# Uptake Mechanism of Technetium-99m-d,l-HMPAO in Cell Cultures of the Dissociated Postnatal Rat Cerebellum

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The accumulation and retention mechanisms of $^{99m}$Tc-d,l-hexamethylpropyleneamine oxime ($^{99m}$Tc-d,l-HMPAO) were investigated in cultures of the dissociated rat cerebellum. Our experiments indicate a linear dependency of the uptake on incubation time and on the concentration of the radioligand. Upon chloroform extraction and distribution between the lipophilic and the hydrophilic phases, we located 69.1% of the retained radioactivity in the hydrophilic phase, 24.1% in a bound state and 6.8% in the lipophilic phase. The water-soluble, unbound radioactive contents of the cultures were identified as $^{99m}TcO_4^-$ by HPLC analysis. Treatment of cultures with diethyl maleate (DEM) inhibited the accumulation of radioactivity along with a reduction of the GSH contents of the cultures. However, even in the absence of GSH, significant amounts of radioactivity were accumulated. DEM reduced the radioactive contents of cultures predominantly by diminishing the aqueous phase of the chloroform-extracted material. By contrast, the metabolic state, manipulated by treating the cultures with oligomycin B or 2,4-dinitrophenol, had no significant effect on the accumulation of radioactivity. Our experiments suggest two major mechanisms for the retention of radioactivity following the exposure of neuronal tissue to $^{99m}$Tc-d,l-HMPAO: Conversion of the lipophilic complex to the hydrophilic product, $^{99m}TcO_4^-$, and binding to non-diffusible cell components.

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After the introduction of $^{99m}$Tc-d,l-HMPAO as a new agent for imaging regional cerebral blood flow with SPECT tomography (*1–4*), its value for routine clinical investigations was soon realized.

Under optimal conditions, a lipophilic (primary) complex is formed in high yield from $^{99m}TcO_4^-$ and d,l-hexamethylpropyleneamine oxime. However, the complex is not stabile and spontaneously degrades into hydrophilic derivatives: The secondary complex, $^{99m}TcO_4^-$ and $TcO_2x2H_2O$ (*2,5–7*). The lipophilic $^{99m}$Tc-d,l-HMPAO freely crosses the blood-brain barrier, but then rapidly gets trapped in the brain. Neirinckx et al. (*5*) proposed GSH as the rate-determining reactant for an intracellular conversion of lipophilic $^{99m}$Tc-d,l-HMPAO into its hydrophilic, non-diffusible form. GSH, a reducing agent, is present in the organism in high intracellular and low extracellular concentrations, matching the profile of a substance which leads to the tissue accumulation of $^{99m}$Tc. Hence, tissue GSH concentrations were found to determine the retention rates in brain, heart and liver (*5*). Likewise, the GSH contents of brain tumors correlate with the uptake of $^{99m}$Tc-d,l-HMPAO (*8*). In vitro studies showed that the instability of the chelate is enhanced in a concentration-dependent manner by an aqueous solution of GSH (*5,9*). Moreover, the endogenous contents of GSH in rat brain homogenates were found to determine the rate constant of disappearance of the lipophilic complex (*5*).

However, a recent report suggests binding to proteins and cell organelles as an alternative mechanism of significance for the retention of $^{99m}$Tc-d,l-HMPAO in the brain (*10*): Following intravenous administration of $^{99m}$Tc-d,l-HMPAO and the subsequent analysis of subcellular fractions of rat brains, a preponderance of the radioactivity in a bound state has been located by the authors. Thus, 61.2% of the radioactivity in the neocortex was associated with cell organelles, 25.2% with cytoplasmic proteins and only 13.6% as found in an unbound state of the cytosol.

In the present study we have investigated the two retention mechanisms of $^{99m}$Tc-d,l-HMPAO, binding and trapping due to a hydrophilic conversion, and their relative contribution to the accumulation of radioactivity. The experiments were performed using cell cultures of the dissociated postnatal rat cerebellum, which offer the advantage that $^{99m}$Tc-d,l-HMPAO can be applied under a stringent control. Preliminary accounts of these experiments have been given previously (*11,12*).

## MATERIALS AND METHODS

### Neuronal Cell Cultures

The cerebellar tissue of 7-day-old HIM:OFA (Sprague Dawley) rats was removed by dissection and a single-cell suspension was

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prepared by published procedures (*13*). Cells were plated in the absence of serum on poly-D-lysine-coated 24 multiwell tissue culture plates (Nunc #168357) or 35 mm tissue culture dishes (Nunc #153060) at a density of $5.2 \times 10^5$/well or $2.6 \times 10^6$/dish, respectively. The culture medium consisted of Eagles basal medium (Gibco #041-01010) supplemented to contain 2 m*M* glutamine, 25 U/ml penicillin/streptomycin, 17 m*M* glucose and 31 μg/ml insulin, except for a short exposure of the cultures to 10% horse serum (Gibco #034-06050) in order to improve the viability of the cells. This technique yields cultures spread as a monolayer of predominantly neuronal cells (>90%) with a plating efficiency of about 60% after 1 wk in vitro (*14*) and average protein contents of 20 μg/well. Experiments were performed with cultures 4–6 days in vitro.

