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Biochemistry

Use of un-derivatized thyroid hormones for photoaffinity labeling of binding proteins

(L-thyroxine/3,5,3′-triiodo-L-thyronine/3,3′,5′-triiodo-L-thyronine/free radicals/rat liver nuclei)

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ABSTRACT **Irradiation of the thyroid hormones thyroxine and 3,5,3′-triiodothyronine in the near UV (>300 nm) causes homolytic fission of C—I bonds in both rings. In the presence of hormone-binding proteins, the phenyl radical thus formed, and possibly also the iodine radical, can establish a covalent bond with certain amino acid residues in the binding site. Most if not all of the iodine radicals, however, appear to be reduced to iodide. Incubation of purified carrier proteins for the thyroid hormones in human serum as well as of an extract of rat liver nuclei or of whole nuclei with trace amounts of ^{125}I- or ^{14}C-labeled hormone, followed by irradiation, resulted in covalent binding. This was proven by gel filtration after boiling with guanidine·HCl and by sodium dodecyl sulfate/polyacrylamide gel electrophoresis of the irradiated solutions or of the excluded-peak material obtained after gel filtration. Sodium dodecyl sulfate/polyacrylamide gel electrophoresis of ^{125}I-labeled irradiated nuclear extracts showed a prominent peak ($M_r \approx 45{,}000$), sometimes with a shoulder or small peak at $M_r \approx 56{,}000$, and a fast-moving peak ($M_r \approx 12{,}000$). Similar patterns were obtained with *N*-bromoacetylthyroxine or *N*-bromoacetyltriiodothyronine without irradiation. When a suspension of whole nuclei was irradiated instead of nuclear extracts, the shoulder also became a prominent peak.**

Affinity labeling of binding sites in proteins is believed to require conversion of the ligand to a reactive derivative that can bind covalently to certain amino acid residues. In the case of photoaffinity labeling, the derivative has to be irradiated *in situ* in order to be converted to a reactive species such as a nitrene or carbene. The thyroid hormones L-thyroxine (T4) and 3,5,3′-triiodo-L-thyronine (T3) have been converted to their *N*-bromoacetyl derivatives for covalent binding to carrier proteins for the hormones in serum (1, 2) or to thyroid hormone receptors in rat liver nuclear extract (NE) (3). Photoaffinity labeling of NE with a carbene-forming derivative of T3 has also been reported.†

We now wish to report that the un-derivatized thyroid hormones can be used for photoaffinity labeling of the carrier proteins albumin, prealbumin, and thyroxine-binding globulin (TBG) from human serum, as well as of receptors in rat liver NE or in intact nuclei.

We had observed in earlier experiments that an analog of T4, 3-[4-(4-hydroxy-3,5-diiodophenoxy)-3,5-dinitrophenyl]-propionic acid, binds covalently to prealbumin upon irradiation with long-wave UV light (4). This observation prompted us to investigate the possibility of using un-derivatized T4 or T3 as photoaffinity labels.

In a preliminary investigation (5), we explored the photolytic behavior of the thyroid hormones. In the present communication, we demonstrate that photoaffinity labeling with the un-derivatized hormones is indeed possible.

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MATERIALS AND METHODS

Reagents. Carrier-free [3′,5′-^{125}I]T4 and [3′-^{125}I]T3 as well as carrier-containing [3′,5′-^{125}I]rT3 (≈1,000 μCi/μg; 1 Ci = 3.7×10^{10} becquerels), rT3 being 3,3′,5′-triiodo-L-thyronine, were from New England Nuclear. [3,5-^{125}I]T4 was synthesized as described (6). [2-^{14}C]T4 was purified by HPLC (5) from a preparation that had suffered appreciable radiodecomposition on storage. We are greatly indebted to Constance S. Pittman (University of Alabama) for this preparation. The product obtained by HPLC was chromatographically pure.

