Inhibition of the Nuclear Entry of 3,3',5-Triiodo-L-thyronine by Monodansylcadaverine in GH\textsubscript{3} Cells*

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We have recently reported that the entry of 3,3',5-triiodo-L-thyronine (T\textsubscript{3}) into mouse 3T3 fibroblasts occurs by receptor-mediated endocytosis (Cheng, S. Y., Maxfield, F. R., Robbins, J., Willingham, M. C., and Pastan, I. (1980) Proc. Natl. Acad. Sci. U. S. A. 77: 3425-3429). In this communication, we evaluated the functional significance of this mode of entry using GH\textsubscript{3} cells, a growth hormone-producing cell line which has a high number of T\textsubscript{3} nuclear receptors. T\textsubscript{3}-specific, saturable membrane uptake systems were demonstrated in GH\textsubscript{3} cells. Monodansylcadaverine, an inhibitor of \(\alpha_2\)-macroglobulin, epidermal growth factor, vesicular stomatitis virus, and T\textsubscript{3} uptake in fibroblasts, blocked virtually all of the cellular uptake of T\textsubscript{3} with a half-maximal concentration of 29 \(\mu\text{M}\). Concomitant with the inhibition of the cellular uptake of T\textsubscript{3}, the accumulation of T\textsubscript{3} into nuclei was reduced. The inhibitory effect of monodansylcadaverine on the reduction of T\textsubscript{3} incorporation into nuclei was not due to the inhibition of binding to nuclear receptors, but probably was due to a decrease in the cytoplasmic availability of T\textsubscript{3} as a result of inhibition of cellular entry. These results indicate that the uptake of T\textsubscript{3} by receptor-mediated endocytosis is a physiologically significant process.

There is a large body of evidence showing that the initiation and expression of the action of thyroid hormone is through the interaction of 3,3',5-triiodo-L-thyronine with nonhistone proteins in chromatin (1-3). The mode of entry of thyroid hormones was long believed to be by passive diffusion. However, recent studies have demonstrated the presence of T\textsubscript{3} or T\textsubscript{4} transport or receptor-binding proteins in rat liver and other tissues (4-9). Using rhodamine-labeled T\textsubscript{3} we have shown that one mechanism by which T\textsubscript{3} enters into cultured cells is by receptor-mediated endocytosis (10, 11). In those studies the physiological significance of such entry was not clearly defined.

One way to evaluate the biological relevance of the uptake of T\textsubscript{3} by receptor-mediated endocytosis is to demonstrate a reduction or total inhibition in the nuclear uptake of T\textsubscript{3} as a consequence of blocking this specific pathway. We have selected GH\textsubscript{3} cells, a rat pituitary cell line, for such studies. GH\textsubscript{3} cells contain approximately 16,000 T\textsubscript{3} nuclear receptor sites/cell which is approximately 3 to 4 times more than mouse 3T3 fibroblasts. The production of growth hormone in these cells is stimulated by thyroid hormone. (12).

Monodansylcadaverine is a potent inhibitor of receptor-mediated endocytosis in fibroblasts. It blocks the cellular internalization of \(\alpha_2\)-macroglobulin (13, 14), low density lipoprotein (15), epidermal growth factor (16, 17), vesicular stomatitis virus' and T\textsubscript{3} in mouse fibroblasts (10). The present study demonstrates that MDC blocks in a dose-dependent manner the receptor-mediated uptake of T\textsubscript{3} by GH\textsubscript{3} cells as well as the accumulation of T\textsubscript{3} in the nucleus. Thus, the data strongly suggest that the availability of T\textsubscript{3} to nuclear receptors is mediated by this pathway.

\textbf{MATERIALS AND METHODS}

\textbf{Reagents}

\[ ^{125}\text{I}T_3 \] (1200 \(\mu\text{Ci/\mu g}\)) and \(L-[\text{\textsuperscript{3}H}]\text{amino-acids (0.1 mCi/ml) were purchased from New England Nuclear. Unlabeled T_3, bovine serum albumin, spermidine, spermine, and 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid were from Sigma. Horse serum, fetal calf serum, and Ham’s F-10 medium were purchased from Grand Island Biological Co. [U-\textsuperscript{3}H]Monodansylcadaverine (7.1 Ci/mmol) was obtained from Amersham Corp. 3,5-Diaminobenzoic acid dihydrochloride was obtained from Nakarai Chemicals, Kyoto, Japan. Dibutyl phthalate and dinonyl phthalate were obtained from Eastman.

