Activation of the Flavin Photodeiodination of Thyroxine by “Thyroxine Deiodinase” and Other Proteins*

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(Received for publication, April 15, 1963)

The deiodination of thyroid hormones in vitro by slices, homogenates, subcellular fractions, and soluble extracts from tissues of animals of different species has received considerable attention and results have been reviewed fairly recently (1). With most tissues side reactions other than deiodination occur concomitantly, but with slices, homogenates, subcellular fractions, and soluble extracts obtained from skeletal muscle, the major metabolic pathway for thyroxine is through deiodinating mechanisms (2, 3). The same is true for homogenates obtained from human leukocytes (4) and from rat and bovine pituitary.1 The main iodinated products of the deiodination of thyroxine by these tissue preparations in vitro are iodide and a moiety with an Rf of zero in several solvent systems, known as “origin material” and considered to be an iodinated protein.

Tata (5) observed that the addition of riboflavin monophosphate (FMN)2 and Fe3+ ions to a skeletal muscle extract increased its thyroxine-deiodinating and origin material-forming activity considerably. Lissitzky, Roques, and Benevent (6) soon confirmed the activating effect of FMN, but not of Fe3+ ions, and realized moreover the importance of the light intensity during the reaction (7, 8). In both laboratories the assay for the determination of the thyroxine-dehalogenating activity of a muscle preparation involved addition of FMN and Fe3+ ions (9, 10) or of FMN alone (8) and a constant bright light supply. The deiodination found in the fully activated system in which the tissue preparation was either boiled or omitted was then subtracted as blank. This assay system appears to have been realized moreover the importance of the light intensity during the reaction: with most proteins origin material is formed and, moreover, the rate of the reaction is accelerated considerably. We indicated that the addition of FMN in the presence of light for the assay of the thyroxine-deiodinating activity of a given tissue preparation might yield misleading results with regard to the deiodinating mechanisms physiologically involved in the tissue.

The present paper extends these results and attempts an interpretation of the possible cause underlying this activating effect of proteins and several amino acids on the rate of deiodination of thyroxine and of other oxidations by FMN and light. Results obtained raise serious doubts as to the physiological significance of a “thyroxine deiodinase” isolated from a tissue by using an assay system supplemented with FMN and light.

**EXPERIMENTAL PROCEDURE**

Deiodination Studies—Under the general procedure, L-thyroxine, labeled with 131I in positions 3' and 5', was obtained from Abbott Laboratories in 50% propylene glycol. Equal volumes of this solution and of one containing 2 mmoles of stable L-thyroxine per 10 µl were mixed and stored at about −15°C until the moment of use.

Unless stated otherwise, 2 mmoles of L-thyroxine-131I were added to 0.5 ml of a buffer, pH 7.4, in a Pyrex glass test tube. Immediately thereafter 10 mmoles of FMN were added and the mixture was incubated at 37°C for 15 minutes in a Warburg apparatus for photosynthesis studies (Braun-Melsungen, Germany)
over two 40-watt daylight Philips fluorescent tubes. The light intensity reaching the reaction mixture was approximately 3600 lux.

When anaerobic conditions were required, the reaction was carried out in small Warburg manometric vessels with two side arms or in Thunberg tubes. Careful deoxygenation was achieved by three prolonged evacuations with a vacuum pump interspaced with the addition of highly purified nitrogen. Care was taken to protect the mixture from light during this procedure. The reaction was started by tipping in the labeled thyroxine and illuminating the vessel, and was stopped either by tipping in plasma from the other side arm or by opening the vessels only under diffuse red light.

Samples (20 to 30 μl) of the reaction mixture were applied to Whatman No. 1 or 3MM filter paper on a spot to which stable iodide (10 μg), thyroxine (40 μg), and propylthiouracil (20 μg) had been applied previously. In other instances the reaction was first stopped by the addition of 0.5 ml of human, rat, or bovine plasma or by placing the reaction mixture in the dark. The samples were then applied to the chromatographic paper in a dark room to which only diffuse red light was admitted for manipulations. The strips were kept in the dark until admission of the chromatographic solvents. In this manner errors due to the oxidation and ensuing deiodination of trace amounts of thyroxine on filter paper exposed to light and air were avoided (15, 16). With such precautions, deiodination data obtained by paper chromatography, electrophoresis, and trichloroacetic acid precipitation were coincident.