### Technetium-99m-d,1-HMPAO (Exametazine) Uptake

The experimental incubations with $^{99m}$Tc-d,1-HMPAO were performed in a radiochemical laboratory at ambient temperature (23 ± 2°C). While outside the $CO_2$ incubator, the cultures were kept in a HEPES-buffered (N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid) medium as described (*15*). $^{99m}$Tc-d,1-HMPAO was prepared by mixing one vial of the commercial product, Ceretec (Amersham) with 15–25 mCi per 5 ml $^{99m}TcO_4^-$, freshly eluted from a $^{99}$Mo/$^{99m}$Tc generator (Mallinckrodt, St. Louis). The time since previous elution of the generator was generally less than 2 hr and never exceeded 4 hr. One vial of Ceretec contains 0.5 mg lyophilized d,1-HMPAO, 7.6 μg stannous chloride dihydrate and 4.5 mg NaCl, stoppered under nitrogen. Given the molecular weights of 268.4 and 99, respectively, d,1-HMPAO exceeds $^{99m}TcO_4^-$ in this formula by a ratio of approximately 15,000 to 1. For experimental incubations with $^{99m}$Tc-d,1-HMPAO, the stock solution was diluted $1:10^2$ to $1:10^6$ with a phosphate-buffered saline PBS solution, supplemented to contain 0.1% bovine serum albumin (PBS-BSA: 152.9 mmol $Na^+$, 4.15 mmol $K^+$, 0.9 mmol $Ca^{2+}$, 0.49 mmol $Mg^{2+}$, 142.4 mmol $Cl^-$, 9.42 mmol $HPO_4^{2-}$ and 1 g/liter BSA, pH 7.4). Since $^{99m}$Tc has a specific activity of 7 pmol/mCi, the 1:100 dilution of a 4-mCi/ml stock solution will yield a final concentration of 280 p*M*. The $^{99m}$Tc-d,1-HMPAO incubations were ended by a rapid wash and cultures were then left with PBS for controlled periods of time (generally for 5 min). The radioactivity, released from cultures subjected to osmotic shock with distilled water, was transferred to test tubes and counted with a gamma counter (Biogammall, Beckmann Inc). Unless otherwise noted, each data point was obtained by incubating at least four individual cultures.

Results were adjusted for the radioactive decay of $^{99m}$Tc ($T_{½}$ = 6 hr). In addition, the instability of the $^{99m}$Tc-d,1-HMPAO complex was taken into account and corrected by a first-order rate constant (k) of 0.152 $h^{-1}$ (see Results) according to the equation:

$$y = y_0 \times e^{-kt},$$

where t is the time between complex formation and the beginning of the experimental incubation. For exposures ≥30 min, t denotes the time until the end of the incubation, since the accumulation of radioactivity proved to be linearly dependent on $^{99m}$Tc-d,1-HMPAO concentrations in the medium.

### Modulation of the $^{99m}$Tc-d,1-HMPAO Uptake by Substances

We investigated the effect of diethyl maleate (DEM), a glutathione (GSH)-depleting substance (*16*), $HgCl_2$ and the metabolically active agents oligomycin B and 2,4-dinitrophenol (DNP). The experiments were conducted as described above except that cultures were treated with the substances prior to the incubations with $^{99m}$Tc-d,1-HMPAO. Stock solutions of DEM (Sigma #D7004, 5 *M* in 70% ethanol), oligomycin (Sigma #05126, 1 *M* in 96% ethanol) and $HgCl_2$ (Sigma #M1136, 1 mmol in distilled water) were diluted with PBS to the final concentrations indicated under Results.

### Determination of the Half-Life of $^{99m}$Tc-d,1-HMPAO Using High-Performance Liquid Chromatography (HPLC)

The spontaneous decomposition of the $^{99m}$Tc-d,1-HMPAO complex in PBS-BSA solution was investigated by HPLC using a Hamilton PRP-1/5 micron 4 × 150 mm reversed-phase column. Test samples injected into the column were eluted at a flow rate of 0.5 ml/min with 50% acetonitrile and 2% methanol in 0.01 *M* $HPO_4^{2-}$, pH 7.0. We either used HPLC systems fitted with on-line sodium iodide radioactivity detection or collected 0.3- or 0.5-ml samples which were counted off-line in a gamma counter (Beckmann Inc, Biogammall). Both on-line and off-line radioactivity detections yielded identical results (Fig. 1A).