The *N*-bromoacetyl derivatives BrAc-[3′,5′-^{125}I]T4 and BrAc-[3′-^{125}I]T3 were synthesized as previously described (3) with some modifications. 1,2-Dimethoxyethane was used instead of dimethylformamide. Triethylamine was omitted and aqueous $NaHCO_3$ (2–5 μl of a fresh solution sufficiently diluted to obtain pH 8.3 ± 0.1) was used instead. This was added to the solution of the labeled hormone [100–150 μCi obtained from the supplier (above)]. In some preparations, the solvent chloroform/methanol/acetic acid, 45:4:1 (vol/vol), was used for thin-layer chromatography instead of 10% acetic acid in ethyl acetate.

Carrier Proteins. Human serum albumin (crystallized four times) was from ICN and prealbumin (≈98% pure) was from Calbiochem-Behring. A purified preparation of TBG was kindly supplied by S. Grimaldi of our institute.

Nuclei and NE. Nuclei from 6 g of rat liver were prepared according to Blobel and Potter (7) except that all solutions contained 1 mM phenylmethylsulfonyl fluoride. The nuclear pellet was washed and, for the preparation of NE, extracted as described (3).

Incubation with Labeled Hormones or Their Bromoacetyl Derivatives. Carrier proteins (5 mg/ml) in 20 mM sodium phosphate/100 mM NaCl, pH 7.4, in a total volume of 0.5–1 ml and human serum (diluted 1:10 with 50 mM Tris·HCl, pH 6.8) were incubated for 15 min at room temperature with 2–5 μCi of [^{125}I]T4 or 2×10^5 cpm (≈5 μCi) of [^{14}C]T4. Aliquots of the NE were incubated with 1–10 nM [3′,5′-^{125}I]T4 or [3′-^{125}I]T3 for 18 hr at 4°C. Suspensions of the washed nuclear pellet (0.9–1.2 mg of protein per ml) in 0.3 M sucrose/4 mM $MgCl_2$/100 mM NaCl/10 mM Tris·HCl, pH 8, were incubated similarly.

Aliquots of NE were also incubated with BrAc-[3′,5′-^{125}I]T4 and BrAc-[3′-^{125}I]T3 in the same manner.

Irradiation. The incubated solutions, except those obtained with the bromoacetyl derivatives, were irradiated with light

Abbreviations: T4, L-thyroxine; T3, 3,5,3′-triiodo-L-thyronine; rT3, 3,3′,5′-triiodo-L-thyronine; BrAc, *N*-bromoacetyl; TBG, human serum thyroxine-binding globulin; NE, nuclear extract.

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† Samuels, H. H., in *Abstracts of the 63rd Annual Meeting of The Endocrine Society*, Cincinnati, OH, June 1981, p. 193 (abstr.).

above 300 nm‡ for various lengths of time (*Results*), using the previously described procedure (5). The solutions were in plastic cups (Caplugs no. 9 from Caplugs, Buffalo, NY). The cups, containing 0.5–1 ml of solution, were cooled in ice-water or in running cold tap water. The milky suspension of whole nuclei (0.5 ml per cup) was magnetically stirred during irradiation.

Gel Filtration. Solid guanidine·HCl was added to irradiated solutions of carrier proteins or to irradiated NE to bring the molarity to ≈6 M. The solutions were then heated for 2 min in a boiling water bath and chromatographed at room temperature on a Sephadex G-25M column (PD-10, Pharmacia; bed volume, 9.1 ml; height, 9.1 cm) that had been equilibrated with the elution buffer, which was either the incubation buffer (above) or 50 mM NH_4HCO_3 (when a volatile buffer was needed for subsequent operations). Fractions (0.9 ml) were collected. All elution profiles showed retarded peaks and an excluded peak of covalently bound radioactive material (*Results*).

Polyacrylamide Gel Electrophoresis. $NaDodSO_4$/polyacrylamide gel electrophoresis was performed according to Laemmli (8) with an acrylamide-to-bisacrylamide weight ratio of 29/1; 10% and 17.5% gels were used. Polyacrylamide gel electrophoresis under nondenaturing conditions was performed similarly, except that $NaDodSO_4$ was omitted and a 7.5% gel was used.