Ascending paper chromatography was carried out in 1-butanol-ethanol-2 N ammonia (5:1:2) or 1-butanol-acetic acid-water (75:100:15). Localization of the radioactive compounds was determined by radioautography, by staining the iodide with palladium chloride and the phenols with diazotized sulfanilic acid, or by scanning the strips automatically with two sensitive Geiger-Müller tubes. The proportion of I\textsuperscript{131} in the different compounds was determined quantitatively by counting the corresponding spots in a well-type scintillation counter or by planimetry of the areas resulting from the automatic scanning.

Proteins—The proteins used in this study were chosen at random. Bovine fibrinogen (Armour Laboratories), three times crystallized egg white lysozyme, twice crystallized bovine pancreas trypsin, five times crystallized bovine pancreas ribonuclease, special purity type V urease, and egg albumin from Sigma Chemical Company, thryglobulin, hemoglobin, bovine β-lactoglobulin, human α-globulins (Fraction IV), salmine trypsin, and twice crystallized edestin from Nutritional Biochemicals Corporation, penicillinase (California Corporation for Biochemical Research), human serum albumin (Protein Foundation), and insulin (Lilly) were freshly dissolved in a buffer at pH 7.4 and added to the reaction mixture before the addition of FMN and L-thyroxine. The final concentration of the protein is indicated for each experiment and usually ranged between 0.1 and 1 mg per ml. When the protein solution was boiled for 15 to 20 minutes, it was rehomogenized before addition to the reaction mixture.

Buffers—Some experiments were carried out in Krebs-Ringer-phosphate buffer, pH 7.4, but in most cases a NaH₂PO₄-Na₂HPO₄ buffer or a citric acid-phosphate buffer of the same pH was used. For experiments carried out at other pH values, citric acid-phosphate mixtures were used between pH 2.4 and 8.0. Sodium Veronal-HCl between pH 8 and 9 and, for higher pH, sodium carbonate-sodium bicarbonate mixtures were also used. Buffer solutions were prepared according to Gomori (17).

Absorption Spectra—For these determinations FMN was added to a phosphate buffer, pH 7.4, at a final concentration of 40 μmols per ml. Part of the solution was kept in the dark and the other part was illuminated for 30 minutes in Warburg manometric vessels and under the same conditions described for the deiodination procedure. The absorption spectra of the dark and illuminated flavin moieties were read in silica cuvettes in a Beckman DB automatic recording spectrophotometer, and the corresponding buffer was read as a blank. When FMN was illuminated in solutions containing amino acids and fresh or boiled proteins, the absorption spectrum of the flavin moiety was read against solutions of these compounds as blanks.

Paper Chromatography of FMN Derivatives—Samples (0.25 ml) of the reaction mixtures were delivered to Whatman No. 3MM paper strips in the dark room under diffuse red light. Ascending chromatography was carried out in 1-butanol-acetic acid-water (4:1:5) and the strips were later left to dry in the dark. Flavin derivatives were then identified by visual inspection over an ultraviolet lamp; their RF values and the color of their fluorescence were used as reference (18).

DPNH Oxidation—The catalytic aerobic oxidation of DPNH by FMN and light was studied under experimental conditions similar to those described by Vernon (19); light intensity reaching the mixture was, however, about 3600 lux. The oxidation of DPNH was followed by measuring the decrease in optical density at 340 mp. The optical densities of solutions illuminated in the same run and containing all the reagents except the DPNH were subtracted as blanks. Proteins and amino acids were added to give final concentrations of 1 mg per ml.

RESULTS

Conditions Leading to Activation of Deiodination of Thyroxine—

The radioautography of a typical paper chromatogram showing the kinetics of the deiodination of I\textsuperscript{131} labeled L-thyroxine by FMN and light in the absence and presence of 10% human plasma has been reported previously (14). In the protein-free medium the main iodinated product of the reaction is iodide,\textsuperscript{3} with some radioactivity appearing at the origin of chromatograms, just above the origin and just below the iodide spot. In the presence of plasma, a large proportion of the radioactivity liberated from the hormone was found as origin material. In neither case was triiodothyronine ever identified clearly as a product of the reaction. When the concentration of plasma in the medium was decreased to about 1 to 2%, not only did appreciable amounts of origin material appear as reaction product, but the rate of the deiodination was appreciably accelerated. Boiling of the plasma eliminated this activating effect and the reaction then proceeded qualitatively and quantitatively as in the absence of plasma. It was also shown (14), with the use of purified bovine fibrinogen, that results were essentially the same as those obtained with plasma; the activity of the fibrinogen