\textbf{Cell Culture}

GH\textsubscript{3} cells were obtained through the courtesy of D. A. Tashjian, Jr. (Harvard Medical School, Boston). Cell cultures were routinely grown in 95% air, 5% CO\textsubscript{2} with Ham’s F-10 medium supplemented with 15% horse serum and 2.5% fetal calf serum. Cells were plated at a density of 2 \(\times\) 10\textsuperscript{5} cells/cm\textsuperscript{2} flask and used in the late logarithmic phase of growth.

\textbf{Binding or Uptake of [\textsuperscript{125}I]T\textsubscript{3}, by Cells}

Two methods were used to analyze the binding and uptake of [\textsuperscript{125}I] T\textsubscript{3} by cells. These two methods differ only in the procedure to separate unbound T\textsubscript{3} from the cells. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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\‡ The abbreviations used are: T\textsubscript{3}, 3,3',5-triiodo-L-thyronine; PBS, phosphate-buffered saline; MDC, monodansylcadaverine; \(\alpha_2\)-M, \(\alpha_2\)-macroglobulin.

\(\textsuperscript{1}\) S. Y. Cheng, and R. Horiuchi, manuscript in preparation.

tubes before the determination of the radioactivity eliminated the contribution of nonspecific adsorption of $^{125}$I T$_3$ in the tubes to the total uptake of $^{125}$I T$_3$ by the cells.

Oil-Centrifugation Method—A modified procedure of Kaneko et al. (18) was adopted. Cells were detached from the flasks and incubated with $^{125}$I T$_3$ similarly as described under “Washing Method.” At the end of incubation, 200 ml of the incubation mixture was layered on top of a three-layer solution in Eppendorf 400 ml microtest tubes (No. 5421). The three layers of solutions were (from top to bottom): 40 ml of dibutyl phthalate:dimonyl phthalate (6:5.3:5, v/v); 150 ml of buffer containing 0.25 mM sucrose, 0.5 mM CaCl$_2$, and 10 mM Tris (pH 7.4); and 30 ml of dibutyl phthalate:dimonyl phthalate (8:2, v/v). At 4 °C cells were rapidly separated from unbound T$_3$ by centrifugation at 12,900 × g for 1 min. The tips of the tubes containing cells in the lower oil solution were cut. The radioactivity associated with the cells was determined with a Beckman Gamma 8000 spectrometer.

The choice of the two methods depended on the purpose of the experiments. Since the oil-centrifugation method resulted in cells pelleting into organic reagents, subsequent isolation of nuclei was obviously impossible. Therefore, if isolation of nuclei was required for quantification of nuclear bound T$_3$, the washing method was used.

Preliminary experiments showed that total uptake T$_3$ by cells at 37 °C differed by less than 10% between these two methods.

Incorporation of $^{125}$I T$_3$ into Nuclei in Intact Cells

After incubation of $^{125}$I T$_3$ with intact cells at 37 or 0 °C and subsequent washings as described above, the pellets were treated twice (2 ml) with a buffer containing 60 mM KCl, 15 mM NaCl, 0.5 mM spermine, 0.15 mM spermidine, 2 mM EDTA, 0.5 mM ethylene glycol bis(β-aminoethoxy) ether)-N,N,N',N'-tetracetic acid, 15 mM mercaptoethanol, and 0.5% Triton X-100 (19) (buffer A) at 0 °C. Nuclei were pelleted at 400 × g for 5 min and the incorporation of $^{125}$I T$_3$ into nuclei was determined as described above.

