Photoaffinity Labeling of Human Thyroxine-Binding Prealbumin with Thyroxine and *N*-(Ethyl-2-diazomalonyl)thyroxine†

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ABSTRACT: To facilitate studies of thyroid hormone-binding proteins, we have synthesized and tested the photoaffinity analogues *N*-(ethyl-2-diazomalonyl)-3,5,3′-triiodothyronine (EDM-T_3) and *N*-(ethyl-2-diazomalonyl)thyroxine (EDM-T_4). The binding affinities of L-EDM-T_4 and D-EDM-T_4 to human thyroxine-binding prealbumin were 4% and 13.2%, respectively, that of L-thyroxine (L-T_4). For comparison the affinities of L-EDM-T_3 and D-EDM-T_3 to crude rat liver nuclear receptor preparation were 0.1% and 0.7%, respectively, that of L-triiodothyronine (L-T_3). Photolysis of prealbumin–[^{125}I]-L-EDM-T_4 complexes at 254 nm resulted in covalent linkage of [^{125}I]-L-EDM-T_4 to prealbumin as judged by sodium dodecyl sulfate gel electrophoresis. Virtually no labeling was observed in the absence of photolysis. Photolabeling of prealbumin was specific for the high-affinity hormone binding site since it was (a) completely blocked during photolysis in the presence of excess 3,5,3′,5′-tetraiodothyroacetic acid, (b) saturated at high [^{125}I]-L-EDM-T_4 concentrations, (c) prevented when the hormone binding site had been previously blocked by dansylation of prealbumin, and (d) blocked competitively by T_3 and T_4 with inhibition constants (K_i) similar to the dissociation constants (K_d) for these ligands. Analysis of prealbumin photolabeling by direct attachment of [^{125}I]-L-EDM-T_4 or attachment of unlabeled L-EDM-T_4 followed by titration of the remaining sites with [^{125}I]-L-T_4 indicated a photolabeling efficiency of 54–61% at 63–67% site occupancy. After destruction of the diazo group by preirradiation, L-EDM-T_4 was found to label prealbumin after further irradiation; 19–26% photolabeling efficiency could be achieved by using preirradiated reagent at 87–91% site occupancy. This carbene-independent photoattachment was also specific for a high-affinity hormone binding site. The mechanism of the carbene-independent process may involve attachment via radical formation following photoinduced loss of the thyronine ring iodine. Accordingly, specific covalent cross-linking of L-T_4 to prealbumin was demonstrated; however, the photolabeling efficiency was much lower than that with preirradiated EDM-T_4. Both EDM-T_4 and T_4 were employed to photolabel prealbumin, thyroxine binding globulin, and albumin in unfractionated human serum.

Thyroid hormones interact with a variety of proteins found in the serum (Woeber & Ingbar, 1968; Nilsson & Peterson, 1975; Andrea et al., 1980), the cytoplasm (Sterling et al., 1974; Surks et al., 1975), the mitochondrial membrane (Sterling et al., 1977), and the nucleus (Samuels et al., 1974; Schuster et al., 1979; Eberhardt et al., 1979) of responsive cells. The interaction of thyroid hormones with these proteins is essential for the transport, metabolism, and expression of the biological activity of the hormones [for reviews, see Baxter et al. (1979) and Eberhardt et al. (1980)]. The nuclear binding proteins have been identified as acidic, nonhistone proteins (Latham et al., 1976; Silva et al., 1977) that bind to DNA (MacLeod & Baxter, 1976; Ricketts et al., 1980) and are likely candidates as mediators of thyroid hormone action at the level of transcription (Eberhardt et al., 1980). The serum binding proteins include thyroxine-binding globulin (Nilsson & Peterson, 1975), thyroxine-binding prealbumin (Andrea et al., 1980), and albumin (Woeber & Ingbar, 1968).

Thyroxine-binding prealbumin (TBPA)[1] is the only thyroid hormone-binding protein to date that has been well characterized. X-ray crystallographic analysis at 1.8-Å resolution (Blake et al., 1978) has revealed details of the T_4 binding site and has disclosed that TBPA contains a putative DNA binding site (Blake & Oatley, 1977), which suggests a possible relationship to the intranuclear receptor. In addition, hormone analogue binding studies demonstrate a high degree of structural homology between the binding domains of TBPA (Andrea et al., 1980) and a crude receptor preparation from rat hepatic nuclei (Bolger & Jorgensen, 1980). Thus, TBPA may be a useful model for studying the mechanism of hormone–receptor interactions.

Specific affinity labels would be potentially useful in the purification and characterization of these proteins and comparative mapping of their hormone binding sites. Cheng et al. (1977) have used the chemical affinity analogue *N*-(bromoacetyl)-L-thyroxine to label TBPA and demonstrated that lysine-9 and lysine-15 residues as well as the N-terminal glycine can interact with the hormone side chain. As an alternative to nucleophilic affinity reagents, photoaffinity probes (Bayley & Knowles, 1977; Chowdhry & Westheimer, 1979) have the potential for offering greater specificity, since, in principle, equilibria can be established before the activation event is initiated and because these agents have shorter reactive half-lives.

In this report, we describe the synthesis of *N*-(ethyl-2-diazomalonyl) derivatives of the L and D isomers of T_4 and T_3. We show that the diazo derivative of T_4 is a highly efficient photoaffinity reagent for TBPA and that T_4 can also serve, although less effectively, as a photoaffinity reagent. We also demonstrate the utility of these compounds for specifically

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[1] Abbreviations: T_4, thyroxine; T_3, 3,5,3′-triiodothyronine; T_4Ac, 3,5,3′,5′-tetraiodothyroacetic acid; T_4Fo, 3,5,3′,5′-tetraiodothyroformic acid; EDM-T_3, *N*-(ethyl-2-diazomalonyl)-T_3; EDM-T_4, *N*-(ethyl-2-diazomalonyl)-T_4; TBG, thyroxine-binding globulin; TBPA, thyroxine-binding prealbumin; $NaDodSO_4$, sodium dodecyl sulfate.

0006-2960/82/0421-5651$01.25/0

labeling thyroxine-binding proteins in unfractionated serum.

Experimental Procedures

Materials. Unlabeled T_3 and T_4 were purchased from Sigma. [^{125}I]-L-T_3 and [^{125}I]-L-T_4 were from New England Nuclear Corp. The specific activities were approximately 520 and 930 Ci/mmol, respectively. Ethyl diazoacetate was obtained from Aldrich and phosgene from Matheson. Preparative thin-layer chromatography (TLC) was performed on 1000-μm PLQF or 250-μm LQ6F plates obtained from Whatman. The solvent system consisted of ethyl acetate–acetic acid (9:1). TBPA was obtained from Dr. R. C. Hevey, Behring Diagnostics, Somerville, NJ. The protein behaved as a homogeneous preparation after NaDodSO$_4$ gel electrophoresis and had an immunological purity of 98%. Crude rat liver nuclear extract was prepared by the method of Latham et al. (1976) and it was used without further purification. TBPA was dansylated with dansyl chloride (Sigma) by using the procedure of Cheng et al. (1975). Unreacted dansyl chloride was removed by dialysis against 0.1 M Tris-HCl (pH 8.0). Partially purified TBG was supplied through the generosity of Dr. Ralph Cavalieri, Veterans Administration Hospital, San Francisco, CA.

Synthesis of Thyroid Hormone Photoaffinity Analogues. Ethyl-2-diazomalonyl chloride was prepared as described previously (Brunswick & Cooperman, 1971) and stored at −90 °C. Six hundred micromoles of T_3 or T_4 was dissolved in 2 mL of dimethyl formamide at 0–4 °C. A 6 molar excess of ethyl-2-diazomalonyl chloride was added, and after 5 min the solution was filtered and the solvent removed under vacuum at 45 °C. The resulting oil was taken up in methanol, applied to a 100-μm PLQF silica plate, and developed by ascending chromatography in ethyl acetate–acetic acid (9:1). The bands containing the products (R_f = 0.6–0.7) were located by exposure of the margins of the plate to UV light, and the products were extracted from the silica with methanol. The solvent was removed under vacuum, and the product was solidified by the addition of cold water and then was washed with water and lyophilized. Typical recoveries were 30–40%. Data for L- and D-EDM-T_3 were as follows: mp 108 °C dec; TLC (UV, ninhydrin negative) R_f 0.62; IR (KBr) 2160 cm^{-1}. Anal. Calcd for $C_{20}H_{16}N_3O_7I_3$: C, H, N. Data for L- and D-EDM-T_4 were as follows: mp 97–98 °C dec; TLC (UV, ninhydrin negative) R_f 0.64; IR (KBr) 2160 cm^{-1}. Anal. Calcd for $C_{20}H_{15}N_3O_7I_4$: C, H, N.