Polyacrylamide gel electrophoresis without $NaDodSO_4$ was used for [3′,5′-^{125}I]T4-labeled human serum that had been irradiated for 1 hr. The gel was cut in 2-mm strips and the radioactivity in each strip was measured to locate the positions of the carrier proteins. The strips were then fixed with trichloroacetic acid and thoroughly washed with acetic acid/methanol/water, 1:2:7 (vol/vol), to remove all noncovalently bound radioactive material, and radioactivity was again measured.

$NaDodSO_4$/polyacrylamide gel electrophoresis of irradiated solutions (carrier proteins, NE) or suspensions (nuclei) was performed after boiling the sample for 2 min in the presence of 1% $NaDodSO_4$ and 1% 2-mercaptoethanol. In some experiments, the irradiated suspension of labeled nuclei was first extracted with 0.4 M NaCl and the extract was boiled with $NaDodSO_4$ and 2-mercaptoethanol as above.

Hydrolysis and HPLC. The ^{125}I-labeled excluded peak material obtained by gel filtration (above) in experiments with human serum albumin was lyophilized and treated with Pronase in 0.2 M Tris·HCl, pH 8, for 18 hr at 37°C. The hydrolysate was filtered through a Sep-Pak C_{18} cartridge (Waters Associates) to remove Pronase. The adsorbed material was eluted with 1% NH_4OH in methanol and the eluate was evaporated. The residue was dissolved in starting buffer for HPLC (5).

RESULTS

Gel Filtration and HPLC. A few examples of gel filtration of labeled, irradiated, and guanidine-treated carrier proteins and of similarly treated NE are shown in Fig. 1. All profiles show an excluded peak of covalently bound labeled material. Covalent binding must have occurred primarily or entirely through the phenyl radicals formed by homolytic fission of C—I bonds (5).

The simultaneously formed iodine radicals must have been largely or completely reduced to iodide, because appreciable incorporation of labeled I· in the protein is not compatible with the high ratios of I^- to protein-bound radioactivity seen in Fig. 1. Furthermore, no iodotyrosines were detected when a Pronase hydrolysate of the excluded-peak material (Fig. 1*A*) was analyzed by HPLC. Finally, the amount of covalently bound radioactivity (size of excluded peak) rose from 25% (Fig. 1*A*) to 46% when phenolic ring-labeled T4 ([3′,5′-^{125}I]T4) was replaced with side chain-labeled T4 ([2-^{14}C]T4) in experiments with human serum albumin. The increase in covalently bound radioactivity must have been entirely due to incorporation of phenyl radicals because iodine radicals formed from side chain-labeled T4 are not labeled.

