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Biochemistry

Synthesis of thyroid hormone metabolites by photolysis of thyroxine and thyroxine analogs in the near UV

(specifically labeled iodothyronines/HPLC)

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ABSTRACT **Photolysis of thyroxine and its analogs in the near UV permitted synthesis in good yield of picogram to gram quantities of thyroid hormone metabolites. Preparation of the same metabolites by classical chemical synthesis requires multistep procedures. Specifically labeled metabolites of high specific activity (e.g., those carrying the label in the nonphenolic ring) were obtained by photolysis of appropriately labeled thyroxine or 3,3',5'-triiodothyronine (reverse triiodothyronine). Some of these labeled metabolites, which are required for metabolic studies (3-iodothyronine and 3,3'-diiodothyronine, labeled in the nonphenolic ring), had not previously been obtained by other methods. Irradiation of thyroxine and reverse triiodothyronine in 150 mM methanolic ammonium hydroxide with >340-nm light caused removal of one iodine atom from the phenolic ring with formation of 3,5,3'-triiodothyronine and 3,3'-diiodothyronine, respectively. Irradiation with higher-energy light (>300 nm) led to stepwise removal of additional iodine atoms. Those in the phenolic ring were removed preferentially, so that 3,5-diiodothyronine and 3-iodothyronine, respectively, were formed. The iodine atoms in the nonphenolic ring were lost more slowly. Tetraiodothyroacetic acid followed a similar photodeiodination pattern. Photolysis with light in the near UV is a simple method for the synthesis of thyroid hormone metabolites.**

Since photolysis of the thyroid hormones thyroxine (T4) and 3,5,3'-triiodothyronine (T3) with short-wave UV light causes rapid and extensive deiodination as well as diphenyl ether splitting (1, 2), we have investigated photolysis in the near UV. Use of this lower-energy light permitted study of the kinetics of the photodegradation of T4 and analogs of T4. These kinetics made it possible to devise a simple procedure for the synthesis of various thyroid hormone metabolites by photolysis. Both unlabeled and specifically labeled metabolites were prepared.

MATERIALS AND METHODS

3'-Iodothyronine (3'-T1) and 3,3'-diiodothyronine (3,3'-T2) were synthesized by iodination of thyronine and 3-iodothyronine (3-T1), respectively. Thyronine, 3-T1, and 3',5'-diiodothyronine (3',5'-T2) were gifts from Paul Block, Jr. All other iodoamino acids and desamino analogs were obtained from commercial sources in the form of the free acids.

[^{125}I]T3 and [^{125}I]T4 labeled exclusively in the phenolic ring (carrier-free) were from New England Nuclear. [^{125}I]T4 of high specific activity labeled exclusively in the nonphenolic ring was synthesized as described (3).

3,3',5'-[^{125}I]Triiodothyronine ([^{125}I]rT3) of high specific activity labeled exclusively in the nonphenolic ring was synthesized as follows. Tyrosine (3 mg) in 10 μl of 15 M ammonium hydroxide and 5 ml of 0.1 M sodium phosphate (pH 7) were thoroughly mixed and then centrifuged. To 100 μl of the clear supernatant (pH 7.5) was added carrier-free Na ^{125}I (≈50 μCi; 1 Ci = 3.7×10^{10} becquerels) and then 10 μl of a freshly prepared aqueous solution of chloramine T (4 mg/ml). The reaction was stopped after 2 min by addition of 10 μl of sodium metabisulfite (a 1:10 dilution of a saturated aqueous solution). The reaction mixture was fractionated by HPLC as described below for the analysis of irradiated solutions. The combined 3,5-diiodotyrosine-containing fractions were desalted (3) and then evaporated. The residue was treated with 4-hydroxy-3,5-diiodophenylpyruvic acid as described for the synthesis of [3,5-^{125}I]T4 (3) except that the pH was adjusted to 8.5 instead of 7.6. After fractionation of the reaction mixture on an AG50W-X4 column (1), the combined rT3-containing fractions were desalted (3) and then evaporated. (The elution volume of rT3 is only very slightly greater than that of T4.)