### HPLC Analysis of the Radioactive Contents of Cultures Exposed to $^{99m}$Tc-d,1-HMPAO

Cerebellar cultures grown on 35-mm tissue culture dishes, were incubated with 261 ± 25 pmol (mean ± s.d., n = 3) $^{99m}$Tc-d,1-HMPAO respectively, for 30 min and rinsed for 5 min with PBS. The radioactive contents of five cultures, pooled and harvested in 2 ml of distilled water, were passed through a 30,000 MW cutoff filter unit (Centricon-30 Microconcentrator, #4208 Amicon, Danvers, MA). Twenty to 500 μl of the filtrate were injected into the HPLC system described above. The radioactivity was determined on-line or off-line in a gamma counter. With either technique, the accumulated radioactivity was identified as $TcO_4^-$ (Fig. 1).

### Chloroform Extraction of the Radioactive Contents of Cultures Exposed to $^{99m}$Tc-d,1-HMPAO

Cerebellar cultures, grown on 35-mm tissue culture dishes, were incubated with $^{99m}$Tc-d,1-HMPAO for 5 min or 30 min, rinsed for 10 min with PBS and harvested in 1 ml per culture of distilled water with a "rubber policeman." One milliliter of chloroform was added and the material was vortexed in a test tube for 1 min. With an additional 1 ml of water and chloroform each, the probes were briefly mixed on a vortex and centrifuged for 3 min at 500 *g*. The aqueous phase ("hydrophilic fraction") was transferred to a counting vial, leaving a denaturated precipitate in the chloroform phase. A "lipophilic fraction" was determined by counting a precipitate-free sample of the chloroform phase and by extrapolating the result to the total volume of chloroform. The "bound fraction" was evaluated by counting the chloroform which contained the precipitate and by subtracting the radioactive contribution of its "lipophilic fraction." the results of two cultures were averaged to obtain one data point.

### HPLC Analysis of the GSH Contents of Cultures

The endogenous GSH contents were assayed in cerebellar cultures grown on 35-mm tissue culture dishes with and without prior exposure to DEM. Cultures placed on ice were harvested in chilled 0.4 *M* perchloric acid with a "rubber policeman." The contents of five cultures were pooled in 1 ml of perchloric acid,

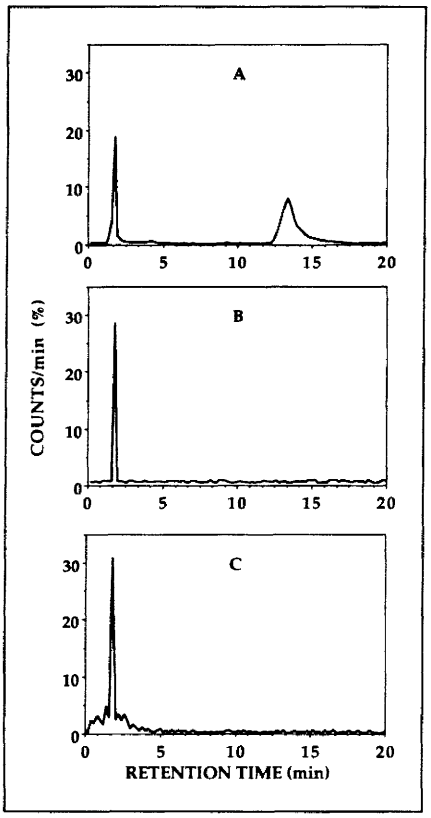


**FIGURE 1.** (A) Chromatogram of $^{99m}$Tc-d, 1-HMPAO degradation products. 120 min after reconstitution of the complex a HPLC column was loaded with 25 $\mu$Ci/5 $\mu$l $^{99m}$Tc-d,1-HMPAO solution (in PBS-BSA) and eluted as described under Material and Methods. Radioactivity, measured on line, is expressed as the percentage of the total radioactivity of the eluate. Peaks correspond to $^{99m}TcO_4^-$, the secondary and the primary complex with retention times of 2 min, 4.2 min, and 13.5 min, respectively. (B) Chromatogram of $^{99m}TcO_4^-$. A 14 $\mu$Ci/5 $\mu$l sample of $^{99m}TcO_4^-$, freshly eluted from a $^{99}$Mo/$^{99m}$Tc generator, was injected into the HPLC column and processed as described above. (C) Chromatogram of the radioactivity accumulated by cerebellar cell cultures. The radioactive contents of cultured cerebellar cells were analyzed after passing the lysate of $^{99m}$Tc-D, 1-HMPAO-loaded cultures through a 30,000-MW cutoff filter unit. A 0.6-$\mu$Ci/500 $\mu$l sample was injected into the HPLC column and processed as described above. Note the single peak coinciding with the retention of $^{99m}TcO_4^-$

frozen in liquid nitrogen and stored for periods of <8 wk at −70°C before being analyzed. Upon thawing, the probes were homogenized by sonication and centrifuged at 17,000 *g* in a chilled rotor. 25-$\mu$l of the supernatant were injected into a HPLC system and analyzed as described previously (*8*). The protein was measured according to Lowry et al. (*17*), and GSH levels are referred to as $\mu$g GSH per mg protein.