A modified procedure was used to synthesize [^{125}I]-L-EDM-T_4. L-T_4 (110 nmol, sodium salt pentahydrate) was mixed with 116 μCi of [^{125}I]-L-T_4 (969 Ci/mmol) in methanol. The solvent was removed under vacuum, the residue was redissolved in 90 μL of dimethylformamide, and a 6-fold molar excess of ethyl-2-diazomalonyl chloride was added. After 2 min the mixture was applied directly to a 250-μm LQ6F TLC plate that was developed with the solvent system described above. The product was extracted from the silica with methanol and stored in methanol protected from light at 4 °C.

Photolysis. Photolysis reactions were carried out at room temperature in 1-cm light path quartz curvettes with a Rayonet mini photochemical reactor equipped with a RPR 2537-Å lamp. In one experiment a Rayonet RPR-100 reactor equipped with 16 RPR 3500-Å lamps was used, and samples were chilled to 0–4 °C with ice water during photolysis.

Reversible Binding Assays. The competition binding assay employing gel filtration described by Latham et al. (1976) was used to measure the binding of T_3 analogues to the nuclear receptor preparation. In this assay analogues are tested for their ability (relative to L-T_3) to displace [^{125}I]-L-T_3 from high-affinity binding sites. A similar method has been described for binding studies with TBPA (Somack et al., 1982). Stock solutions of analogues were prepared and stored in methanol and diluted to final concentration in assay buffer. The TBPA assay mixture contained 10^{-8} M TBPA, 1.25×10^{-8} M [^{125}I]-L-T_4 (approximately 0.15 μCi/reaction), varying concentrations of analogue, and 0.1 M Tris-HCl (pH 8.0) containing 0.1 M NaCl and 1 mM EDTA in a volume of 0.5 mL. After incubation for 30 min at 25 °C, tubes were chilled to 0 °C and protein-bound [^{125}I]-L-T_4 was isolated by gel filtration on Sephadex G-25 columns (bed volume of 2.0 ± 0.1 mL) equilibrated with Tris buffer. In this procedure a 0.4-mL aliquot of the reaction is applied to the column, which is rinsed with 0.4 mL of buffer. The protein fraction is eluted with 0.8 mL of additional buffer. Free, radioactive iodide (approximately 5–16% of the total radioactivity in a typical commercial lot of [^{125}I]-L-T_4) elutes from the column directly after the protein and was estimated in some experiments by collecting the following 1.6 mL of column eluate. Free hormone binds tightly to the gel matrix and does not elute in the volumes used. So that receptor–hormone dissociation on the column during gel filtration could be minimized, samples were filtered at 0–4 °C. The entire procedure could be performed in less than 2 min. Since the half-life of the dissociation of the TBPA–T_4 complex at 4 °C measured by this technique was 13 min, the extent of dissociation of bound [^{125}I]-L-T_4 in the equilibrium competition assays during gel filtration was minimal. Doubling the elution time had no significant effect on the association constants obtained for the analogues tested (Somack et al., 1982).

The concentration of T_4 binding sites was measured directly from scatchard analysis (Scatchard, 1949) using the equation $\bar{\nu}/\mathrm{TF} = K_a(n - \bar{\nu})$ where $\bar{\nu}$ is the molar ratio of bound ligand to TBPA, TF is the concentration of free ligand, K_a is the apparent association constant for each binding site, and n is the number of binding sites with the value K. Assay conditions for Scatchard analysis were equivalent to those used in competition assays except that the concentration of unlabeled T_4 was varied from 2.5×10^{-9} to 1×10^{-7} M.

Photoaffinity Labeling of TBPA. Two methods were employed to measure the extent of photoaffinity labeling of TBPA. In the first method, [^{125}I]-L-EDM-T_4 was employed and labeled protein was collected by gel filtration. Reactions (0.5 mL) contained 40 nM TBPA and [^{125}I]-L-EDM-T_4 in 43 mM Tris-HCl buffer (pH 8.0). The concentration and specific activity of [^{125}I]-L-EDM-T_4 varied in the individual experiments and is indicated in the figure legends. After 15 min at 25 °C, reactions were photolyzed and the high-affinity ligand, T_4Ac, was added at a final concentration of 5×10^{-5} M to displace noncovalently bound EDM-T_4. After 45 min at 25 °C, ^{125}I-labeled protein was collected by the gel filtration procedure described above. Since free radioactive iodide elutes from the column directly after the protein peak and the iodide level is further increased by UV irradiation, control reactions containing no protein were used to correct for free iodide that might contaminate the protein peak and contribute to an overestimation of labeled sites.

Alternatively, the extent of photolabeling was determined by photolysis of TBPA with unlabeled EDM-T_4. Noncovalently bound ligands were then displaced from the protein by incubation with T_4Ac followed by gel filtration and dialysis. The concentration of photolabeled sites was then measured by determining the high-affinity L-T_4 binding sites remaining by titration with [^{125}I]-L-T_4 using the Scatchard analysis described above. This method yields a more accurate estimate

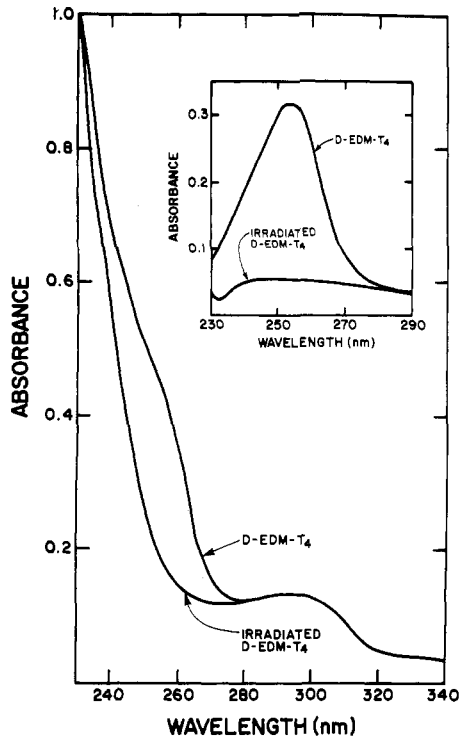

FIGURE 1: UV absorption spectra of D-EDM-T_4 and photolyzed D-EDM-T_4. Photolysis was carried out for 15 s at 254 nm at 25 °C in quartz cuvettes as described under Experimental Procedures. Reference cuvette solutions were treated identically. D-EDM-T_4, present at 2.5×10^{-4} or 2×10^{-5} M (inset), was dissolved in Tris-HCl (pH 8.0) assay buffer. The reference cuvette for the data shown in the inset also contained 2×10^{-5} M L-T_4.

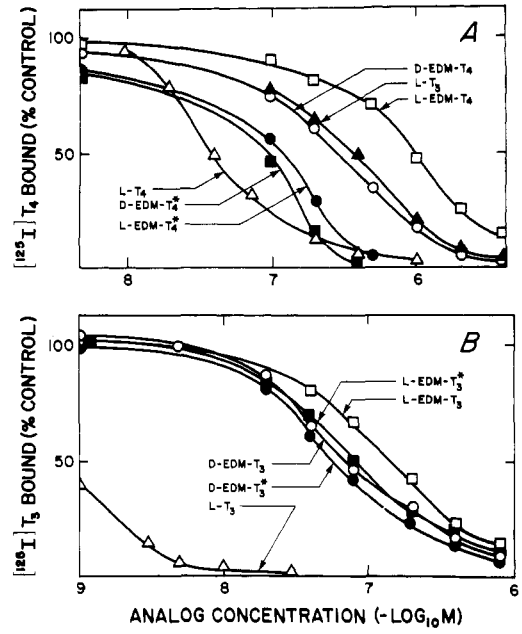

FIGURE 2: Competition binding assays for determining the affinity of EDM-T_4 analogues to TBPA (A) and EDM-T_3 analogues to nuclear receptor (B). Experimental details are described under Experimental Procedures. Where indicated by the asterisk (*), the hormone analogue was photolyzed for 10 s at 254 nm in Tris-HCl (pH 8.0) buffer before assaying. Each point is the average value of two determinations.

of the extent of specific labeling, since it measures the actual concentration of high-affinity sites that have lost the capability of binding L-T_4.