Photolysis. Labeled or unlabeled T4 or analogs of T4 were irradiated in 150 mM methanolic ammonium hydroxide with light from a Canrad–Hanovia (Newark, NJ) lamp 697A-36. This lamp emits light over a wide wavelength range. However, since the lamp was placed inside a double-walled immersion well of borosilicate glass,† virtually all <300-nm light was filtered out (1% transmission at 300 nm). Cooling was provided by circulating tap water.

In experiments in which less energetic light was desired, a Corning filter 0-52 (17 × 25 × 2 mm, glass no. 7380) was taped onto the outside wall of the well. No matter whether the filter was used or not, the remainder of the well was covered with aluminum foil for more efficient use of light. With the filter in place, virtually all <340-nm light was filtered out (1% transmission at 340 nm).‡

The solution to be irradiated (0.2–2 ml) was in a small plastic cup (15 mm diameter, 15 mm high), which was placed in a bath of ice water. The lamp–immersion well assembly was placed horizontally above the cup and touching it. In some experiments, the cup was replaced with a tightly stoppered spectrophotometric quartz cell (1-cm light path) that permitted flushing of the sample solution with virtually oxygen-free nitrogen (4) before irradiation.

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Abbreviations: T4, thyroxine; T3, 3,5,3'-triiodothyronine; rT3, 3,3',5'-triiodothyronine; 3,3'-T2, 3,3'-diiodothyronine; 3,5-T2, 3,5-diiodothyronine; 3',5'-T2, 3',5'-diiodothyronine; 3-T1, 3-iodothyronine; 3'-T1, 3'-iodothyronine. All amino acids were *L*(*S*)enantiomers.

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† This home-made well was similar to one that can be obtained from Ace Glass (7857E).

‡ Transmission data were determined by placing two pieces of glass of the same composition and thickness (4 mm) as used in making the immersion well, with and without an additional Corning filter 0-52, in the cell compartment of a recording spectrophotometer.

In large-scale experiments, the lamp–immersion well assembly, without filter and aluminum foil, was inserted vertically into a cylindrical flask by means of a large ground glass joint. This flask, which, after insertion of the immersion well, had a capacity of 400 ml, contained the solution to be irradiated and had gas inlet and outlet tubes for slow nitrogen bubbling during photolysis. This ensured good mixing and uniform irradiation. Since nitrogen bubbling causes evaporation of ammonia, methanolic ammonium hydroxide (see above) was replaced by methanolic sodium hydroxide.

Analysis of Irradiated Solutions. Aliquots of irradiated solutions containing trace amounts of carrier-free radioactive solutes or ≈2 μg of unlabeled solute were analyzed by HPLC. The instrument and the μBondapak C_{18} column used were from Waters Associates (see Fig. 2). For analysis of desamino analogs of iodothyronines, elution was continued isocratically after completion of the gradient. Photolysis products were obtained by collecting the eluate corresponding to a given absorption peak (280 nm). Labeled photolysis products were analyzed by collecting the eluate in 0.6-ml fractions and assaying them in a gamma scintillation counter.

RESULTS

Since compounds can be photolyzed only to the extent to which they absorb light, it was essential to determine the absorption spectra of various iodothyronines in 150 mM methanolic ammonium hydroxide, the solvent normally used.

The absorption spectra of T4, T3, and 3,5-diiodothyronine (3,5-T2) and the wavelength regions in which virtually no light reached the sample when Corning filter 0-52 was used and when this filter was omitted are shown in Fig. 1. The absorption spectrum of rT3 (not shown) is similar to that of T4. Above 340 nm (with filter), nearly all the absorbed light is taken up by T4 (or rT3), while T3 receives much less and T2 receives virtually none. This explains why >340-nm light photolyzed T4 much more readily than T3, while 3,5-T2 was resistant to photolysis (see below). In contrast, all three compounds absorb >300-nm light (without filter) and can therefore by photolyzed. Even in this case, the rate of photodegradation decreased in the order T4(rT3) > T3 > 3,5-T2, as could be expected from the absorption spectra.