### Correlation of the Endogenous GSH Contents with $^{99m}$Tc-d,1-HMPAO Uptake

Control cultures as well as cultures exposed to DEM concentrations ranging from 0.025 mmol to 5 mmol for various periods of time were harvested for measurements of endogenous GSH contents as described above. Sister cultures, maintained in 24 multiwell tissue culture plates and subjected to identical schemes of DEM treatment, were exposed the same day to 2.37 ± 0.32 pmol (mean ± s.d., n = 3) $^{99m}$Tc-d,1-HMPAO for 5 min and processed as described above. Data points were correlated by linear regression using the least squares method.

### $^3$H2-deoxyglucose 2-DG Uptake

We determined the metabolic activity of cerebellar cell cultures in the presence of oligomycin B, 2,4-dinitrophenol (DNP), $HgCl_2$ and DEM by the 2-DG technique. Experiments were performed on cultures grown in 24 multiwell tissue culture plates. Prior to experimental incubations, the bicarbonate-buffered culture medium was replaced by the HEPES-buffered medium described above. Cultures were exposed to the substances for 15 min, incubated with 2-DG for 15 min in the presence or absence (controls) of the substances and rinsed for 1 min with PBS. The radioactive contents of cultures subjected to osmotic shock were harvested and counted with 5 ml Atomlight (NEN) solution in a liquid scintillation counter (Packard Tricarb 460CD) (counting efficiency 30%). 2-(1,2-$^3$H)deoxy-D-glucose, specific activity 30.2 Ci/mmol (NEN) was diluted 1:500 from a 1-mCi/ml stock solution with 6.62 $\mu M$ cold 2-deoxyglucose (Sigma #D8375) in PBS. Thus, 1 ml of the incubation solution (the volume offered to the individual cultures) contained 6.62 $\mu M$ 2-DG with 2 $\mu$Ci radioactivity.

## RESULTS

### Inherent Instability of the $^{99m}$Tc-d,1-HMPAO Complex

Technetium-99m-d,1-HMPAO spontaneously degrades to hydrophilic derivatives. The rate of this reaction depends primarily upon the time since previous elution of the generator, the amount of added radioactivity and the pH of the solution (*2,6,7*).

Using HPLC, we assessed the radiochemical purity of a 2.54 pmol $^{99m}$Tc-d,1-HMPAO (0.349 $\mu$Ci/ml PBS-BSA) solution 5, 35, 65 and 95 min after kit formulation and determined a half-life of the primary, lipophilic complex of 4.56 hr (k = 0.152 $h^{-1}$), which is in line with previous reports (*2,6*). At any time given, $^{99m}TcO_4^-$ was the major degradation product. The secondary, hydrophilic complex never contributed more than 2.5% to the total radioactivity (Fig. 1A). Uncomplexed $^{99m}TcO_4^-$ was not accumulated by the cerebellar cultures (data not shown). We therefore corrected all data presented in this study not only for the radioactive decay of $^{99m}$Tc, but also for the instability of the complex (see Material and Methods).

### Fate of Accumulated $^{99m}$Tc-d,1-HMPAO

The time-dependent release of radioactivity was studied in cerebellar cultures loaded by a 5-min incubation with $^{99m}$Tc-d,1-HMPAO (Fig. 2). Note that after 120 min of washing, the radioactive contents of cultures were 84% of the value following a 15-sec wash, which was the shortest time we could handle with some accuracy (Fig. 2).

The radioactivity retained by cerebellar cultures was analyzed by a two-fold approach.

1. Cell cultures were extracted with chloroform in order to assess three compartments that accommodate the radioactivity: a hydrophilic, a lipophilic and a bound

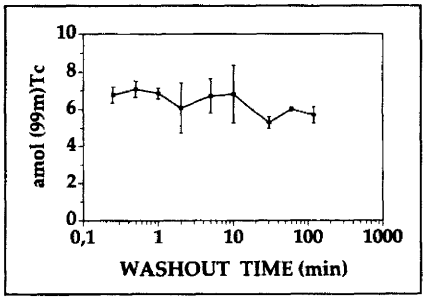


**FIGURE 2.** Time-dependent release of accumulated $^{99m}$Tc. Cerebellar cultures exposed to 2.3 pmol ± 0.03 (mean ± s.d., n = 3) $^{99m}$Tc-d,1-HMPAO for 5 min were rinsed once and then maintained for indicated periods of time in PBS. The figure contains the data (mean ± s.e.m.) of three different dissections, each with 4–6 individual cultures per point.

phase (Fig. 3). The hydrophilic phase of cultures incubated for 30 min with $^{99m}$Tc-d,1-HMPAO occupied 69.1% ± 1.5% (mean ± s.d., n = 5) (Fig. 3, column 2) of the overall radioactivity. The bound fraction contained 24.1% ± 1.2%, leaving 6.8% ± 0.6% for the lipophilic fraction. Cultures incubated with $^{99m}$Tc-d,1-HMPAO for just 5 min displayed an identical distribution of the three compartments (Fig. 3, column 1).