$NaDodSO_4$–Polyacrylamide Gel Electrophoresis. Following photolysis, free hormone was removed from protein samples by gel filtration on Sephadex G-25. The samples were subsequently dialyzed against distilled water, lyophilized, and dissolved in $NaDodSO_4$ sample buffer [60 mM Tris-HCl (pH 6.8), 2% (w/v) $NaDodSO_4$, 5% (v/v) β-mercaptoethanol, and 10% (v/v) glycerol]. The samples were heated at 95 °C for 5 min before electrophoresis on 10–16% polyacrylamide exponential gradient slab gels (Laemmli, 1970). Proteins were stained and fixed with 50% trichloroacetic acid–0.01% Coomassie brilliant blue, destained in 7% acetic acid, dried, and subjected to autoradiography using Kodak X-O-Mat or Kodak X-AR-5 film and intensifying screens. For experiments involving photolabeling of human serum, 2.5 mm thick, 7.5–17.5% exponential gradient polyacrylamide gels were electrophoresed 18 h at 10 mA.

Physical Methods. Melting points were determined with a Hoover apparatus. Microanalyses were performed by the Microanalytical Laboratory, Department of Chemistry, University of California at Berkeley. Infrared spectra were obtained by using a Perkin-Elmer Model 457 IR spectrometer and UV spectra with a Cary Model 118-C recording spectrophotometer.

Results

Characterization of Thyroid Hormone Photoaffinity Analogues. Both EDM-T_4 and EDM-T_3 appeared to be stable in aqueous solutions buffered between pH 4 and pH 10 since no change in the UV spectra or binding affinities could be detected after incubation under these conditions for at least 4 h at room temperature. Solutions stored in methanol for up to 2 weeks at 4 °C showed no evidence of degradation on the basis of thin-layer chromatography and binding affinity. Brief exposure to either 1 N HCl or 1 N NaOH, however, caused degradation as judged by the disappearance of the parent compound and the concomitant appearance of both faster and slower migrating components on thin-layer chromatograms.

Figure 1 shows the UV absorption spectra of D-EDM-T_4 before and after exposure to UV light at 254 nm. Identical spectra were obtained for L-EDM-T_4 and L-EDM-T_3 (data not shown). The UV absorption shoulder with a maximum at 254 nm corresponds to the known strong absorption of diazo esters (Hexter & Westheimer, 1971) and is clearly evident in the difference spectrum between EDM-T_4 and T_4 (Figure 1, inset). The extinction coefficient calculated for the ethyl-2-diazomalonyl group in Tris-HCl buffer (pH 8) at 254 nm is 10 800 cm^{-1} M^{-1}. Brunswick & Cooperman (1973) reported an extinction coefficient of 7100 cm^{-1} M^{-1} for this group at pH 7. A 10-s photolysis period was sufficient for complete decomposition of the diazo group using the Rayonet reactor (Figure 1, inset). The photolysis products have not been characterized; however, it is known that the carbene generated upon photolysis of the diazo group reacts with water to produce the corresponding ethyl-2-hydroxymalonyl derivative or alternatively undergoes a Wolff-type rearrangement to yield a ketene that could react with water in the absence of other available nucleophiles (Chaimovich et al., 1968). The latter product, if present, would probably be a minor product since substitution of diazo compounds with electron-withdrawing groups has been reported to minimize this rearrangement (Hexter & Westheimer, 1971; Chowdhry et al., 1976).

Photoaffinity Analogue Binding to TBPA and Nuclear Receptor. The relative binding affinities of EDM-T_3, EDM-T_4, and their respective photolysis products for the nuclear receptor and TBPA were obtained from the competition binding assays shown in Figure 2 and Table I. The affinities

Table I: Binding Affinities of EDM-T_3 Analogues to Nuclear Receptor and EDM-T_4 Analogues to TBPA

stereoisomer	prephotolysis	binding affinity[a] TBPA, EDM-T_4 (% L-T_4)	binding affinity[a] receptor, EDM-T_3 (% L-T_3)
D	−	13.2	0.7
L	−	4.0	0.1
D	+	40.0	1.0
L	+	28.4	0.7

[a] The relative binding affinities of D- and L-EDM-T_3 and D- and L-EDM-T_4 were determined (see Experimental Procedures) for the nuclear receptor and TBPA, respectively. Values were calculated from the competition binding curves in Figure 2 and are expressed relative to L-T_3 ($K_d = 8 \times 10^{-10}$ M) or L-T_4 ($K_d = 4 \times 10^{-8}$ M) = 100%.

of the L- and D-T_3 analogues for the nuclear receptor were low (1% or less that of L-T_3) compared to the affinities of the L- and D-T_4 analogues for TBPA (4% and 13.2% that of L-T_4). Furthermore, the D isomers were more active than the corresponding L isomers with both proteins. A similar reversal in D and L selectivity has recently been reported for the binding of the pimelate and glutarate amides of T_3 to the receptor (Latham et al., 1981). This finding is significant in view of the fact that the L forms of all other analogues with free α-amino groups show higher binding to TBG, TBPA, and the receptor than the corresponding D forms. We have observed this reversal in D and L selectivity with the binding of other *N*-acyl-T_4 analogues to both the receptor and TBPA (Somack et al., 1982).

The photolysis products of the diazo T_3 and T_4 analogues also showed higher binding affinities for the receptor and TBPA, respectively, than the native compounds (Figure 2). If the major photolysis products formed in aqueous buffer are *N*-ethyl-2-(hydroxymalonyl)-T_4 and -T_3 (resulting from carbene insertion into the OH bond of H_2O), then the higher binding may result from additional hydrogen bonding between the side chain hydroxyl group and either charged or polar protein groups. In the case of TBPA, such interactions could involve lysine, histidine, or glutamic acid residues in the region of the binding channel occupied by the hormone side chain (Blake et al., 1978; Blake & Oatley, 1977; Cheng et al., 1977).

Photoattachment of L-EDM-T_4 to TBPA. The ability of [^{125}I]-L-EDM-T_4 to bind irreversibly to TBPA was examined by using gel filtration to remove free ligands. As shown in Table II, a 15-s photolysis of TBPA in the presence of [^{125}I]-L-EDM-T_4 irreversibly labeled 38% of the total sites occupied by ligand (63%). Furthermore, photolysis of protein alone had no effect on reversible binding of [^{125}I]-L-T_4 (data not shown). Labeling was dependent on ultraviolet light since it was reduced to less than 2% in the absence of light. The data in Table II also indicate that the presence of excess T_4Ac during photolysis prevented virtually all irreversible binding. This result suggests that photoattachment of L-EDM-T_4 is specific for a site normally occupied by a high-affinity ligand. The data in Table II show that preirradiation of L-EDM-T_4 did not completely destroy the ability of the reagent to photolabel TBPA. Thus, prephotolysis of [^{125}I]-L-EDM-T_4 for a time sufficient to destroy the diazo group (Figure 1) followed by photolysis in the presence of TBPA resulted in photolabeling 13% of the total sites occupied by the ligand (87%). Consequently, there appear to be at least two independent photoreactions with EDM-T_4, i.e., a carbene-dependent and a carbene-independent reaction. Further experiments addressing the carbene-independent photolabeling and the specificity of

Table II: Ultraviolet Irradiation Dependent Irreversible Binding of [^{125}I]-L-EDM-T_4 to TBPA[a]

reaction	irradiation (254 nm)	[^{125}I]-L-EDM-T_4 bound (nM)	photoattachment efficiency[c] (%)
1	+	9.7 ± 0.6 (4)[b]	38.4 ± 2.5
2	−	0.3 ± 0.1 (3)	1.2 ± 0.4
3[d]	+	0.2 ± 0.1 (3)	0.8 ± 0.4
4[e]	+	4.4 ± 1.2 (3)	12.6 ± 3.4