HPLC of the photolysates perfectly resolved tyrosine, thyronine, and nine iodoamino acids (Fig. 2), as well as 3,5,3′,5′-tetraiodothyroacetic and 3,5,3′,5′-tetraiodothyropropionic acid and their less iodinated congeners (not shown). Only 3,3′-T2 and 3′5′-T2 were incompletely separated from each other. The desamino analogs had appreciably longer retention times than the iodothyronines (see ref. 5).

Photolysis of T4. The photodegradation of T4, when irradiated with >300-nm light for various periods of time, is shown in Fig. 3. A small amount of T3 has been formed after 1 min while, after 5 min more, T3 and a small amount of 3,5-T2 were present in the reaction mixture. With further irradiation, the stepwise deiodination of T4 continued so that, after 15 min, almost no T4 and only little T3 remained. A small amount of 3,3′-T2 also was present in the 15-min photolysate, but the major reaction products were 3,5-T2 and 3-T1.

Iodine atoms were removed much faster from the phenolic than from the nonphenolic ring of T4. No more than traces of rT3 and 3′,5′-T2 were present in any of the photolysates. Preferential loss of iodine atoms in the phenolic ring also applies to T3, the primary photolysis product of T4. Only a small amount of 3,3′-T2 and no 3′-T1 were present in the 10-min photolysate. The major photodegradation product was 3,5-T2, which has no iodine atoms remaining in the phenolic ring and hence has its absorption peak at a lower wavelength than T4 or T3. Nevertheless, it absorbs some >300-nm light (Fig. 1) and therefore slowly underwent further deiodination to 3-T1, which was present, together with a larger amount of 3,5-T2, in the 15-min hydrolysate.

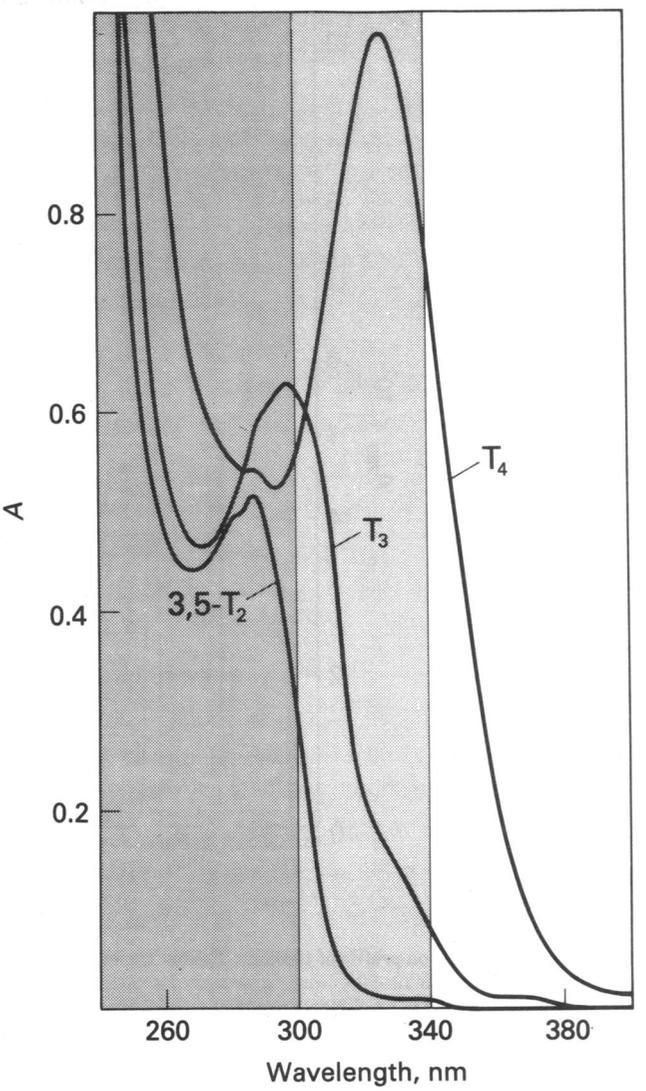

FIG. 1. Absorption spectra of 153 μM T4, T3, and 3,5-T2 in 150 mM methanolic ammonium hydroxide. The shaded areas indicate wavelength regions in which virtually no light is received by the sample during photolysis: ▩, photolysis with a Corning filter 0-52; □, photolysis without a filter.