\textsuperscript{3} Iodine would have an RP similar to that of iodide in the butanol-ethanol-ammonia system; the possibility that the I\textsuperscript{131} with this RP is in the form of iodine is not very likely, considering that no radioactivity could be extracted from the reaction mixture with organic solvents after acidification, except when an oxidizing agent such as FeCl₃ or H₂O₂ is also added.
**Protein Activation of Thyroxine Deiodination and Muscle Deiodinase**

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Contrc~ll:T;~'

**L**

131 T4 +FMN+ Lytozyme

168

Control 2: in+ FMN* boiled Lyrozyme

CL1 mg

AdTLzl&

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FIQ. 1. Automatic scanings of paper chromatograms, developed in 1-butanol-ethanol-ammonia, of samples containing 131-labeled L-thyroxine, FMN, Krebs-Ringer-phosphate buffer, pH 7.4, and increasing amounts of fresh or boiled egg white lysozyme. The amounts of each reactant were added as described under "Experimental Procedure." The bars at the bottom of the figure indicate the location of chemically identified iodide and thyroxine and of the origin of the chromatograms. T4 denotes thyroxine in this and subsequent figures, wherever it appears.

**FIQ. 2.** Activating effect of urease and trypsin on the rate of deiodination of thyroxine by FMN and light. Experimental conditions were as indicated for Fig. 1, except that the buffer was citric acid-phosphate, pH 7.4. The degree of deiodination observed in protein-free samples was subtracted as blank.

Preparation as catalyzer of the deiodination of thyroxine by FMN and light was proportional to its concentration within the range studied. Fibrinogen therefore appeared to behave as a "thyroxine deiodinase" in an FMN-supplemented assay system.

We have observed similar results with other purified proteins. The addition of purified egg white lysozyme, for instance (Fig. 1), results in the appearance of origin material and in the acceleration of the reaction rate, these effects being more intense as the protein concentration increases. With boiled lysozyme, no origin material appears and the rate of deiodination proceeds as in the absence of the protein. Qualitatively similar results obtained with urease are shown in Fig. 2. With such an FMN + light-supplemented assay system, a direct relationship would be found between the "deiodinating activity" of urease and its concentration. The same was found with proteins other than fibrinogen, lysozyme, and urease; namely, with thyroglobulin, hemoglobin, penicillinase, and low concentrations of human or rat plasma. The intensity of the activating effect of these proteins and the proportion of iodine found as origin material depend on the protein used. Thyroglobulin and urease were found to have an especially high origin material-forming activity: about 30% of the iodine liberated from the hormone was found at the origin of the chromatograms.

Boiling does not always eliminate the capacity of a protein to activate the deiodination of thyroxine by FMN and light. Boiled trypsin, for instance, accelerates the deiodination rate more intensely and has a higher origin material-forming activity than the fresh preparations (Fig. 2). In the case of insulin, glucagon, and salmon protamine, for instance, the fresh and boiled preparations have approximately the same activating effect on the rate of deiodination. Other proteins, such as ribonuclease and β-lactoglobulins, which do not increase the rate of deiodination of thyroxine by FMN and light, acquire this property after boiling for 10 to 15 minutes.

Although most of the amino acids tested so far (methionine, leucine, glycine, valine, cystine, and oxidized glutathione) have no effect on the rate and products of this deiodination reaction, it was found that histidine and tryptophan have a most intense activating effect, as shown in Fig. 3.
In both the absence and presence of "activating" proteins, an increase in the concentration of FMN or in the intensity of illumination results in an acceleration of the reaction rate (Fig. 4). An increase of the hormone concentration causes an increase of the total amount of thyroxine degraded, within the concentration range studied, although the proportion of hormone undergoing deiodination decreases progressively (Fig. 4). The activating effect of proteins becomes less and less apparent as the concentration of thyroxine or of FMN or the light intensity increases. Results described here are therefore only reproducible under experimental conditions similar to those employed for the present experiments. The activation spectrum of the reaction was not determined, and the possibility that the wave length spectrum of the light source employed also influences results was therefore not excluded.

