlated with increased hepatotoxicity [13] and higher hepatic cancer incidence [14] during *N*-OH-AAF administration. When *N*-OH-AAF sulfotransferase inhibitors, viz. acetanilide or pentachlorophenol (PCP), are administered with AAF or *N*-OH-AAF, hepatic cancer incidence decreases [15–18] as does carcinogen binding to macromolecules and evidence for hepatotoxicities [19–22].

Experiments to assess the role of *N*-OH-AAF sulfotransferase activity in the carcinogenic and cytotoxic actions of AAF showed that rats undergo a rapid loss of *N*-OH-AAF sulfotransferase activity when fed a diet containing AAF [23]. AAF-mediated losses in sulfotransferase activity were dose dependent, reversible, and occurred without evidence of accompanying AAF