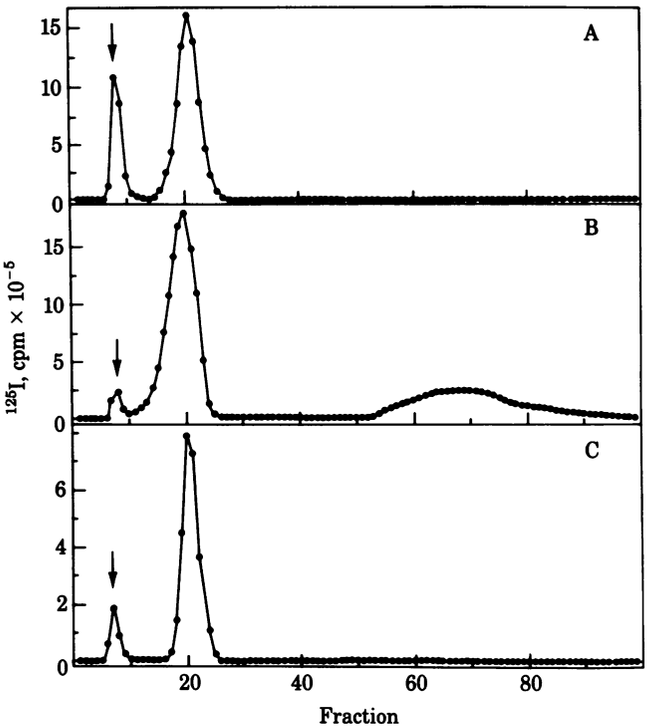

FIG. 1. Sephadex G-25 elution profiles of binding proteins after irradiation in the presence of trace amounts of [3′,5′-^{125}I]T4, followed by heating with guanidine·HCl. The excluded peaks represent covalently bound radioactive material, the retarded peaks represent iodide, and the still more retarded peak (in *B* only) represents free T4. Proteins and irradiation times: *A*, albumin, 30 min; *B*, prealbumin, 18 hr; *C*, NE, 1 hr. Radioactivity distribution (percent of total, peaks from left to right): *A*, 25%, 75%; *B*, 4%, 56%, 40%; *C*, 12%, 88%. Arrows indicate void volume V_o.

Replacement of [3′,5′-^{125}I]T4 with [3′-^{125}I]T3 resulted in a decrease of the covalently bound radioactive material from 12% (Fig. 1*C*) to 4% in experiments with NE. This shows that fission must have occurred at least to some extent in the nonphenolic ring because exclusive fission of [3′-^{125}I]T3 in the phenolic ring produces only unlabeled phenyl radicals, as can be seen from the theoretical yields of labeled phenyl radicals shown in Table 1.

Nevertheless, fission in the phenolic ring must have been predominant, because replacement of [3′,5′-^{125}I]T4 with [3,5-^{125}I]T4 in experiments with human serum albumin resulted in an increase of covalently bound radioactive material from 25% (Fig. 1*A*) to 40%. If fission had taken place primarily or entirely in the nonphenolic ring, a decrease rather than an increase would have been observed (Table 1). It should be noted that, in our previously reported studies on the photolytic behavior of T4 and T4 analogs (5), we also have observed predominant, but not exclusive, fission in the phenolic ring.

T4 has a higher affinity for prealbumin than for albumin (9); yet a comparison of Fig. 1*A* with Fig. 1*B* shows that more of the total radioactive material was covalently bound to albumin (25%) than to prealbumin (4%). Furthermore, covalent binding

‡ More energetic and hence more destructive light (e.g., 254 nm) is not required for photoaffinity labeling.

Table 1. Dependence of radioactivity yield of phenyl radicals on site of labeling and site of fission

Ligand	Site of labeling	Site of fission	Radioactivity yield, %*
T4, rT3	Phenolic ring	Phenolic ring	50
T4, T3, rT3	Phenolic ring	Nonphenolic ring	100
T4, T3, rT3	Nonphenolic ring	Phenolic ring	100
T4, T3	Nonphenolic ring	Nonphenolic ring	50
T4, T3, rT3	Side chain	Phenolic ring	100
T4, T3, rT3	Side chain	Nonphenolic ring	100
T3	Phenolic ring	Phenolic ring	0
rT3	Nonphenolic ring	Nonphenolic ring	0

* These theoretical yields are calculated as follows: (radioactivity of phenyl radical formed/radioactivity of ligand used) × 100.

of 4% of the radioactivity to prealbumin required irradiation for 18 hr and, even after that period of time, 40% (90% after 30 min) was still in the form of free T4, whose identity was proven by HPLC (5) after elution with 0.1 M NaOH. In contrast, covalent binding to albumin (Fig. 1*A*) and to NE (Fig. 1*C*) was complete (no free T4 remaining) after 30 min and 1 hr, respectively.

This suggests that covalent binding is not exclusively a function of the affinity, but also depends on the close proximity in the binding site of certain amino acid residues with which a phenyl radical can form a covalent bond. These should be mainly aromatic residues (*Discussion*).

Polyacrylamide Gel Electrophoresis. $NaDodSO_4$/polyacrylamide gel electrophoresis after irradiation of [3′,5′-^{125}I]T4 or [3,5-^{125}I]T4 in the presence of human serum albumin, TBG, or prealbumin confirmed covalent binding. The peaks of covalently bound radioactive material coincided with the areas stained by Coomassie blue.