Binding of $^{125}$I T$_3$ to Isolated Nuclei

GH$_3$ cells were detached from the flask as described above. Cell suspensions were incubated with 1 ml Tris-HCl, pH 7.4, at 0 °C for 4 min. Cells were homogenized with a Dounce homogenizer (5 strokes). To this cell suspension was added immediately 3 volumes of a buffer containing 67 mM KCl, 20 mM NaCl, 23 mM KHO$_3$, 30 mM KH$_2$PO$_4$, 15 mM MgCl$_2$, 10 mM KOH, 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid pH 7.1, (buffer B). Nuclei were isolated by centrifugation at 1000 × g for 3 min. The nuclei were immediately suspended in buffer N and incubated with 0.5 nm $^{125}$I T$_3$ at 37 °C for 30 min in the presence or absence of MDC. After incubation the nuclei were pelleted by centrifugation. The nuclei were washed once with buffer N and further washed with buffer A as described above. The radioactivity was determined similarly as described.

Uptake of $[^{3}H]$ monodansylcadaverine by GH$_3$ Cells

The cells (2.7 × 10$^5$ cells) in 60-mm dishes were rinsed 3 times with PBS and incubated with various concentrations of $[^{3}H]$ monodansylcadaverine as designated in the figure legends. After incubation for 30 min at 37 °C, the cells were washed 4 times with PBS and harvested with a rubber policeman. One-tenth of the cell suspension was dissolved in 1 ml of solubilizer (NCS, Amersham Corp.). Ten ml of the scintillant Biofluor (New England Nuclear) was added and the radioactivity was determined with a Packard Tri-Carb 460CD scintillation spectrometer. Nuclei were isolated from nine-tenths of the cell suspension as described above, and the radioactivity associated with the nuclei was determined.

The uptake experiments were also carried out in suspended cells. GH$_3$ cells were detached from the flask as described above. Cell suspensions were incubated with various concentrations of $[^{3}H]$ monodansylcadaverine similarly as described above. The cell pellets were chilled and pelleted for 3 min (200 × g), and the cell pellet was washed 3 times with PBS (1 ml) at 0 °C. All further steps were carried out at 0 °C. The cell pellets were suspended in 1 ml of PBS. One-tenth of the cell suspension was dissolved in 0.2 ml of 5 N NaOH, 0.7 ml of H$_2$O, and 10 ml of Aquasol (New England Nuclear). The radioactivity was determined as described above. Nuclei were isolated from the remaining nine-tenths of the cell suspension as described above, and the radioactivity associated with the nuclei was determined similarly.

Cellular Protein Synthesis

GH$_3$ cells (1.5 × 10$^6$ cells/tube) were incubated with 1 ml of Ham’s F-10 medium containing 0.2 μCi of L-[14C]amino-acids and various concentrations of MDC for 30 min at 37 °C. At the end of incubation, the cells were chilled to 0 °C. An equal volume of 1.2 n perchloric acid was added to the cell suspension and allowed to stand for 1 h at 0 °C. The suspension was filtered through Whatman GF/C glass fiber filter paper. The paper was washed 4 times with 0.6 n perchloric acid (2 ml each) and 2 times with 95% ethanol. After drying, the paper was put into a vial containing Aquasol-2 (New England Nuclear) and counted with a Beckman scintillation spectrometer.

Determination of DNA

Cells or isolated nuclei were washed once with 5% trichloroacetic acid at 0 °C. DNA was extracted by incubation of cells or isolated nuclei for 30 min at 90 °C. The suspension was cooled at 0 °C and centrifuged at 1500 × g for 15 min. DNA content was determined according to the method of Setaro and Morley (20).

RESULTS

Uptake of $[^{125}I]$ T$_3$ by Cells and Its Incorporation into Nuclei

At 37 °C—At 37 °C, the uptake of T$_3$ increased with time and reached a plateau after 2 h (Fig. 1). The incorporation of $[^{125}I]$ T$_3$ into nuclei followed similar kinetics. About 10% of the total cellular $[^{125}I]$ T$_3$ taken up is incorporated into nuclei. The kinetics of the binding of T$_3$ to nuclei of intact cells was identical to that reported previously for GH$_3$ cells (21).

In Fig. 2 the binding of T$_3$ by the nuclei of intact cells is shown plotted by the method of Scatchard (22). The solid lines in the Scatchard plot are the theoretical curves calculated from a nonlinear least squares fit assuming one class of binding sites using the computer procedures described by Johnson et al. (23). The data fit the calculated curves to give an apparent K$_o$ of 0.071 nm and a binding capacity of 64 fmol/100 μg of DNA. The inset in Fig. 2 shows a plot of $[^{125}I]$ T$_3$ bound versus free $[^{125}I]$ T$_3$ concentrations. The amount of $[^{125}I]$ T$_3$ bound reached a plateau when the free $[^{125}I]$ T$_3$ concentration was at 0.5 nm.