The chloroform extraction protocol was also applied to cultures pretreated for 30 min with DEM or $HgCl_2$. Both substances decreased the accumulation of radioactivity in a concentration-dependent manner (see below). Treatment with $HgCl_2$ diminished the radioactive contents by affecting all compartments alike (Fig. 3, column 5), whereas DEM predominantly reduced the aqueous compartment, bringing about a relative increase of the bound fraction (Fig. 3, columns 3 and 4). However, in absolute terms DEM also diminished the bound fraction (0.5 mmol by 2.1%, not significant, $p > 0.05$; 5 mmol by 8.1%, significantly different from cntrol, $p < 0.01$).

2. The water-soluble radioactive contents were analyzed by harvesting cultures in distilled water and injecting the lysate into a Hamilton PRP-1 HPLC column, after proteins and cellular fragments had been removed through ultrafiltration. The HPLC analysis of the filtrate revealed a single, well separated peak coinciding with $^{99m}TcO_4^-$ (Fig. 1B-C).

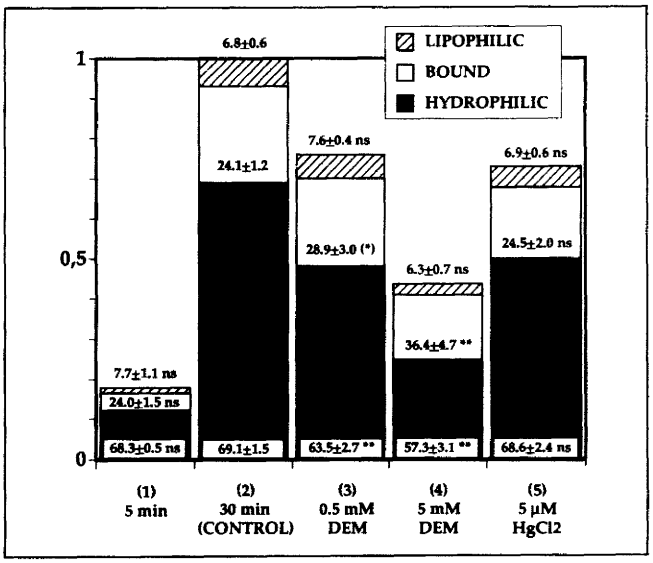


**FIGURE 3.** Chloroform extraction of radioactivity from cultures loaded with $^{99m}$Tc-d,1-HMPAO. Cultures incubated with 32.3 ± 3 pmol (mean ± s.d., n = 5) $^{99m}$Tc-d,1-HMPAO for 5 min (column 1) or 30 min (columns 2–5) were harvested and subjected to the chloroform extraction protocol. Cultures of columns 3–5 were pretreated for 30 min with 0.5 m*M* DEM, 5 m*M* DEM, or 5 µ*M* $HgCl_2$, respectively. The height of each column represents the ratio of its total radioactivity to the control (column 2). The chloroform extraction allows to identify three different fractions: a lipophilic, a bound, and a hydrophilic phase. The figures associated with the columns represent the contribution of each fraction (lipophilic, bound, hydrophilic, mean ± s.d.) to the total radioactivity of the column. The fractions of column 2 (control) were compared to the fractions of columns 1 and 3–5 using the Student's t-test for unpaired samples. (ns): $p > 0.05$; (*): $p < 0.05$; (**): $p < 0.01$.

### Time and Concentration Dependence of the $^{99m}$Tc-d,1-HMPAO Uptake

Cultures exposed to $^{99m}$Tc-d,1-HMPAO for periods ranging from 30 sec to 2 hr showed a linear dependency of the uptake on the time of incubation (Fig. 4A). Likewise, the uptake was linearly related to concentrations ranging from 27 f*M* to 272 p*M* with incubation periods kept constant (Fig. 4B).

### Correlation of Endogenous GSH Levels with the Uptake of $^{99m}$Tc-d,1-HMPAO

Endogenous GSH has been attributed a crucial role in the retention of $^{99m}$Tc-d,1-HMPAO by mediating the hydrophilic conversion of the chelate (*5,9*). We tested this hypothesis by treating cerebellar cultures with the GSH-depleting agent DEM and subsequently measuring the accumulated $^{99m}$Tc (Fig. 5).