Plotting data such as those shown in Fig. 4 according to the method of Lineweaver and Burk (20) permitted the calculation of apparent $K_m$ constants for trypsin and lysozyme, for instance. It was found that they varied with the concentration of FMN employed in the assay system.

The importance of the pH of the reaction mixture for the ensuing deiodination rate is shown in Fig. 5, in the absence of proteins, the maximal rate of deiodination of thyroxine by FMN and light is observed at pH 7.4 to 8.0. The half-maximal rate of reaction occurs at a pH coincident with the $pK$ of the phenolic hydroxyl group of thyroxine, as occurs with other protein-free deiodinating systems (16, 21). When proteins such as lysozyme and tryptophan were present during the deiodination reaction, a maximal peak of activity was found in the alkaline range, as in the case of the protein-free reaction. However, a peak of deiodinating activity was also obtained at about pH 4, several pH points below the $pK$ of the phenolic hydroxyl group of thyroxine. Similar peaks of activity in the acid pH range were also found with dilute plasma or an amino acid, tryptophan. However, it was not observed with other proteins and amino acids which also activate the deiodination of thyroxine by FMN and light, such as urease and histidine. The pH of the reaction mixture was controlled before and at the end of each experiment; the appearance of a peak of activity in the acid pH range did not depend on the buffer anion employed.

None of the proteins and amino acids used for the present study had any thyroxine-deiodinating or origin material-forming activity in the absence of FMN, light, or both.

Conditions Which Decrease or Inhibit Deiodination Rate—As already indicated (14), high concentrations in the reaction medium of proteins with thyroxine-binding power, such as human serum albumin, decrease the rate of deiodination of thyroxine by FMN and light and may block it completely. Under experimental conditions such as those described here, substitution of 30% of the buffer by human plasma completely inhibits the deiodination reaction. If the FMN concentration is increased, however, the amount of plasma necessary to achieve the same result has to be increased also. Reduced glutathione ($10^{-3}$ M), $10^{-3}$ M cysteine, $10^{-3}$ M serotinin, $10^{-3}$ M ascorbate, $10^{-3}$ M diethylstilbestrol, and $10^{-4}$ M cytochrome c (oxidized) were also found to inhibit the reaction.

Exclusion of light or admission of only red diffuse light stops immediately the deiodination of thyroxine by FMN. Admission of daylight starts the reaction again. Formation of origin material takes place only during the period of illumination, if a protein is added in the dark to a mixture of thyroxine and FMN which has previously been illuminated so as to insure about 50% deiodination, no origin material is formed unless light is again admitted to the system and further deiodination takes place.

Under the experimental conditions described here (2 mmoles of thyroxine and 10 mmoles of FMN in 0.5 ml of buffer), thyroxine is not deiodinated anaerobically. However, when the concentration of FMN is increased considerably to compensate for the fact that FMN cannot act catalytically under anaerobic conditions, deiodination was repeatedly observed and was found to be more intense in the presence of urease and trypophan, for instance, than in their absence. For this experiment, 200 mmoles of FMN, 0.1 mmmole of thyroxine, and 0.1 and 0.5 mg of trypophan or urease, respectively, in 0.5 ml of buffer, pH 7.4

![Fig. 5. The effect of the pH of the reaction medium on the rate of deiodination of thyroxine by FMN and light, in the absence and presence of proteins. Experimental conditions were as described for Figs. 2 and 4.](http://www.jbc.org/)

![Fig. 4. The effect of increasing the concentration of FMN or of thyroxine, or the light intensity, on the rate of deiodination of thyroxine by FMN and light, in the absence and presence of proteins. The latter were added at a concentration of 1 and 0.2 mg per 0.5 ml of buffer for lysozyme and trypsin, respectively. Experimental conditions were as described for Fig. 2.](http://www.jbc.org/)
were incubated and illuminated as usual. We have not observed any deiodination of thyroxine when FMN was first reduced anaerobically by light in the absence or presence of DPNH, EDTA, proteins, etc., and thyroxine was then added to the system in the dark during the phase of reoxidation of the flavin by oxygen or cytochrome c.