[a] Reactions contained 40 nM TBPA, 9.5×10^{-7} M [^{125}I]-L-EDM-T_4 (856 μCi/μmol) and 43 mM Tris-HCl (pH 8.0) buffer in 0.5 mL. After 15 min at 25 °C, reactions were photolyzed at 254 nm for 15 s, and then 20 μL of 10^{-3} M T_4Ac was added to displace noncovalently bound [^{125}I]-L-EDM-T_4. After 45 min, radiolabeled protein was collected by gel filtration. [b] Values are expressed ± SD followed by the number of determinations in parentheses. [c] Defined as the concentration of sites covalently labeled divided by the concentration of sites initially occupied. The concentration of sites initially occupied was calculated from the mass action equation for equilibrium binding by using the K_d values for L-EDM-T_4 and prephtolyzed L-EDM-T_4 derived from Figure 2. [d] T_4Ac (4×10^{-5} M) was present during photolysis. [e] [^{125}I]-L-EDM-T_4 was prephotolyzed for 20 s in Tris buffer before adding TBPA. Subsequent treatment was identical with the conditions in footnote *a*.

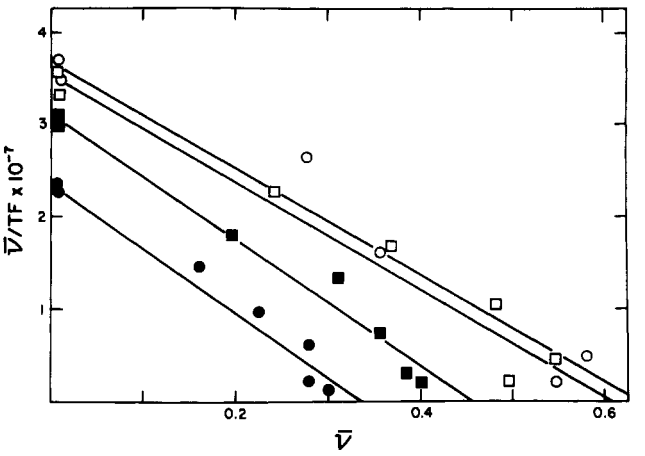

FIGURE 3: Photolabeling of TBPA with L-EDM-T_4 estimated by Scatchard analysis. Reactions containing 2×10^{-7} M TBPA were incubated for 15 min with 2×10^{-6} M L-EDM-T_4 (circles) or 2×10^{-6} M L-EDM-T_4 that had been prephotolyzed for 15 s in Tris-HCl (pH 8.0) buffer (squares). The reactions were then either photolyzed for 15 s (closed symbols) or not photolyzed further (open symbols). After T_4Ac (5×10^{-5} M) addition and exhaustive dialysis, the concentration of high-affinity L-T_4 binding sites remaining was estimated by Scatchard analysis.

the photolabeling of the thyroid hormone binding site of TBPA are described separately below.

Carbene-Dependent and -Independent Labeling of TBPA with L-EDM-T_4. As discussed above, prephotolysis of [^{125}I]-L-EDM-T_4 followed by further photolysis in the presence of TBPA lead to substantial incorporation of radioactivity. We examined this phenomenon further, using unlabeled L-EDM-T_4 in experiments where reversibly bound ligands were removed after photolysis by dialysis followed by direct estimation of the remaining available sites by Scatchard analysis. The analysis (Figure 3) confirmed that prephotolyzed reagent retained photolabeling activity upon further photolysis. This cannot be explained by incomplete carbene formation since a 10-s photolysis was sufficient for complete destruction of the diazo group (Figure 1). Doubling the prephotolysis time did not decrease the extent of photolabeling, and no further increase in the carbene-independent labeling was observed after exposure to TBPA using longer irradiation times (data not shown).

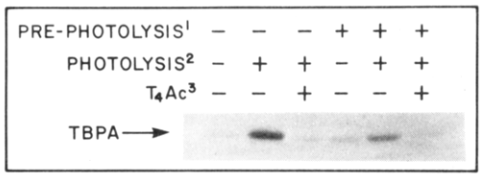

FIGURE 4: $NaDodSO_4$ gel electrophoresis of TBPA photolyzed in the presence of [^{125}I]-L-EDM-T_4 or prephotolyzed [^{125}I]-L-EDM-T_4. Reactions containing 1.25×10^{-7} M TBPA and either 5×10^{-7} M [^{125}I]-L-EDM-T_4 (0.2 μCi) or prephotolyzed [^{125}I]-L-EDM-T_4[1] were incubated for 15 min at 25 °C and photolyzed[2] in the presence or absence of 5×10^{-5} M T_4Ac[3]. Photolysis was carried out for 15 s at 254 nm in tris-HCl buffer. T_4Ac was then added to all reactions at a final concentration of 5×10^{-5} M to ensure displacement of unbound radioactive ligand. After removal of free ligand by gel filtration and dialysis, the protein fractions were electrophoresed in $NaDodSO_4$ as described under Experimental Procedures.

From five separate determinations with L-EDM-T_4 using the Scatchard method to measure photolabeling, the extent of labeling of occupied sites (67% of the total available sites) was 61.1 ● 8.9% (SD). From three separate determinations using prephotolyzed L-EDM-T_4, the extent of labeling of occupied sites (91% of total available sites) was 25.6 ± 3.2% (SD). The carbene-dependent and -independent photoaffinity labeling efficiencies (sites irreversibly labeled/sites occupied) measured by the Scatchard approach (Figure 3) are higher than the values obtained by measuring the irreversible attachment of [^{125}I]-L-EDM-T_4 (Table II). In the direct attachment assay, however, the total sites available for photolabeling were calculated on a weight basis, whereas in the Scatchard analysis they are measured directly. When the photolabeling efficiencies obtained from direct assays (Table II) are adjusted to reflect the concentration of occupied sites determined by Scatchard analysis, the values for the carbene-dependent and -independent processes were 54% and 19%, respectively. Since the photolabeling efficiency determined by the direct attachment assay depends upon incorporation of radiolabeled iodide, the lower corrected values obtained in this assay may be explained by loss of label due to photoinduced outer ring deiodination (discussed below). Alternatively, it is possible that photoinitiated radicals derived from the bound hormone participate in destruction or modification of sites without covalent attachment of ligand.

Scatchard analysis (Figure 3) also confirmed that the site-directed photoaffinity labeling with both EDM-T_4 and prephotolyzed EDM-T_4 was light-dependent since no high-affinity sites were blocked irreversibly in the absence of irradiation. Moreover, the decrease in the ability of TBPA to bind T_4 after photolysis with EDM-T_4 or prephotolyzed EDM-T_4 cannot be explained by photoinactivation of TBPA since irradiation of TBPA alone had no effect on reversible binding to L-T_4 (data not shown).

The observations discussed above for carbene-dependent and -independent photolabeling using gel filtration and Scatchard analysis were confirmed by $NaDodSO_4$ gel electrophoresis of the Sephadex column eluates of reactions containing TBPA photolyzed with [^{125}I]-L-EDM-T_4 (Figure 4). Eluates were treated with $NaDodSO_4$ under conditions sufficient to dissociate TBPA into individual 14 000 dalton subunits (see $NaDodSO_4$–Polyacrylamide Gel Electrophoresis under Experimental Procedures). Both carbene-dependent and -independent labeling processes were UV light dependent and were blocked by the presence of T_4Ac during photolysis. Sixty-five percent of the radioactivity in the applied samples was recovered in the TBPA monomer bands excised from the gel. Therefore, about 35% of the photolabeled adducts that were resistant to either gel filtration or dialysis appeared to dissociate during $NaDodSO_4$ gel electrophoresis. This difference could be due to incomplete recovery of sample upon resuspension and loading or incomplete migration of sample into the gel. Alternatively, some of the covalent bonds formed may be unstable under the conditions of electrophoresis. For example, certain covalent bonds such as those formed by formaldehyde cross-linking are unstable in the presence of $NaDodSO_4$ (Jackson, 1978). Furthermore, if some of the labeling were derived from photoinduced generation of iodine radicals (see below) and subsequent incorporation into protein heteroatoms, then it is possible that this fraction might be released after exposure to mercaptoethanol during sample preparation prior to $NaDodSO_4$ electrophoresis.