The kinetics of the photolysis of T4 over more extended periods of time are shown in Table 1. Irradiation with >300-nm light for 20–30 min led to gradual decrease of 3,5-T2 and a concomitant increase of 3-T1. The former could not be replenished due to exhaustion of T4 and its primary photolysis product T3. Further deiodination of 3-T1 to thyronine proceeded very slowly.

Irradiation of T4 with lower-energy light (>340 nm) yielded the primary deiodination product T3 much more slowly, but in higher yield because, under these conditions, T3 was fairly resistant to further deiodination as expected from the spectrum (Fig. 1). After 40 min, 42% of the T4 used was present in the photolysate as T3 and only 13% as 3,5-T2.

The importance of the light energy is demonstrated in Fig. 4 which shows the HPLC profiles of two T4 photolysates obtained under identical conditions except that >300-nm light was used in one case and >340-nm light was used in the other. With

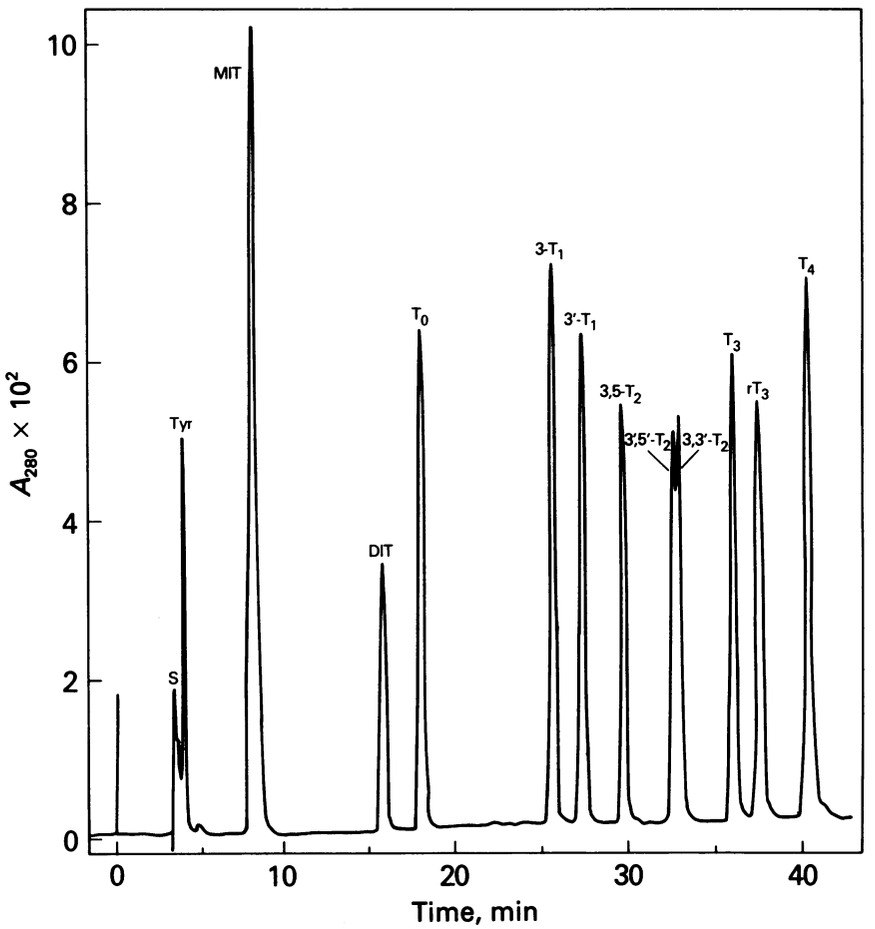

FIG. 2. HPLC elution profile of tyrosine, T_0, and various iodoamino acids. A μBondapak C_{18} column was used; elution for 40 min at a flow rate of 1 ml/min was with a 10–50% linear gradient of acetonitrile in 15 mM ammonium acetate, adjusted to pH 4 with acetic acid. S, solvent front; MIT, 3-iodotyrosine; DIT, 3,5-diiodotyrosine; T_0, thyronine.

the higher-energy light, virtually all T4 was converted to 3,5-T2 and 3-T1 after 20 min while, with the lower-energy light, the major photodegradation product after the same period of time was T3.