Polyacrylamide gel electrophoresis under nondenaturing conditions of [3′,5′-^{125}I]T4-labeled irradiated human serum, followed by fixation and thorough washing, gave the profile shown in Fig. 2. TBG and albumin account for most of the covalently bound radioactivity and prealbumin only for very little, which is in agreement with the results obtained in gel filtration (Fig. 1 *A* and *B*). This again suggests that covalent binding partly depends on the close proximity of certain amino acid residues in the binding site.

$NaDodSO_4$/polyacrylamide gel electrophoresis after irradiation of [3′-^{125}I]T3 in the presence of NE (Fig. 3*A*) reveals covalent binding primarily to proteins of M_r ≈45,000 and ≈12,000.

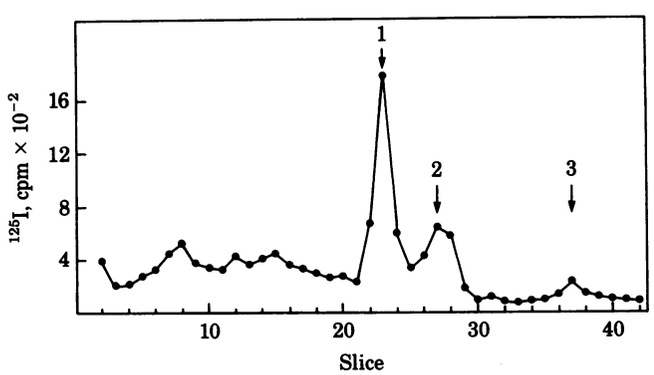

FIG. 2. Polyacrylamide gel electrophoresis (without $NaDodSO_4$) of human serum (diluted 1:10) after irradiation with trace amounts of [3′,5′-^{125}I]T4. The gel (7.5%) was sliced and each slice was fixed with 10% trichloroacetic acid and washed to remove all noncovalently bound radioactive material. The radioactivity of each slice was then measured. Numbers: 1, TBG; 2, albumin; 3, prealbumin.

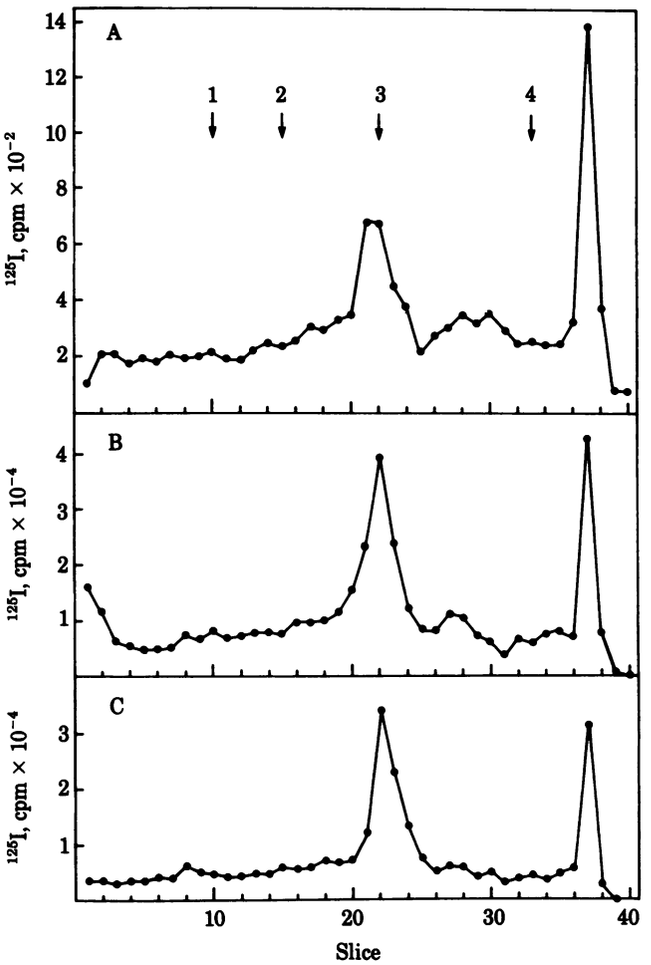

FIG. 3. $NaDodSO_4$/polyacrylamide gel electrophoresis (10% gel) of NE covalently labeled with ^{125}I. Ligands and irradiation times were: *A*, [3′-^{125}I]T3, 1 hr; *B*, BrAc-[3′-^{125}I]T3, not irradiated; *C*, [3′,5′-^{125}I]T4, 1 hr. Marker proteins: 1, phosphorylase *b* (M_r, 96,000); 2, bovine serum albumin (M_r, 68,000); 3, ovalbumin (M_r, 43,000); 4, carbonic anhydrase (M_r, 30,000).