Specificity of Photolabeling of TBPA with L-*EDM-*T_4. As discussed previously, photolabeling of TBPA with L-EDM-T_4 could be eliminated by including excess T_4Ac during the photolysis (Table II), suggesting that photoattachment of the ligand was at the high-affinity hormone binding site. Further evidence that photoaffinity labeling of TBPA with L-EDM-T_4 was specific for this binding site was obtained from experiments employing dansyl-TBPA. Dansyl chloride competes irreversibly with T_4 for the same binding site on TBPA and has been employed as an affinity reagent to label the lysine-15 residue that is located in the T_4 binding domain (Cheng et al., 1975). As shown in Figure 5A, photolabeling of dansyl-TBPA with [^{125}I]-L-EDM-T_4 produced only 10% the incorporation of radiolabel as with TBPA. Quantitation of the binding sites of dansyl-TBPA by Scatchard analysis is shown in Figure 5B and indicates that 5.6% of the T_4 binding sites remained undansylated. Thus, the residual incorporation of [^{125}I]-L-EDM-T_4 by dansylated TBPA may be accounted for by undansylated binding sites. These data provide strong support for the concept that photoattachment of L-EDM-T_4 is to the hormone binding site of TBPA.

The specificity of carbene-dependent and carbene-independent photolabeling was further investigated by irradiating TBPA with either [^{125}I]-L-EDM-T_4 or prephotolyzed [^{125}I]-L-EDM-T_4 in the presence of varying concentrations of L-T_4 and L-T_3. The photolysis reactions were subjected to $NaDodSO_4$ electrophoresis, the gels were autoradiographed, and the radioactivity in each excised TBPA monomer band was plotted as a function of the concentration of competing hormone as shown in Figure 6. From the competition curves, inhibition constants for photolabeling (K_I) of 6.6×10^{-8} M for L-T_4 and 3.9×10^{-7} M for L-T_3 were obtained for the carbene-dependent process (Figure 6A). The corresponding K_I values for the carbene-independent process were 7.2×10^{-8} M for L-T_4 and 6.6×10^{-7} M for L-T_3 (Figure 6B). Since these values are similar to the dissociation constants for L-T_4 and L-T_3 derived from the equilibrium competition assays (Figure 2), we conclude that photoattachment of EDM-T_4 by both labeling mechanisms is specific for the high-affinity hormone binding site of TBPA.

Photoaffinity Labeling of TBPA with L-T_4. The observation that irradiation of TBPA using prephotolyzed L-EDM-T_4 resulted in irreversible attachment of ligand suggested the involvement of photoreactive regions of the hormone other than the diazo group. Photoinduced cleavage of haloaryl compounds, including T_3 and T_4, to yield halogen atoms and aryl radicals has been described (Turro, 1978; van der Walt & Cahnmann, 1982). In addition, the covalent attachment of 5-iodo-2′-deoxyuridine to thymidine kinase was reported to involve the pyrimidine moiety (Cysyk & Prusoff, 1972). We, therefore, considered the possibility that the carbene-in-

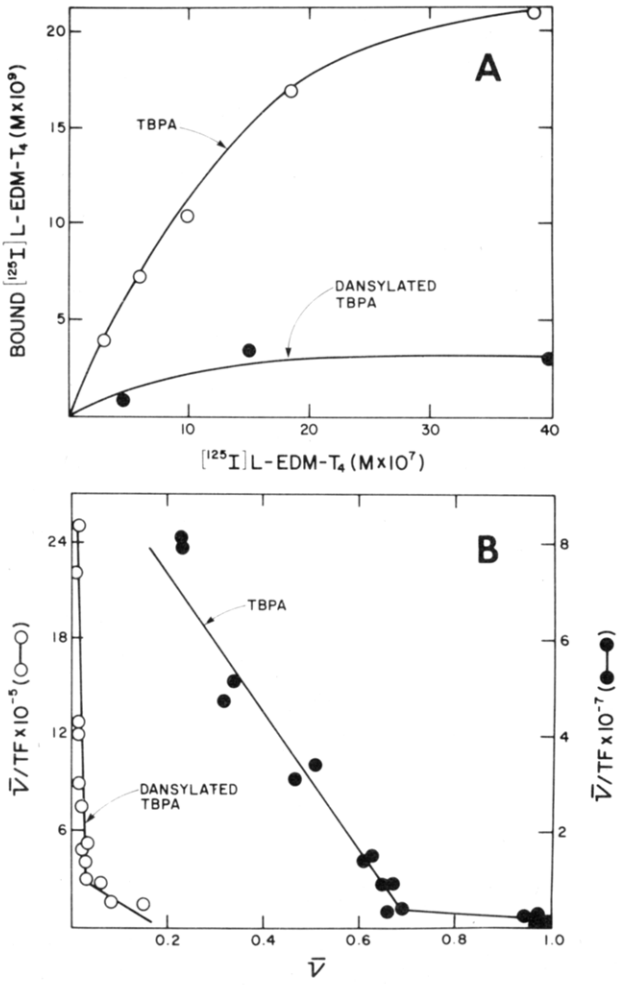

FIGURE 5: Effect of [^{125}I]-L-EDM-T_4 concentration on photoaffinity labeling of TBPA and dansylated TBPA (A). [^{125}I]-L-EDM-T_4 (662 Ci/mmol) was incubated at the indicated levels with either 40 nM TBPA (○) or dansylated TBPA (●) and photolyzed at 254 nm (see Experimental Procedures). After photolysis T_4Ac was added to displace noncovalently bound ligands and ^{125}I-labeled protein collected by gel filtration as described under Experimental Procedures. Scatchard analysis of [^{125}I]T_4 binding to TBPA and dansylated TBPA (B). TBPA (●) or dansylated TBPA (○) was present at 1×10^{-8} M.

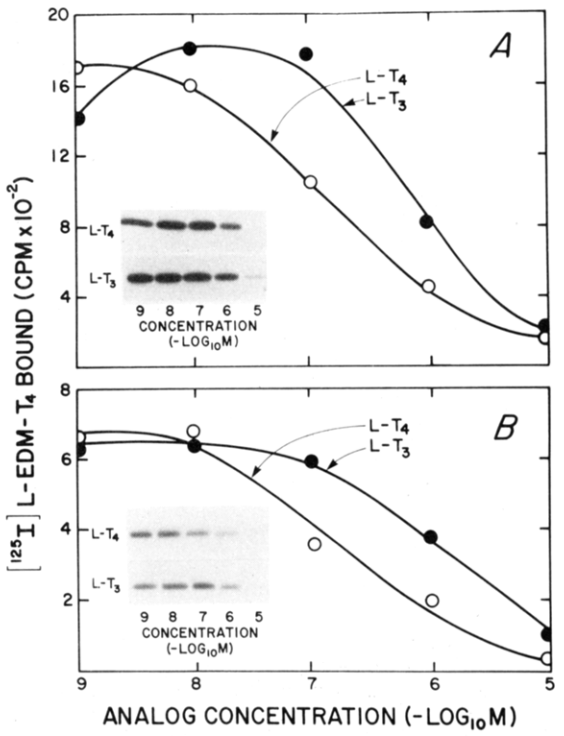

FIGURE 6: $NaDodSO_4$ gel electrophoresis of TBPA photolabeled with [^{125}I]-L-EDM-T_4 (A) or prephotolyzed [^{125}I]-L-EDM-T_4 (B) in the presence of T_3 and T_4. Reactions [in 0.5 mL of Tris-HCl (pH 8.0) buffer] containing 6.17×10^{-8} M TBPA, 2.2×10^{-7} M [^{125}I]-L-EDM-T_4 (1540 Ci/mmol) or prephotolyzed (15 s at 254 nm) [^{125}I]-L-EDM-T_4, and the indicated concentration of unlabeled L-T_4 or L-T_3 were photolyzed at 254 nm for 15 s. T_4Ac was added, and the reactions were desalted and electrophoresed in $NaDodSO_4$ as described in the legend for Figure 4. The total cpm in each TBPA monomer subunit was plotted as a function of the concentration of unlabeled T_4 or T_3 present during photolysis. Autoradiograms of gel sections containing the TBPA subunit are shown in the insets. The photolabeling inhibition constant (K_I) is defined as the concentration of nonradioactive T_3 or T_4 required to produce 50% inhibition of photolabeling (C_{50}). Since K_I will vary with the total radioactive ligand concentration, the values were corrected by using the following equation derived from mass action considerations: $K_I = C_{50}K_T/(K_T + T)$, where T = concentration of [^{125}I]-L-EDM-T_4 that competed and K_T = dissociation constant for L-EDM-T_4 (4.3×10^{-7} M). K_T was obtained from the relationship $[K_{d(1)}/K_{d(2)}]K_dT_4$, where $K_{d(1)}$ and $K_{d(2)}$ are the equilibrium dissociation constants for L-EDM-T_4 and L-T_4, respectively, calculated from the equilibrium competition curves in Figure 2 and K_dT_4 is the average value for the equilibrium dissociation constant for L-T_4 (2.15×10^{-8} M) calculated from the Scatchard analyses shown in Figures 3, 5, and 8.