Temperature-dependent Uptake of T$_3$ by Cells and Its Incorporation into Nuclei—Both the uptake of T$_3$ by intact cells and its subsequent incorporation into nuclei were temperature-dependent (Fig. 3). When cells were incubated with 0.1 μM $[^{125}I]$ T$_3$ at 0 °C for 30 min, total uptake by the cells was

*In this system where T$_3$ first interacts with plasma membrane receptors and later is transferred to the nucleus, the reversibility of the binding has not been established. Consequently, it is not necessarily proper to use an analysis based on equilibrium thermodynamics, i.e., Scatchard analysis. However, because a Scatchard-type analysis is the standard method employed in this field, we have used it to characterize the binding of T$_3$. 

**Figu1. Time-dependent uptake of $[^{125}I]$ T$_3$ by GH$_3$ cells (O) and its incorporation to nuclei (C). Cells (2 × 10$^6$ cells) were incubated with 0.5 nm $[^{125}I]$ T$_3$ at 37 °C. At the times indicated they were washed, and the nuclei were isolated as described under “Materials and Methods.” Each time point represents the mean of duplicate determinations with variations less than 5%.
were incubated with 

Each time point represents the mean of duplicate determinations 

were isolated as described under “Materials and Methods.” The lines are theoretical 

37°C-Fig. 2. Scatchard analysis of nuclear binding of [125I]T3 in 

Competitive Inhibition of the Binding of [125I]T3 by Unlabeled T3—Fig. 4 shows the time-dependent binding of 

TABLE I 

only 15% of that at 15°C and 3% of that at 37°C. When cells were incubated with [125I]T3 at 0°C and the cells disrupted and the nuclei isolated, radioactive was not detectable in the nuclei. At 15°C incorporation of T3 into nuclei was less than 

Competitive Inhibition of the Binding of [125I]T3 by Unlabeled T3 at 0°C—Fig. 4 shows the time-dependent binding of 

Fig. 3. Temperature-dependent uptake of [125I]T3 by GH3 cells and its incorporation into nuclei. GH3 cells (6.8 X 106 cells) were incubated with 0.1 nM [125I]T3 for 30 min at 0, 15, or 37°C. Unbound [125I]T3 was separated from cells by oil-centrifugation and the nuclei were subsequently isolated. The radioactivity associated with cells (●) and nuclei (▲) was determined as described under “Materials and Methods.” 

15 μM unlabeled T3 for 45 min; 74% of [125I]T3 was dissociated into the medium. In contrast, when cells were treated similarly at 37°C, only 16% was dissociated into the medium. These results showed that binding of [125I]T3 to cell surface receptors is not only saturable but also reversible at 0°C. At 37°C [125I]T3 is internalized and not reversibly displaced by the large excess of unlabeled T3. 

Transfer of Plasma Membrane-Bound [125I]T3 into the Nuclei—To demonstrate the functional significance of the receptors, the following experiments were carried out. GH3 cells were incubated with 0.5 nM [125I]T3 for 2 h at 0°C. Under these conditions, [125I]T3 was not incorporated into the nuclei (see above). After the 2-h incubation, the cells were thoroughly washed as described under “Materials and Methods.” The cells were then warmed to 37°C for various lengths of time. Fig. 5 shows that the ratio of T3 incorporated into the nuclei to total T3 associated with the intact cells increased linearly with time and reached a plateau after 60 min. The warming of the [125I]T3-bound cells from 0 to 37°C resulted in the dissociation of 35% of the bound radioactivity at 5 min and 40% after 10 min. No additional dissociation of [125I]T3 into the medium was detected with longer incubation indicating that probably all the remaining bound [125I]T3 was already internalized. These results strongly suggest that the T3 membrane receptors mediate the translocation of membrane-bound T3 into the nuclei. 