The radioactive material bound to the protein of M_r ≈12,000 (determined with a 17.5% gel) could not be washed out by exhaustive destaining. In control experiments in which NE was incubated with BrAc-[3′-^{125}I]T3 without irradiation (Fig. 3*B*), similar radioactivity profiles were obtained. This suggests that the same receptors may have been covalently labeled with underivatized T3 and with BrAc-T3. In some experiments with NE, a shoulder, and occasionally even another peak, was seen, corresponding to M_r ≈56,000.

Covalent labeling of NE with [3′,5′-^{125}I]T4 (Fig. 3*C*), with BrAc-[3′,5′-^{125}I]T4, or with [3′,5′-^{125}I]rT3 (not shown) also gave similar radioactivity distributions after $NaDodSO_4$/polyacrylamide gel electrophoresis. The incorporation of radioactivity was 1–2 orders of magnitude higher with T4 (Fig. 3*C*) than with T3 (Fig. 3*A*). This is supposedly due to predominant fission of C—I bonds in the phenolic ring, which leads to a much lower concentration of *labeled* phenyl radicals when T3 is used (Table 1) and for this and other reasons (see *Discussion*) does not permit conclusions about the relative affinities of these two ligands. A considerably higher incorporation may be expected with T3 in which the nonphenolic ring is labeled, due to predominant fission of C—I bonds in the phenolic ring (Table 1).

When a suspension of whole nuclei was used instead of NE, the profile (Fig. 4) always showed an additional labeled protein

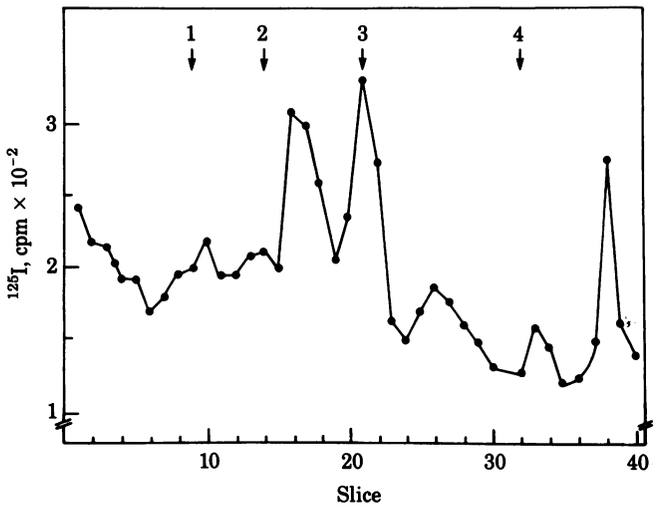

FIG. 4. NaDodSO$_4$/polyacrylamide gel electrophoresis (10% gel) of NE obtained by extracting [3',5'-^{125}I]T4-labeled irradiated whole nuclei. Irradiation time, 1 hr. For marker proteins see legend to Fig. 3.

with M_r ≈56,000. In the experiment shown in the figure, the irradiated nuclei were extracted and the extract was used for polyacrylamide gel electrophoresis. In other experiments in which the irradiated nuclei were treated directly with NaDodSO$_4$ and mercaptoethanol, similar profiles were obtained. The low efficiency of covalent labeling with suspensions of nuclei (Fig. 4 compared to Fig. 3C) is most likely due to light scattering by the milky suspension.

DISCUSSION

Our experimental data suggest that covalent labeling is primarily due to the phenyl radical formed during C—I bond fission and that I· is largely or entirely reduced to I$^-$. They also confirm our previous observation (5) that C—I fission takes place predominantly, but not exclusively, in the phenolic ring.