Photolysis of rT3. The kinetics of the photolysis of rT3 are shown in Table 2. As in the case of T4, deiodination took place almost exclusively in the phenolic ring. The primary and secondary photodegradation products were 3,3'-T2 and 3-T1, respectively. The latter was resistant to further deiodination by >340-nm light while >300-nm light caused slow deiodination to thyronine as observed in the photolysis of T4.

Photolysis of 3,5-T2. 3,5-T2 is hardly affected by >340-nm light. Irradiation with >300-nm light led to removal of one iodine atom, followed by slower removal of the second one. The kinetics of these deiodinations, as observed in a large-scale experiment, are shown in Table 3. Photolysis of 3,5-T2 for 2.5–3 hr gave 3-T1 in 67% yield. Hence, the synthesis of 3-T1 by photolysis compares favorably with that by chemical methods (6), which requires more synthetic steps.

Photolysis of 3,5,3',5'-Tetraiodothyroacetic Acid. The photolytic breakdown pattern was analogous to that of T4. The primary photolysis product was 3,5,3'-triiodothyroacetic acid, which then underwent further stepwise deiodination at a slower rate. 3,5,3',5'-Tetraiodothyropropionic acid behaved similarly.

Labeled Metabolites of T4. Labeled metabolites of high specific activity carrying the label exclusively in the nonphenolic ring were synthesized in the following manner. [3,5-^{125}I]T3 was obtained by photolysis of [3,5-^{125}I]T4 for 40 min with >340-nm light. [3-^{125}I],3'-T2 was obtained when [3-^{125}I]rT3 was treated similarly. Irradiation of [3,5-^{125}I]T4 for 15 min with >300-nm light gave [3,5-^{125}I]T2 and similar treatment of [3-^{125}I]rT3 for 20 min gave [3-^{125}I]T1. The yields were similar to those shown in Tables 1 and 2 for the corresponding unlabeled compounds.

DISCUSSION

Advantages of the photolytic method of preparing thyroid hormone metabolites are its simplicity and the possibility of synthesizing several metabolites in a single photolysis run. One or the other of these can be obtained preferentially depending on the light filter used and the irradiation time. The various metabolites are easily separated from each other by HPLC, which also can be used for the isolation of small amounts of chromatographically pure metabolites. Another advantage of the photolytic method is that it is equally applicable to the photolysis of small and large amounts (picograms to grams) of iodothyronines.

Furthermore, certain specifically labeled iodothyronines of high specific activity, which are required for metabolic studies, can be prepared by photolysis. Thus, 3-T1 and 3,3'-T2, labeled exclusively in the nonphenolic ring, were obtained by photolysis of similarly labeled rT3. Photolysis of T4 labeled in the nonphenolic ring also gave labeled 3-T1, together with labeled 3,5-T2. When the photolysis was carried out using a filter that eliminates virtually all <340-nm light, the major photolysis product was T3 labeled exclusively in the nonphenolic ring. Synthesis of this compound by photolysis is simpler than the