In a series of three experiments, we compared the GSH contents of cerebellar cultures to the uptake of $^{99m}$Tc, both manipulated by identical schemes of exposures to DEM (Fig. 6). Note that the regression line intersects the ordinate at 2.92 mmol $^{99m}$Tc, indicating a significant accumulation of radioactivity even in the absence of GSH.

### Altered Metabolic Activity and the Uptake of $^{99m}$Tc

We examined whether altered metabolic activity would affect the accumulation of $^{99m}$Tc and used the uptake of 2-DG as an assay to monitor the metabolic state of the cultures. 2-DG accumulation was severely impaired by treating the cultures with oligomycin B and DNP (Fig. 7), whereas the accumulation of $^{99m}$Tc was not significantly affected. $HgCl_2$, on the other hand, limited the accumulation of both 2-DG and $^{99m}$Tc, whereas DEM only reduced the uptake of $^{99m}$Tc-d,1-HMPAO.

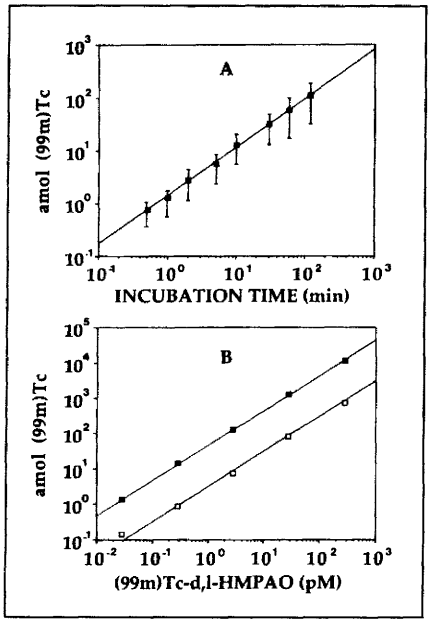


**FIGURE 4.** (A) Time-dependent uptake of $^{99m}$Tc-d,1-HMPAO. Multiwell cultures were incubated with 1 ml of 2.41 ± 0.28 pmol/l; mean ± s.d., n = 3) $^{99m}$Tc-d,1-HMPAO for indicated periods of time. The data (mean ± s.e.m.) are three different dissections, each with four individual cultures per point. Points were fitted to a linear regression line by the method of least squares. (B) Concentration-dependent uptake of $^{99m}$Tc-d,1-HMPAO. Multiwell cultures were incubated with 1 ml of $^{99m}$Tc-d,1-HMPAO with concentrations ranging from 27 fmol/l to 272 pmol/l for 5 min (lower line) or 120 min (upper line) and processed for the determination of radioactivity as described in Material and Methods. Each point represents the mean of four individual cultures (s.d.'s < 10%). Data points were fitted to a linear regression line by the method of least squares. The slopes of the two functions do not differ significantly one from another.

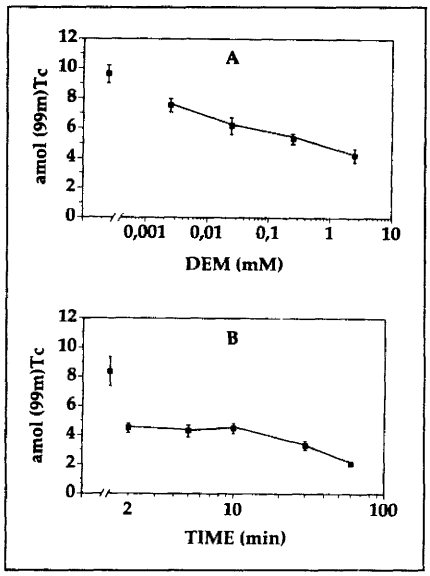


**FIGURE 5.** Effect of diethyl maleate (DEM) on $^{99m}$Tc-d,1-HMPAO uptake. (A) Dose-effect relationship: Controls and cerebellar cultures were treated with indicated concentrations of DEM for 30 min and exposed to 2.31 pmol $^{99m}$Tc-d,1-HMPAO for 5 min. Each data point represents the mean ± s.d. of the radioactive contents of four multiwell cultures. (B) Time-dependence of DEM effects: Cerebellar cultures treated with 5 m*M* DEM for indicated periods of time were rinsed with PBS (1 min) and exposed to 2.23 pmol $^{99m}$Tc-d,1-HMPAO for 5 min. Each data point represents the mean ± s.d. of the radioactive contents of four multiwell cultures.