In contrast to nitrenes and carbenes, phenyl radicals cannot insert themselves in any C—H bond, but they can react with unsaturated compounds. A mechanism for covalent bond formation with aromatic compounds has been suggested (10, 11). The absence of aromatic residues from the binding site of prealbumin (12) may explain, at least in part, the slow covalent bond formation with prealbumin. The low degree of freedom of movement of T4 within the binding site (13) also may reduce covalent binding. A lessened possibility of interaction of phenyl radicals with the protein will favor their recombination with iodine radicals to reform the ligand used (reverse reaction). Because light below 300 nm splits C—I bonds much faster than does light in the near UV, it is likely that photoaffinity labeling with carbene- or nitrene-forming derivatives of the thyroid hormones is partly mediated by C—I fission.

Molecular weights for the nuclear receptor for T3 in the range of 50,000 to 65,000 have been observed under nondenaturing conditions by various investigators (14–16). Under denaturing conditions (NaDodSO$_4$/polyacrylamide gel electrophoresis), Samuels† found a labeled receptor (M_r, 46,000) after photoaffinity labeling of NE with a derivative of T3 and an additional one (M_r, 56,000) after photoaffinity labeling of whole nuclei. Our own results, which were obtained with un-derivatized T3 and T4, fully confirm his findings.

In our previous work (3) in which a receptor of M_r ≈56,000 with a shoulder at ≈45,000 was observed in NaDodSO$_4$/polyacrylamide gel electrophoresis, performed after fractionation of the NE by HPLC, as well as in recent unpublished work, the recovery of radioactivity in HPLC was very low. Furthermore, various degrees of aggregation, as determined by NaDodSO$_4$/polyacrylamide gel electrophoresis of the HPLC eluates, were observed. The bulk of the radioactive material could be eluted only with 6 M guanidine·HCl. It is, therefore, conceivable that a labeled protein with M_r ≈45,000 was present, but only very little of it was eluted.

In our present work with NE and T3, BrAc-T3, T4, BrAc-T4, or rT3, two main peaks (M_r ≈ 12,000 and ≈ 45,000) were observed. In various experiments, an additional shoulder or minor peak (M_r ≈ 56,000) was also seen.

The fact that more than one labeled protein was found in our experiments with whole nuclei or NE could possibly be due to a labile bond in the receptor, resulting in its easy breakdown, or to the existence of different receptors (17). An exploration of these possibilities requires additional experimentation.

Experiments to better characterize the binding proteins in nuclei or NE are desirable. Preliminary experiments in which NE was incubated with labeled hormone and increasing concentrations of unlabeled hormone suggest the presence in NE of a number of binding proteins over a wide affinity range.

Thorough washing of the nuclei with buffer containing or not containing Triton X-100 did not appreciably alter the radioactivity ratio of M_r ≈45,000 to M_r ≈56,000. Furthermore, in previous experiments (18), in which nuclei had been washed with buffer that did not contain Triton X-100, addition of ^{125}I-labeled malic enzyme, a marker for cytoplasm, to the liver homogenate used in the preparation of nuclei resulted in the recovery of only minute amounts of radioactivity in the washed nuclei. These findings make it appear unlikely that the major labeled proteins in NE are cytoplasmic contaminants.

The main purpose of our present work was to investigate the possibility of using the un-derivatized thyroid hormones for photoaffinity labeling. Although it is now clear that the unaltered hormones are excellent photoaffinity labels, it should be kept in mind that photoaffinity labeling with un-derivatized iodothyronines is not well suited for the determination of relative binding affinities. This is due to a combination of various factors: irreversibility of binding, dependence of the extent of radioactivity incorporation in the protein on the position of the label (Table 1) as well as on the nature of the binding site, and differences in the kinetics of photodegradation of different iodothyronines (5). However, the use of un-derivatized iodothyronines provides an excellent method for covalent labeling of receptors and other binding proteins. The great advantage of using the hormones themselves lies not only in their easy availability but even more in the full preservation of their affinity and specificity.

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