Effects of Monodansycadaverine on the Internalization of T3—To evaluate the effect of MDC on the internalization of T3, GH3 cells were incubated with 0.5 nM [125I]T3 in the presence of increasing concentrations of MDC at 0°C for 2 h.
Effect of Monodansylcadaverine on Nuclear Entry of T3

After unbound T3 was removed by extensive washing, the cells were warmed to 37 °C. As shown in Table II, translocation of T3, from the plasma membrane to the nucleus was progressively reduced by MDC. At 50 μM MDC the translocation of T3 from the plasma membrane to the nucleus was completely blocked. MDC could be acting to reduce the accumulation of T3 in the nucleus by decreasing binding to the membrane, decreasing translocation of T3 into the cell (inhibition of endocytosis), decreasing translocation of intracellular T3 to the nucleus, or directly decreasing nuclear binding. To distinguish among these possibilities the following experiments were performed.

Whether MDC directly inhibited the binding of T3 to plasma membrane receptors was evaluated by incubating [125I]T3 cells and nuclei was decreased in the presence of MDC at 0 °C. As shown in Fig. 6, binding of [125I]T3 to membrane receptors was not altered by the presence of MDC up to 200 μM. Thus, the reduction in the translocation of T3 into nuclei was not due to the inhibition of T3 binding to membrane receptors.

The effect of MDC on the internalization of T3 was evaluated by incubating the cells with 0.5 nM [125I]T3 at 37 °C for 30 min in the presence of increasing concentrations of MDC.

As shown in Fig. 7, MDC inhibited the internalization of T3 in a dose-dependent manner with a half-maximal inhibitory concentration of 29 μM. Fig. 7 further demonstrates the corresponding reduction in nuclei-bound [125I]T3. These results clearly show a correlation between the membrane internalization event and the subsequent nuclear incorporation. Whether MDC is only affecting internalization or also affecting some intracellular event is discussed further below.

To evaluate possible effects of MDC on nuclear binding, the uptake of MDC by the cells and its possible interaction with the nucleus was assessed. Furthermore, the binding of [125I]T3 to isolated nuclei in the presence of increasing concentrations of MDC was evaluated. As shown in Table III, in the presence of 0.28, 10, and 100 μM MDC, 3.5, 2.8, and 1.8% of MDC was taken up by the cells. No difference in the uptake was detected whether the incubations were carried out using monolayer or suspended cells. However, no MDC was detected in isolated nuclei. Moreover, as shown in Table IV, MDC at concentrations up to 100 μM did not affect the binding of [125I]T3 to isolated nuclei.

![Graph showing time-dependent translocation of plasma membrane-bound [125I]T3 to nuclei of GH3 cells.](http://www.jbc.org/)

### Table II

Effect of monodansylcadaverine on the translocation of cellular bound [125I]T3 to nuclei

<table>
<thead>
<tr>
<th>Monodansylcadaverine (μM)</th>
<th>[125I]T3 nuclear-bound/cell-bound</th>
<th>Nuclei-bound [125I]T3</th>
<th>Cellular protein synthesis</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2.2</td>
<td>0%</td>
<td>100%</td>
</tr>
<tr>
<td>10</td>
<td>0.6</td>
<td>73%</td>
<td>99%</td>
</tr>
<tr>
<td>50</td>
<td>0</td>
<td>100%</td>
<td>96%</td>
</tr>
<tr>
<td>100</td>
<td>0</td>
<td>100%</td>
<td>90%</td>
</tr>
</tbody>
</table>

4 GH3 cells (1 × 10⁶ cells) were incubated with 1 ml of [125I]T3 (0.5 nM) in serum-free Ham's F-10 medium for 2 h at 0 °C. Unbound [125I]T3 was separated from cells by the washing method. After cells were subsequently warmed up to 37 °C at various lengths of time, nuclei were isolated. The radioactivity associated with cells and nuclei was determined as described under "Materials and Methods."

4 GH3 cells (3 × 10⁶ cells) were incubated with 1 ml of [125I]T3 (0.5 nM) in serum-free Ham's F-10 medium for 2 h at 0 °C. Unbound [125I]T3 was separated from cells by the washing method. After cells were subsequently warmed up to 37 °C to allow the internalization to take place. Isolation of the nuclei and the determination of nuclei-bound [125I]T3 were described in detail under "Materials and Methods." Values are averages of triplicate determinations and the variations were less than 5%.