**Reaction Products**—As already indicated, the major radioactive products of the deiodination of 1-thyroxine labeled in the 3'- and 5'-positions of the phenolic ring are iodide and origin material, whenever the reaction is carried out in the presence of proteins which activate the reaction rate. The radioactivity found on chromatograms as a material with an $R_f$ of zero in several solvent systems coincides with the bromphenol blue-staining material, migrates with the protein moiety on paper electrophoresis, precipitates with proteins on addition of plasma and trichloroacetic acid, is not separated from the protein with an anion exchange resin (22), and is nondialyzable and nonextractable with 1-butanol, chloroform, or ether. The radioactivity is dissociated from the protein moiety only on destruction of the latter by pancreatic hydrolysis. In the hydrolysates obtained with origin material formed during the deiodination of thyroxine by FMN and light in the presence of several different proteins, only iodide was consistently identified. With certain proteins, such as thyroglobulin, for instance, iodotyrosines could be clearly demonstrated, apart from iodide. More detailed results regarding the nature and possible significance of origin material will be described elsewhere.

**Influence of Proteins and Amino Acids on Absorption Spectrum of Illuminated FMN**—When FMN is illuminated under aerobic conditions in a phosphate buffer at pH 7.4 (Fig. 6), the absorption spectrum in the visible region is altered as compared to that of the solution incubated in the dark; the absorption maxima at about 440 and 370 m$\mu$, decrease, and the absorption minimum at 400 m$\mu$ practically disappears. FMN solutions containing proteins or amino acids give an absorption spectrum identical with that obtained with FMN alone when protected from light. Illumination of such solutions, however, induces the appearance of three main types of alterations in the absorption spectrum of the flavin moiety: with some compounds, i.e. reduced glutathione and cysteine, there is hardly any difference between the absorption spectra of the dark and illuminated solutions (Type A); with some proteins and amino acids, i.e. fresh and boiled globulins, glycine, and methionine, the absorption spectrum found after illumination is essentially the same as that obtained after illumination of FMN in solutions not containing these compounds (Type B); with other proteins and amino acids, however, i.e. fresh lysozyme, fresh and boiled trypsin, histidine, and tryptophan, the absorption spectrum of the flavin moiety after illumination usually shows an over-all increase of the absorbancy, especially between 420 and 300 m$\mu$, as compared to that of FMN illuminated in their absence (Type C).

We have found a good degree of correlation between the type of alteration induced by a given protein or amino acid in the absorption spectrum of the illuminated flavin moiety and its effect on the rate of deiodination of thyroxine by FMN in the presence of light. Alterations of Type A were found with compounds which inhibit the deiodination reaction, Type B alterations were found with proteins and amino acids which do not alter the deiodination rate, and alterations designated as Type C were observed with proteins and amino acids which activate the deiodination of thyroxine by FMN and light. A good example of this correlation is afforded by ribonuclease; the fresh protein affects neither the rate of deiodination nor the absorption spectrum of illuminated FMN, and boiling of ribonuclease causes both an intense activation of the deiodination reaction and the appearance of an over-all increase of light absorption. Although thyroxine-protein interactions might also play a role in determining the deiodination rate, an increased light absorption by the FMN-supplemented system and an increase in the rate of deiodination of thyroxine appear to be concomitant findings.

On visual inspection of chromatograms developed in 1 butanol acetic acid-water (4:1:5) in the dark room over an ultraviolet lamp, it was observed that the fluorescence of the spot corresponding to FMN was much brighter, and the pale blue fluorescence due to lumichrome much weaker, with illuminated FMN solutions containing compounds such as tryptophan or tryptosin, which activate the deiodination rate, than with FMN solutions illuminated in their absence. Moreover, preliminary incubation of FMN in the presence of light before addition of the hormone and onset of the reaction resulted in a decreased deiodination rate; this decrease was less intense when preliminary incubation was carried out in the presence of "activating" proteins and amino acids. It was observed that in the latter case the

![Fig. 6. Absorption spectrum of the flavin moiety in FMN solutions kept in the dark (-----) or illuminated (---) in the absence or presence of proteins and amino acids. The absorption spectra were read against blanks containing all the components except FMN and compared to those of unilluminated FMN solutions. The concentrations of proteins and amino acids were 1 mg per ml. The other experimental conditions are described in "Experimental Procedure."](http://www.jbc.org/)
shows results obtained with this preparation in our laboratory; it activates the deiodination of thyroxine in the presence of FMN and light, gives rise to Type C alterations in the illuminated spectrum of the flavin moiety, and catalyzes intensely the rate of oxidation of DPNH by FMN and light.