dependent labeling might result from ultraviolet irradiation induced deiodination followed by insertion of either a thyronine or iodine radical. In Figure 7 we measured the photosensitivity of T_4 to light by irradiating under the conditions employed in the above experiments. The data indicate that the kinetics of free iodide generation from [^{125}I]-L-T_4 paralleled the loss in ability of irradiated hormone to bind reversibly to TBPA. Approximately 80% destruction of L-T_4 binding was observed after photolysis for 100 s at 254 nm. This is not surprising since the presence of iodine on inner and outer ring 3, 5, 3′, and 5′ positions is an essential determinant in hormone binding to TBPA. For example, 3,5,3′-triiodothyronine (T_3) binds with only 10% the affinity of T_4 (Figure 2) and 3,5-diiodothyronine (T_2) binds with 0.32% the affinity of T_4 (Andrea et al., 1980).

Direct evidence that L-T_4 can itself function as a photoaffinity reagent was obtained by irradiating [^{125}I]-L-T_4 in the presence of TBPA followed by $NaDodSO_4$ gel electrophoresis of the products (Figure 7, inset). The kinetics of photolabeling of TBPA with [^{125}I]-L-T_4 as judged by autoradiography parallels the photodegradation of T_4 as judged by the appearance of free iodide and loss of binding affinity to TBPA. The kinetics of labeling using a 350-nm light source (see Experimental Procedures) were slower; however, the same extent of photolabeling could be achieved after 25 min of irradiation (data not shown).

In Figure 8 the photolabeling of TBPA was examined by using nonradioactive L-T_4 as the photoligand and measuring the actual hormone binding sites remaining by Scatchard analysis. With L-T_4 some degree of inactivation (about 7%) was observed with 2.5×10^{-6} M L-T_4. However, this concentration is approximately 5 times that required to achieve total saturation of binding sites. Significant inactivation of binding (36%) could only be demonstrated by using L-T_4 at a much higher level (2×10^{-5} M). If the carbene-independent photolabeling with EDM-T_4 is derived from deiodination alone, then it is not apparent why much more photolabeling resulted with prephotolyzed EDM-T_4 than with T_4 when tested at subsaturating levels of ligand.

The specificity of photolabeling with T_4 was tested by irradiating TBPA with [^{125}I]-L-T_4 in the presence of competing concentrations of nonradioactive L-T_3 and L-T_4, followed by electrophoresis in $NaDodSO_4$ (Figure 9). From the compe-

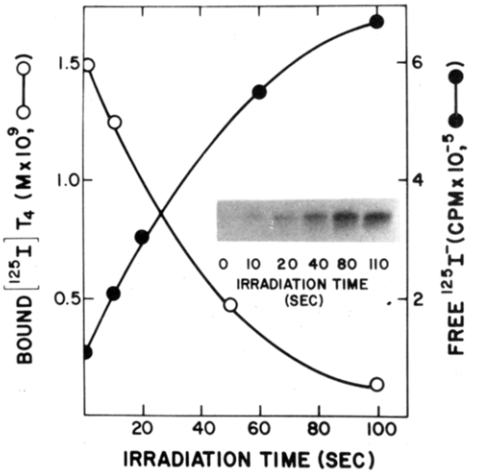

FIGURE 7: Time course of UV-dependent iodide generation from T_4 and photolabeling of TBPA with [^{125}I]-L-T_4. Reactions (0.5 mL) containing 5×10^{-7} M [^{125}I]-L-T_4 (8.5×10^5 cpm) were irradiated at 254 nm in Tris-HCl (pH 8.0) buffer for the indicated times, and free iodide-125 (●) was estimated by gel filtration as described under Experimental Procedures. For determination of the ability of prephotolyzed T_4 to bind reversibly to TBPA, reactions (0.5 mL) containing 2.5×10^{-9} M [^{125}I]-L-T_4 (1.8×10^5 cpm) were similarly irradiated and TPBA was then added at a final concentration of 5×10^{-8} M. After 15 min at 25 °C, protein-bound [^{125}I]-L-T_4 (O) was determined by gel filtration. The inset shows an autoradiogram of the section of an NaDodSO$_4$ gel containing the [^{125}I]-L-T_4-labeled TBPA monomers derived from TBPA irradiated at 254 nm in the presence of [^{125}I]-L-T_4 for the indicated time periods. Reactions contained 1.3×10^{-7} M TBPA and 1.4×10^{-6} M [^{125}I]-L-T_4 (5×10^5 cpm) in 0.38 mL. After irradiation T_4Ac was added, and the samples were desalted and electrophoresed in NaDodSO$_4$ as described in the legend to Figure 4.

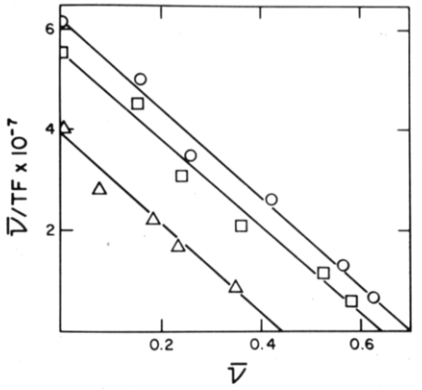

FIGURE 8: Photolabeling of TBPA with L-T_4 estimated by Scatchard analysis. TBPA was irradiated in the presence of either 2.5×10^{-6} M T_4 (□) or 2.0×10^{-5} M T_4 (△), and the concentration of remaining high-affinity L-T_4 binding sites was measured by Scatchard analysis. A control reaction (O) contained 2×10^{-5} M T_4 in the absence of light.

tition curves, inhibition constants (K_I) of 2×10^{-8} M and 1.5×10^{-7} M were calculated for L-T_4 and L-T_3, respectively. These values are similar to the K_d values for these compounds derived from the equilibrium competition assays (Figure 2). Therefore, as in the case with EDM-T_4, the photoattachment of T_4 to TBPA is specific for the high-affinity hormone binding site. In this experiment approximately 10% of the binding sites were reversibly occupied with hormone before photolysis. From the amount of radioactivity recovered from the gel derived from reactions without unlabeled competing hormone, it was estimated that 0.25% of these sites had been covalently labeled. However, since the [^{125}I]-L-T_4 is radiolabeled exclusively in the outer ring 3′ and 5′ positions, if some of the photoattachment involves the photoinduced loss of these iodines, then the specific radioactivity of bound ligand would be reduced and the extent of photoattachment by this method would be underestimated. Indeed, such a mechanism could explain why somewhat higher labeling efficiencies with EDM-T_4 were observed with the Scatchard method than with the direct incorporation procedure. The Scatchard approach, which measures total inactivation might detect labeling that would originate from incorporation of any of the four iodine atoms or an unlabeled thyronine residue, while the direct assay would only detect attachment or a ^{125}I-labeled species.