![Graph showing effect of monodansylcadaverine on the binding of [125I]T3 to GH3 cells.](http://www.jbc.org/)

![Graph showing inhibition of cellular and nuclear uptakes of [125I]T3 by monodansylcadaverine.](http://www.jbc.org/)
Effect of Monodansylcadaverine on Nuclear Entry of T₃

TABLE III

Uptake of [U-¹²⁵I]monodansylcadaverine by GH₃ cells

GH₂ cells (2.7 × 10⁶ cells) were incubated with 1 ml of serum free Ham’s F-10 medium containing various concentrations of [U-¹²⁵I]monodansylcadaverine for 30 min at 37°C. Cellular and nuclear uptakes of MDC were determined as described under “Materials and Methods.” The values represent the mean ± SD of three experiments, two of which were from the results using suspended cells. More than duplicate determinations were used in each experiment and the variations were less than 10%.

<table>
<thead>
<tr>
<th>[U-¹²⁵I]monodansylcadaverine</th>
<th>Cellular uptake</th>
<th>Nuclear uptake</th>
<th>Cellular protein synthesis*</th>
</tr>
</thead>
<tbody>
<tr>
<td>µM</td>
<td>nmoles/2.7 × 10⁶</td>
<td>% total</td>
<td>% control</td>
</tr>
<tr>
<td>0.28</td>
<td>0.009 ± 0.0002</td>
<td>3.5 ± 0.4</td>
<td>Non-detectable</td>
</tr>
<tr>
<td>10</td>
<td>0.28 ± 0.017</td>
<td>2.8 ± 0.2</td>
<td>Non-detectable</td>
</tr>
<tr>
<td>100</td>
<td>1.76 ± 0.31</td>
<td>1.8 ± 0.4</td>
<td>Non-detectable</td>
</tr>
</tbody>
</table>

*GH₂ cells (1.5 × 10⁶ cells) were incubated with 1 ml of L-[¹⁴C]amino-acids mixture in the presence of 10 or 100 µM unlabeled MDC for 30 min at 37°C. The values are means of triplicate determinations from two experiments. The variations were less than 5%.

TABLE IV

Effect of monodansylcadaverine on the binding of T₃ to isolated nuclei of GH₂ cells

Nuclei were isolated from cells (2.6 × 10⁶) and incubated with 0.5 nM [¹²⁵I]T₃ at 37°C for 30 min. Nuclei-bound [¹²⁵I]T₃ was determined as described under “Materials and Methods.” Values are mean ± S.D. (n = 5).

<table>
<thead>
<tr>
<th>Monodansylcadaverine</th>
<th>[¹²⁵I]T₃ bound</th>
<th>Bound % control</th>
</tr>
</thead>
<tbody>
<tr>
<td>µM</td>
<td>fmol/17 µg DNA</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>1.11 ± 0.06</td>
<td>100</td>
</tr>
<tr>
<td>50</td>
<td>1.08 ± 0.10</td>
<td>97.2 ± 8.9</td>
</tr>
<tr>
<td>100</td>
<td>1.06 ± 0.04</td>
<td>95.5 ± 3.2</td>
</tr>
</tbody>
</table>

isolated nuclei. Thus, MDC is not concentrated in nuclei, and even if it were, it would not affect the binding of T₃ to the nucleus.

Possible Toxicity of MDC—To evaluate toxic effects of MDC, cells were incubated with MDC under various experimental conditions. No effect of MDC was observed on protein synthesis at concentrations up to 100 µM (Tables II and III). At 200 µM MDC, 10% inhibition of protein synthesis was observed. Examination of cells treated with 100 µM MDC for 1 h by phase microscopy showed no obvious change in cell shape or permeability to trypan blue.

DISCUSSION

The presence of T₃-specific plasma membrane receptors has been documented in rat liver cells (8, 9), human erythrocyte ghosts (5), rabbit adipocytes (6), and mouse fibroblasts (10, 11). The present study shows that T₃-specific and saturable membrane receptors are also present in GH₂ cells. The demonstration of the presence of T₃ membrane receptors in the GH₂ cell is significant in view of its large number of T₃ nuclear receptors and its responsiveness to physiological concentrations of thyroid hormone in the production of growth hormone. Thus, the GH₂ cell line appears to be a good model system for the study of the mechanism of T₃ uptake and its regulation.