## DISCUSSION

The present study investigates the uptake and retention mechanisms of $^{99m}$Tc-d,1-HMPAO in cell cultures of the dissociated postnatal rat central cerebellum. We incubated cultures with $^{99m}$Tc-d,1-HMPAO for periods ranging from 30 sec to 2 hr. Our data show an uptake strictly linear with time, indicating that the reactions leading to the accumulation of radioactivity did not reach a state of equilibrium. Likewise, the quantity of radioactivity accumulated by the cultures was linearly dependent on the concentration of $^{99m}$Tc-d,1-HMPAO in the incubation medium. If the uptake was linked to an enzymatic reaction or a carrier-mediated phenomenon, we would expect the uptake curve to reach a plateau at high substrate concentrations. However, due to the stringent procedures for reconstituting the complex (see Material and Methods), the maximal concentration we could apply was 235 pmol, which most likely, was not sufficient to test the hypothesis of saturable binding sites.

We examined the radioactive contents of cultures that had been exposed to $^{99m}$Tc-d,1-HMPAO by a two-fold approach: First, by extracting cultures with chloroform,

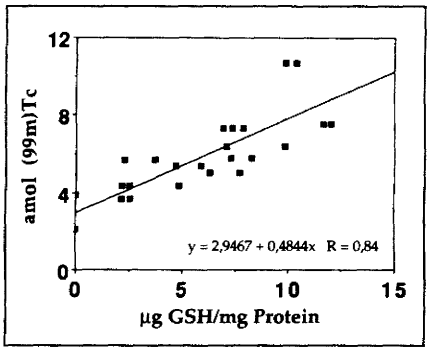


**FIGURE 6.** Correlation of endogenous glutathione (GSH) levels with the uptake of $^{99m}$Tc-d,1-HMPAO. Cerebellar cultures from three different dissections were exposed to 2.37 ± 0.32 pmol (mean ± s.d., n = 3) $^{99m}$Tc-d,1-HMPAO for 5 min and processed for the determination of their radioactive contents as described under Material and Methods. The averaged data of four multiwell cultures were used to plot the data points. HPLC determinations of the GSH contents were performed with the pooled material of five individual cultures grown in 35-mm tissue culture dishes. The GSH levels (abscissa) were plotted against the $^{99m}$Tc contents (ordinate) of identically treated sister cultures. Endogenous GSH contents were reduced by treating cultures with DEM concentrations ranging from 0.025 to 5 m*M*. The data points at the intersection with the ordinate are derived from cultures exposed to 5 m*M* DEM for 30 min. Data were fitted to a linear regression function with a correlation coefficient of 0.84.

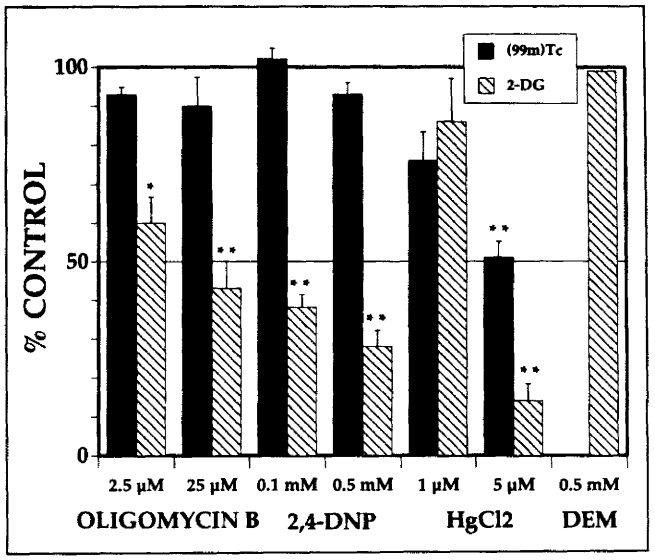


**FIGURE 7.** Metabolic state and $^{99m}$Tc accumulation. $^{99m}$Tc: Cerebellar cultures, treated for 30 min with indicated concentrations of substances, were exposed to 2.53 ± 0.16 pmol $^{99m}$Tc-d,1-HMPAO for 5 min. Bars contain the data (mean radioactive contents ± s.e.m.) of at least three different dissections, each with 4 individual multiwell cultures. 2-DG: Cerebellar cultures, treated for 15 min with indicated concentrations of substances were exposed for 15 min to 2 µCi/ml 2-DG in the presence or absence of the substance and processed for the determination of their radioactive contents as described under Material and Methods. Bars contain the data (mean ± s.e.m.) of at least three different dissections, each with four individual multiwell cultures. Drug effects were compared to controls using the Student's t-test for unpaired samples. (ns): $p > 0.05$; (*): $p < 0.05$; (**): $p < 0.01$.

and second, by analyzing the unbound, water-soluble radioactivity with HPLC.