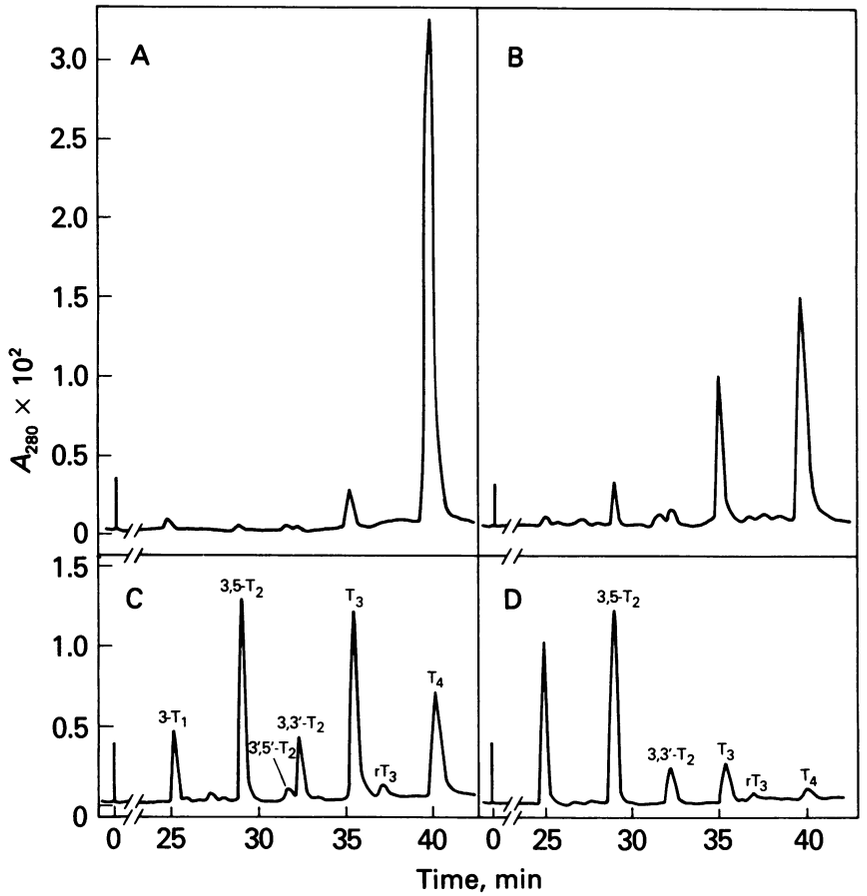

FIG. 3. HPLC elution profiles of photolysates of T4 obtained by irradiation with >300-nm light for 1 min (*A*), 5 min (*B*), 10 min (*C*), and 15 min (*D*).

previously reported one by chemical methods (7), which requires more steps.

While the kinetics of the photodegradation of various iodothyronines largely depends on the relative amounts of light absorbed by them (Fig. 1), the structure of the photodegradation products produced is greatly influenced by the nature of the solvent. When hydrogen-donating solvents such as aliphatic alcohols were used, stepwise deiodination took place almost exclusively and only small amounts of unknown products were

Table 1. Kinetics of photodegradation of T4

Wavelength, nm	Time, min	Yield, % of total					
		T4	T3	3,3'-T2	3,5-T2	3-T1	T_0
>300	1	**88**	7	0	1	2	1
>300	5	46	**31**	4	9	2	1
>300	10	14	26	9	**27**	10	1
>300	15	2	7	6	**36**	30	4
>300	20	1	3	3	30	**35**	6
>300	25	0	1	2	24	**39**	10
>300	30	0	0	1	18	**40**	13
>340	20	35	**40**	4	5	1	0
>340	40	13	**42**	9	13	4	0
>340	60	5	**34**	12	20	7	1

Yields were determined by comparing the heights of the various absorption peaks (280 nm) obtained by HPLC of the photolysates. (The molar extinction coefficients of various iodothyronines at 280 nm do not differ greatly; Fig. 1.) rT3, 3',5'-T2, 3'-T1, 3,5-diiodotyrosine, 3-iodotyrosine, and tyrosine are not included because yields never exceeded 4%. T_0, thyronine. Boldface numbers indicate highest yield obtained for given irradiation time.