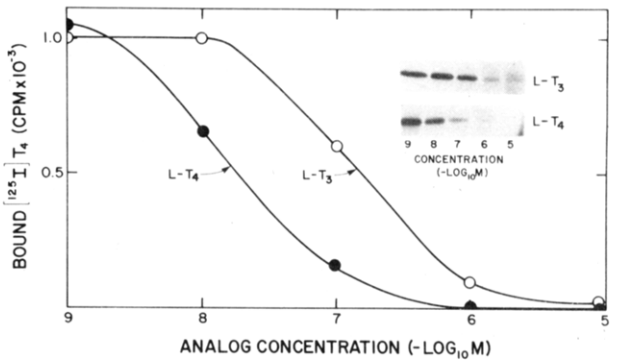

FIGURE 9: NaDodSO$_4$ gel electrophoresis of TBPA photolabeled with [^{125}I]-L-T_4 in the presence of T_3 and T_4. Reactions [0.5 mL in Tris-HCl (pH 8.0) buffer] containing 10^{-8} M TBPA, 3×10^{-9} M [^{125}I]-L-T_4 (4×10^6 cpm), and the indicated concentration of unlabeled L-T_4 or L-T_3 were irradiated at 254 nm for 80 s at 25 °C. T_4Ac was added, and the reactions were desalted and electrophoresed in NaDodSO$_4$ as described in the legend for Figure 4. The total cpm in each monomer band is plotted as a function of the concentration of unlabeled T_3 or T_4 present during photolysis as indicated in the figure. Autoradiograms of those sections of the gels containing the TBPA monomer subunits are shown in the inset.

Photoaffinity Labeling of Serum Proteins with [^{125}*I*]-L-T_4 *and* [^{125}*I*]-L-*EDM-*T_4. Photoaffinity labeling is potentially very useful in the identification and characterization of proteins comprising a small fraction of the total (Nordeen et al., 1981). To explore this potential we investigated the use of EDM-T_4 and T_4 in photolabeling the thyroid hormone binding proteins of unfractionated human serum.

In these experiments diluted serum was incubated with 1 nM [^{125}I]-L-T_4 or 100 nM [^{125}I]-L-EDM-T_4, photolyzed, desalted, and electrophoresed on denaturing NaDodSO$_4$–polyacrylamide gels before autoradiography. Photolysis of EDM-T_4 (Figure 10, lane a) resulted in the radiolabeling of a number of protein bands in approximate proportion to their abundance. With the exception of one band, these bands were nonspecifically labeled; i.e., the inclusion of excess cold T_4 failed to prevent labeling (lane b). This specifically labeled band, migrating near the dye front, corresponds to the position of authentic TBPA monomer standard (lane l). Although TBG binds approximately 4 times more T_4 than TBPA in vivo (Woeber & Ingbar, 1968), we observed no labeling of TBG with EDM-T_4. Other experiments (data not shown) with partially purified TBG indicated that EDM-T_4 binds poorly to TBG (<2% T_4). Presumably, the low occupation of TBG binding sites accounts for this inability of EDM-T_4 to photolabel TBG in serum.

Photolysis of serum in the presence of [^{125}I]-L-T_4 presents a rather different situation. At the low concentration of [^{125}I]-L-T_4 used (1 nM) with 50-fold diluted serum it can be calculated that most of the added hormone must be bound to TBG (due to its high binding affinity) and a small amount to TBPA. Use of a lower concentration was feasible because of the higher affinity of T_4 relative to that of EDM-T_4 for the binding proteins and because [^{125}I]-L-T_4 is available at much

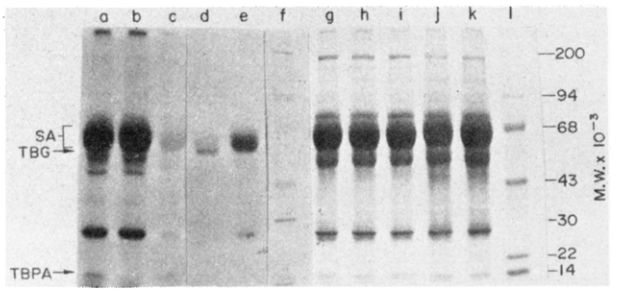

FIGURE 10: Photolabeling of thyroid hormone binding proteins in human serum. Fresh fasting human serum was diluted 50-fold into phosphate-buffered saline. [^{125}I]-L-EDM-T_4 (100 nM) or [^{125}I]-L-T_4 (1 nM) was added in the presence or absence of 2 μM unlabeled T_4. After incubation for 30 min at 20 °C, photolysis was carried out for 30 s (EDM-T_4 or 240 (T_4) at 254 nm. These longer photolysis times were chosen to compensate for UV absorption by serum proteins. After photolysis the samples were desalted by gel filtration and lyophilized before resuspension in $NaDodSO_4$–sample buffer and subsequent electrophoresis. Lanes a–f are the autoradiograms, lane g–l are the corresponding Coomassie blue stained gels before autoradiography. Lane a, [^{125}I]-L-EDM-T_4; lane b, [^{125}I]-L-EDM-T_4 plus unlabeled T_4; lane c, [^{125}I]-L-EDM-T_4, no irradiation; lane d, [^{125}I]-L-T_4; lane e, [^{125}I]-L-T_4 plus unlabeled T_4; lane f, ^{14}C-labeled standard proteins [myoglobin, M_r 200 000; phosphorylase *b* (doublet), M_r 94 000; bovine serum albumin, M_r 68 000; ovalbumin, M_r 43 000; carbonic anhydrase, M_r 30 000; lysozyme, M_r 14 000]. Lane l, unlabeled protein standards (phosphorylase *b*; bovine serum albumin; ovalbumin; soybean trypsin inhibitor, M_r 22 000; TBPA, M_r 14 000). Lanes d and e represent a 40-h exposure and lanes a–c and f represent a 14-day exposure of the same gel.

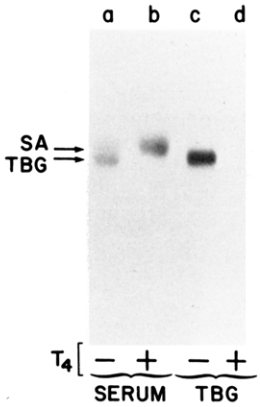

FIGURE 11: Photolabeling of partially purified TBG and serum binding proteins with [^{125}I]-L-T_4. A 100-fold dilution of human serum was incubated with 1 nM [^{125}I]-L-T_4 in the absence (lane a) or presence (lane b) of 2 μM unlabeled T_4. The incubation mixtures were then photolyzed, desalted, and electrophoresed as described in the legend to Figure 10. Partially purified TBG was incubated similarly in the absence (lane c) or presence (lane d) of 2 μM unlabeled T_4. All samples were irradiated for 100 s, and the autoradiogram was exposed for 24 h.

higher specific activity than we have been able to synthesize [^{125}I]-L-EDM-T_4. Upon photolysis, one band was strongly labeled (lane d). This band, which runs just ahead of the overloaded serum albumin band, is appropriate for the reported size estimates of TBG (Nilsson & Peterson, 1975; Gershengorn et al., 1977; Korcek & Tabachnick, 1974). A light background due to labeling of serum albumin was visible in this lane as well. In longer exposures, which overexpose the TBG–albumin region, specific photolabeling of TBPA can be visualized (data not shown). When excess cold T_4 was included in the incubation, the [^{125}I]-L-T_4 was displaced and TBG and TBPA were not labeled (lane e). However, due to the large increase in the free [^{125}I]-L-T_4 pool, due to displacement from TBG, photoattachment to more abundant proteins, particularly albumin, was increased (lane e).

The correct identification of the TBG band is supported by the result that labeling of this band was poorly competed by T_4Fo, an analogue that binds well to TBPA but poorly (<0.1% T_4) to TBG (Hao & Tabachnick, 1971). Unlike the result with T_4, inclusion of cold T_4Fo did not result in increased background labeling of albumin since the [^{125}I]-L-T_4 was not displaced from TBG where most of it was bound (data not shown). The identification of the photolabeled TBG band is confirmed by the experiment shown in Figure 11. Thus, photolabeling of a preparation of partially purified TBG with [^{125}I]-L-T_4 resulted in labeling of the same band that was strongly photolabeled in crude serum with [^{125}I]-L-T_4. Here, where TBG represents a major fraction of the total protein, background labeling was undetectable even when the bound [^{125}I]-L-T_4 was displaced from TBG with T_4. In this partially purified preparation the photolabeled band was completely resolved from the contaminating serum albumin.