At 0°C T₃ bound to the plasma membrane, but no T₃ was internalized as evidenced by its nearly completely reversible dissociation from the binding sites (Table I) and the absence of T₃ in the nuclei (Fig. 3). Earlier, Samuels and Tsai (21) reported a similar finding that the incorporation of T₃ into nuclei of GH₂ cells was absent at 0°C. Thus, the internalization of T₃ is a temperature-dependent process. This result is consistent with the receptor-mediated endocytosis of other ligands (24).

In fibroblastic cells ligands that enter the cells via receptor-mediated endocytosis accumulate in clusters bound to their receptors in bristle-coated pits prior to cellular entry (25). Shortly after cellular entry the ligands are found in uncoated vesicles with characteristic morphological features that we have termed receptosomes (26). Using rhodamine-labeled T₃ it has been possible to follow the entry of T₃ into receptosomes (10). However, preliminary experiments using rhodamine-labeled T₃ to follow the uptake of T₃ in GH₂ cells have proven to be technically impractical. This is because of the difficulties in resolving individual intracellular organelles due to the round shape and thickness of these cells. We presume that T₃ enters GH₂ cells in these structures.

The effect of MDC and other amines has been studied in most detail on the entry of αM into fibroblastic cells. Derivatives of αM that are intensely fluorescent or readily visualized at the electron microscopic level are easy to prepare (25). Further, fibroblasts have large numbers of αM receptors which facilitate binding studies (27). MDC has been found to inhibit the internalization of αM (14) epidermal growth factor (17) and vesicular stomatitis virus at a half-maximal inhibitory concentrations of 150, 450, and 50 µM, respectively. Furthermore, MDC has been found to inhibit the presence of vesicular stomatitis or αM-M-Receptor complexes in bristle-coated pits. Thus, it has been suggested that MDC acts by preventing clustering of vesicular stomatis or αM-M-Receptor complexes in coated pits. Prolonged treatment of cells with MDC and other amines can lead to a decrease in the number of receptors on the cell surface and thereby affect entry (28). In the current studies, cells were not pretreated with MDC prior to the binding studies. Further, MDC treatment was not found to affect the number of T₃ receptors. Therefore, the action of MDC is on some step other than binding to the cell surface. Because total cellular uptake is markedly inhibited by MDC (Fig. 7), its action appears to be on the transfer of T₃ from the cell surface to the cell interior. In analogy to the action of MDC on αM entry, it seems possible that MDC acts by inhibiting accumulation to T₃-Receptor complexes in coated pits.

We have compared the effect of various concentrations of MDC on inhibition of cellular and nuclear uptake. As shown in Fig. 7, total cellular uptake is more sensitive than nuclear uptake to the inhibitory effect of MDC. There are a number of possible explanations for this finding. One is that once T₃ is internalized, it is very efficiently transferred to the nucleus so that inhibition of nuclear uptake is only evident when cellular uptake is markedly restricted. MDC itself did not have a direct action on nuclear binding of T₃ by isolated nuclei and was not itself concentrated in the nucleus of intact cells. In addition to inhibiting ligand entry, it is also possible that MDC affects other cellular processes involving the translocation of cytoplasmic T₃ to the nucleus. Presently, since the mechanism of the translocation of cytoplasmic T₃ to nucleus is unknown, we cannot evaluate these possibilities.

The present study has demonstrated the functional significance of T₃ membrane receptors and physiological relevance of the receptor-mediated endocytic pathway in the uptake of T₃. Many questions remain, including how the T₃ is transferred from cytoplasm into the nucleus and precisely where it
bends and initiates its biological function. Studies with T₃ conjugated to molecules that can be observed in the electron microscope may help elucidate some of these questions.

Acknowledgment—We are grateful to Dr. M. Johnson for performing the computer analysis of the data in the binding of T₃ to nuclei of intact cells.

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Inhibition of the nuclear entry of 3,3',5'-triiodo-L-thyronine by monodansylcadaverine in GH3 cells.
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