Chloroform, along with ethyl acetate, has been used for the separation of the lipophilic $^{99m}$Tc-d,1-HMPAO from its hydrophilic degradation products (*18*). In a two-phase system with water, chloroform can be expected to dissolve lipids, denature proteins and to accumulate the lipophilic $^{99m}$Tc-d,1-HMPAO. The hydrophilic degradation products of the chelate will occupy the aqueous phase.

Following the extraction with chloroform, we recovered 69.1% of the radioactivity in the aqueous phase of the cultures, in which 24.1% was associated with denatured cell components and the complementary 6.8% was found dissolved in the lipophilic phase. These ratios were identical for cultures exposed for 5 min or 30 min and suggest two primary mechanisms for the retention of $^{99m}$Tc: The conversion of the lipophilic complex into a hydrophilic, non-diffusible product which gets trapped inside the cells, and binding of radioactivity to cell organelles or proteins. The former mechanism has been proposed by Neirinckx et al. (*5*) and appears to be largely GSH-dependent (see below), whereas binding as a major principle for the accumulation of radioactivity was recently proposed by Costa et al. (*10*).

The nature of this binding is yet unknown. It seems remarkably stable, since $^{99m}$Tc appears still associated with trichloroacetic acid-precipitated brain cell organelles and cytoplasmic proteins when rats had been injected with $^{99m}$Tc-d,1-HMPAO (*10*). Using HPLC, we identified $^{99m}TcO_4^-$ as the only water-soluble, unbound radioactive product of cultures exposed to $^{99m}$Tc-d,1-HMPAO. However, the HPLC technique requires the exclusion of macromolecules before injecting a sample into the column, and $^{99m}$Tc-d,1-HMPAO or degradation products other than $^{99m}TcO_4^-$ may remain undetected in a bound state. Due to its hydrophilic properties, $^{99m}TcO_4^-$ is neither taken up by brain cells in vitro (this study), nor does it pass the intact blood brain barrier in vivo.

A previous report has shown that the secondary complex, which elutes in our HPLC system well separated from the primary complex and pertechnetate, is the main decomposition product following incubations of $^{99m}$Tc-d,1-HMPAO with 0.1 mmol GSH or 10% rat brain homogenate (*5*). The reason for this variance is unknown, but it is conceivable that intact cells handle the chelate complex in a way different to homogenized tissue. If the hypothesis of a GSH-mediated hydrophilic conversion of $^{99m}$Tc-d,1-HMPAO holds true, depletion of the intracellular GSH should lead to a reduced accumulation of radioactivity in our culture system. We followed this approach by treating cerebellar cultures with two substances of a diverse mode of action: DEM and $HgCl_2$. Whereas DEM specifically depletes tissue GSH levels by an enzyme-mediated reaction (*16,19*), $HgCl_2$ has additional effects on cell functions due to the high affinity of mercuric ions to thiol and disulfide residues (*20*). Our data indicate that DEM, along with reducing the GSH contents of the cultures, also lessens the accumulation of radioactivity. This reduction primarily originates from a decrease of the hydrophilic phase, as revealed by our experiments using the chloroform extraction protocol, and supports the above mentioned hypothesis of a GSH-mediated hydrophilic conversion of $^{99m}$Tc-d,1-HMPAO (*5*). DEM also decreased the bound radioactive contents of the cultures, but this effect had significantly less impact on the overall retention of $^{99m}$Tc. $HgCl_2$, on the other hand, affected all three phases alike, underlining the poor specificity of the substance.

Following a completely different approach, we were curious whether an altered metabolic state would affect the accumulation of $^{99m}$Tc. We treated cultures with oligomycin B, which blocks, or DNP, which uncouples oxidative phosphorylation (*21*). Effects of these substances have been described in cell suspensions harvested from monolayer cultures of the rat cerebellum, where oligomycin B and DNP cause decrease or increase of respiratory rates, respectively (*22*). We observed pronounced effects by both substances on energy metabolism, monitored by the uptake of 2-DG, whereas the accumulation of $^{99m}$Tc was not significantly affected. DEM, on the other hand, had no effect on the metabolic state. Given the dependence

of $^{99m}$Tc accumulation on the GSH levels, we may therefore conclude by indirect evidence that metabolic changes caused by DNP or oligomycin B do not significantly alter the GSH levels of the cultures. This observation is in line with in vivo experiments, where it requires drastic measures of hypoxia in order to observe measurable changes of the GSH contents (*23,24*).

In conclusion, our cell culture experiments suggest a two-fold retention mechanism for $^{99m}$Tc-d,l-HMPAO and provide an estimate for the relative impact of each of these factors. Previous compartment models for the central nervous system have only considered the (GSH-dependent) hydrophilic conversion as the rate-limiting step for the accumulation of $^{99m}$Tc (*5,25,26*). However, since the binding by nervous tissue significantly contributes to the overall radioactivity, it will have to be considered in such models.

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