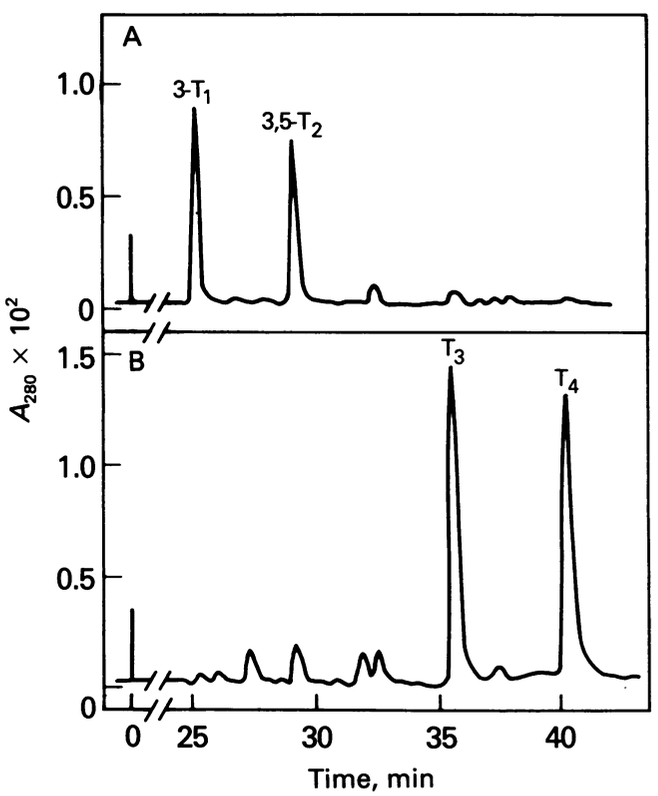

FIG. 4. HPLC elution profiles of photolysates of T4 obtained by irradiation for 20 min with >300-nm light (*A*) or >340-nm light (*B*).

Table 2. Kinetics of photodegradation of rT3

Wavelength, nm	Time, min	Yield, % of total				
		rT3	3,3'-T2	3-T1	T_0	MIT
>300	1	**88**	12	0	0	0
>300	5	43	**35**	15	1	2
>300	10	16	34	**36**	4	3
>300	15	6	21	**50**	8	5
>300	20	3	10	**53**	13	7
>300	25	1	5	**49**	21	5
>340	20	34	**50**	5	0	2
>340	40	12	**56**	15	1	4
>340	60	4	18	**25**	2	4

Yields were determined as in Table 1. 3',5'-T2, 3'-T1, and tyrosine are not included because yields never exceeded 3%. T_0, thyronine. Boldface numbers indicate highest yield obtained for given irradiation time.

Table 3. Kinetics of photodegradation of 3,5-T2 with >300-nm light

Time, hr	Yield, % of total		
	3,5-T2	3-T1	T_0
0.5	**65**	28	0
1.0	35	**52**	2
1.5	21	**62**	5
2.0	14	**65**	9
2.5	8	**67**	13
3	4	**67**	17
4	0	**58**	27
5	0	**48**	35
8	0	25	**49**

Yields were determined as in Table 1. Kinetics were determined in a large-scale (1 g of 3,5-T2) experiment. T_0, thyronine. Boldface numbers indicate highest yield obtained for given irradiation time.

formed. Irradiation of T4 or an analog of T4 in the near UV causes homolytic fission of a C—I bond. The iodothyronine radical thus formed abstracts a hydrogen atom from the solvent to form a C—H bond (see ref. 8). The photolyses described in this communication were carried out in methanol containing 1% concentrated ammonium hydroxide.

When acetonitrile (which is not a hydrogen-donating solvent) containing 5% 150 mM methanolic ammonium hydroxide for better solubility was used, stepwise deiodination took place at a slower rate and a large number of unknown products was formed. In water or aqueous buffer at neutral or alkaline pH, photodegradation took an entirely different course. Although T4 was degraded rapidly, numerous unknown products were formed and virtually no known metabolites of T4 were present in the photolysates.

Various other solvents were tried (methanol acidified with concentrated hydrochloric acid, 98% formic acid, dimethyl sulfoxide, 1-propanol/1% ammonium hydroxide), but none gave as good yields of T4 metabolites as 150 mM methanolic ammonium hydroxide. Neither changes in temperature between 2°C and 30°C nor exclusion of oxygen by nitrogen bubbling appreciably altered photolytic degradation patterns.

Although, in alkaline methanol, iodine atoms are removed from both rings of iodothyronines or their desamino analogs, deiodination of the phenolic ring is by far predominant. The rates of deiodination at various positions decrease in the order $5' > 3' > 5 > 3$.

This means that deiodination can, through proper choice of substrate, light filters, and irradiation time, be controlled in such a manner that a desired thyroid hormone metabolite can be obtained in optimal yield. Thus, photolysis of T4 or T4 analogs in the near UV promises to become the method of choice for the synthesis of unlabeled or labeled metabolites that are difficult to prepare by chemical methods.

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