It is difficult to judge whether any of the labeling of albumin seen in the serum experiments was binding site specific due to the levels of nonspecific labeling. Serum albumin possesses a single medium-affinity T_4 binding site as well as a large number of lower affinity sites (Steiner et al., 1966; Tabachnick, 1967). In preliminary experiments with very low concentrations of [^{125}I]-L-T_4 and purified bovine serum albumin there was no apparent diminution of photolabeling by excess unlabeled T_4 as judged by autoradiography. Indeed, electrophoresis and autoradiography of a cyanogen bromide digest of albumin photolabeled with [^{125}I]-L-T_4 revealed that each of the fragments was labeled. However, the labeling of only one of these fragments, which comprises the amino-terminal 88 amino acids, was specifically abolished when photolysis was performed in the presence of excess unlabeled T_4 (data now shown), suggesting that the medium-affinity site resides on this portion of the protein.

Discussion

In order to facilitate the isolation and characterization of thyroid hormone binding proteins, we have synthesized specific photoaffinity derivatives of thyroid hormones. The N-substituted diazomalonyl derivatives of T_3 and T_4 were prepared and tested, since this group has been successfully employed in photoaffinity labeling of other proteins (Bayley & Knowles, 1977). Substitution with the diazomalonyl group has proven to be more effective than substitution with diazoketone or diazoacetyl groups. The diazomalonyl group is more stable in aqueous solutions in the neutral pH range and shows less tendency to undergo nonproductive intramolecular Wolff rearrangements after photolysis than the latter groups (Baron et al., 1973). The stability in the neutral pH range observed for the *N*-(ethyl-2-diazomalonyl) analogues of T_3 and T_4 also supports earlier findings that pH-dependent Dimroth rearrangements are not a problem with ethyl-2-diazomalonyl derivatives of amines (Brunswick & Cooperman, 1971) and facilitates the use of these derivatives in biological applications.

Two types of light-dependent affinity labeling of TBPA with EDM-T_4 have been demonstrated at subsaturating levels of ligand: carbene dependent and carbene independent. Both types have been measured by using [^{125}I]-L-EDM-T_4 in direct attachment assays as well as by using unlabeled reagent in inactivation assays where blocked high-affinity T_4 binding sites were estimated by titration of the remaining sites with [^{125}I]-L-T_4. The carbene-dependent photoattachment efficiency (sites covalently labeled/sites initially occupied) was 54–61% and the carbene-independent photoattachment efficiency was 19–26%. Approximately 65% of the photoaddition products of both photolabeling processes that are resistant to gel fil-

tration and dialysis were recovered in TBPA monomer subunits after $NaDodSO_4$ gel electrophoresis. One explanation for this observation is that some of the photoaddition products may not be stable covalent adducts.

The carbene-dependent and carbene-independent photolabeling processes were both specific for the high-affinity hormone binding site of TBPA. It was shown that the carbene-dependent process reached saturation at high ligand concentration, was completely blocked when photolysis was carried out in the presence of excess L-T_4Ac, and could not be demonstrated after T_4 binding sites were previously blocked with the chemical affinity reagent, dansyl chloride. For both types of photolabeling processes, the concentrations of T_4 and T_3 required to reduce covalent attachment by 50% were similar to the equilibrium dissociation constants for these ligands.

With regard to the carbene-independent photolabeling reported here, similar examples of nonchromophore, yet light-dependent attachments are known. Carbene-independent labeling using diazomalonyl derivatives of cAMP has been described by Guthrow et al. (1973) and nitrene-independent labeling using an azido derivative of angiotensin II by Galardy & LaVorgna (1981). Moreover, many aromatic ligands lacking either carbene- or nitrene-generating groups have been employed in photoaffinity labeling of proteins [for a review, see Chowdhry & Westheimer (1979)]. The carbene-independent labeling described in the present report may involve covalent attack of protein residues by means of radicals generated by light-catalyzed iodine loss at inner or outer thyronine ring positions. Aryl halogen containing compounds, such as thyroxine, are known for their photoreactivities (van der Walt & Cahnmann, 1982). For example, Cysyk & Prusoff (1972) reported that ultraviolet irradiation of 5-iodo-2′-deoxyuridine results in reactive free radicals of deoxyuridine and iodide: when 5-iodo-2′-deoxyuridine is bound to thymidine kinase, photolysis of the complex causes the irreversible attachment of the pyrimidine moiety of the photoreactive substrate to the enzyme. In support of this mechanism, we demonstrated specific, site-directed, covalent labeling of TBPA using L-T_4. The efficiency of photolabeling with T_4 was, however, much lower than the efficiency with prephotolyzed EDM-T_4. The reason that more carbene-independent photolabeling resulted with EDM-T_4 than with L-T_4 is not apparent. The ring systems in EDM-T_4 might be oriented in the binding site in a more favorable position for radical attack on protein residues than T_4, or bound EDM-T_4 may be more susceptible to irradiation damage than T_4. Another explanation that could account for this result are differences in the dissociation rates of the ligands from TBPA relative to the kinetics of radical formation and attack. We cannot rule out the additional possibility that the carbene-independent labeling is due to a unique photoactivation of the ethyl-2-diazomalonyl photoproduct.

It is important to note that virtually no photoattachment could be detected when either EDM-T_4 or the photolysis product of EDM-T_4 was exposed to TBPA without further irradiation. This distinguishes the photolabeling reactions reported here from "pseudophotoaffinity" mechanisms that involve dark reactions between protein nucleophiles and long-lived electrophilic photoproducts generated in solution (Ruoho et al., 1973; Katzenellenbogen et al., 1977; Payne et al., 1980). For example, 90% of the photoaffinity labeling of rat α-fetoprotein with 16-diazoestrone was actually attributed to an electrophilic intermediate formed by photolysis in solution (possibly a reactive ketene) rather than to the intact diazo group of the reagent (Payne et al., 1980). The possibility that the labeling of TBPA reported here involves free, electrophilic photoproducts would appear to be excluded since 0.1 M Tris-HCl, which was present in all reactions, would have functioned as an effective nucleophilic scavenger (Marver et al., 1976). In addition, in other experiments (data not shown) we found that both the carbene-dependent and -independent photoattachment processes proceeded to the same extent in the presence of 20 mM β-mercaptoethanol, a more commonly used scavenger (Payne et al., 1980). Until the photoattachment products between EDM-T_4 and TBPA are identified we cannot rule out the possibility that a ketene from Wolff rearrangement of the α-diazo ester (Chaimovich et al., 1968) or a short-lived reactive species other than a ketocarbene generated within the binding site may participate in the labeling of TBPA reported here.

Photoaffinity labeling has potential for identifying and characterizing minor or unstable components, such as hormone acceptors, that are difficult to obtain in a pure, biologically active form. As a first step toward determining the feasibility of labeling the thyroid hormone nuclear receptor we have utilized EDM-T_4 and T_4 to photolabel thyroxine-binding proteins in unfractionated human serum. We demonstrated labeling of TBPA with EDM-T_4 that is prevented by competition with excess cold T_4. EDM-T_3 binds to the nuclear receptor, albeit weakly (0.7% of L-T_3), but progress with photolabeling is limited by the relatively low specific activity we have thus far been able to obtain for [^{125}I]-L-EDM-T_3. With T_4 itself as a photoaffinity probe, we have specifically photolabeled both TBG and TBPA in crude serum. TBG is present in plasma at only about 1 part in 6000. Thus, the ability to detect photolabeling of such a minor component suggests that the use of T_3 as a photoaffinity probe for the nuclear thyroid hormone receptor is feasible. Ongoing studies indicate that this is possible, and the results of these investigations are reported in a separate communication (David-Inouye et al., 1982).

Acknowledgments

We express our appreciation to Nancy Malich for providing technical assistance and to Dorothea Faber and Andrea Mazel for assistance in the preparation of the manuscript. We also acknowledge the helpful advice of Dr. John Baxter. We are deeply indebted to the late Dr. Eugene Jorgensen, who provided expert advice and encouragement during the course of these studies. We share with others within the field of thyroid hormone action a profound sense of loss in Dr. Jorgensen's intimely death.

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