**DISCUSSION**

Results previously described (14) and those presented here show that the deiodination of thyroxine by FMN-supplemented tissue preparations assayed in the presence of light may be mimicked both qualitatively and quantitatively by numerous compounds tested at random. If any one of a number of purified proteins is added during the deiodination of thyroxine by FMN and light, iodide and "origin material" appear as the main iodinated reaction products and in many cases the rate of the reaction is considerably accelerated.

As regards the reaction products, the origin material formed by the system described in the present study is not distinguishable, on the basis of paper chromatography and electrophoresis dialysis, precipitation with trichloroacetic acid, hydrolysis, etc.

FMN solutions were more intensely yellow at the end of the illumination period, whereas in the case of solutions containing FMN alone, the color was much paler.

All of these observations would indicate that the proteins and amino acids which were found to activate the deiodination of thyroxine by FMN and light protected FMN from photodegradation, stabilized some flavin intermediate, or both, the end result being similar to that which would have been expected by increasing the concentration of FMN or an active derivative thereof, or both, and increasing the absorption of light by the system.

**Influence of Proteins and Amino Acids on Rate of DPNH Oxidation**—We found that proteins and amino acids which activate the deiodination of thyroxine by FMN and light also activate the rate of oxidation of DPNH by the same system, whereas compounds which do not influence deiodination hardly altered the rate of DPNH oxidation. A typical example of each case is shown in Fig. 8; tryptophan activates both DPNH oxidation and thyroxine deiodination (Fig. 3), whereas α-globulins do not.

**Results Obtained with Muscle "Thyroxine Deiodinase"**—A preparation of muscle "thyroxine deiodinase" was very kindly and generously supplied by Professor S. Lissitzky; it corresponded to one described by Lissitzky, Roques, and Benevent (7). Fig. 8 from the origin material formed during the deiodination of thyroxine by FMN-supplemented "thyroxine deiodinase" preparations (11, 23), by nonsupplemented tadpole liver homogenates (24), and by other deiodinating systems. The kinetics of the deiodination of thyroxine by a muscle "thyroxine deiodinase" preparation assayed in an FMN-supplemented system in the presence of bright light may be reproduced by adding any one of a series of numerous proteins and some amino acids to the same assay system. As indicated in our present study, bovine fibrinogen, egg lysozyme, and highly purified urease behave both qualitatively and quantitatively as do the "thyroxine deiodinase" preparations described by others (7, 9) in an FMN-supplemented assay system incubated in the presence of light. Urease, for instance, would have an even higher "deiodinating activity" than the preparations partially purified from skeletal muscle by Tata (9) and by Lissitzky et al. (7) on the basis of millimicromoles of thyroxine degraded during equal time intervals with equal amounts of protein.

**FIG. 7.** The effect of the presence of α-globulins or of tryptophan on the rate of oxidation of DPNH by FMN and light. The reaction mixture contained approximately 0.7 μmole of DPNH and 30 μmoles of FMN in total volume of 3 ml of phosphate buffer, pH 7.4. Other experimental conditions are described in "Experimental Procedure."

**FIG. 8.** The effect of thyroxine deiodinase isolated from skeletal muscle by Lissitzky et al. (7) on the rate of deiodination of thyroxine by FMN + light in the absence (open bar) and presence (striped bar) of 1 mg of protein, on the absorption spectrum of illuminated (---) FMN as compared to solutions kept in the dark (--.--), and on the oxidation of DPNH. All experimental conditions are indicated in "Experimental Procedure."

It will be shown elsewhere that origin material is formed when proteins with a low thyroxine-binding capacity are present during the deiodination of thyroxine by oxidizing "systems," such as intense light, peroxidases, for example, ferrocyanide, ferrocyanide, etc.
It should also be considered that none of the proteins or amino acids used for the present study has any deiodinating activity in the absence of FMN, light, or both. Neither do the “thyroxine deiodinase” preparations obtained from rabbit skeletal muscle by using the FMN-supplemented system for the assay of its deiodinating activity. From the data reported by Tata (9) it would appear that his preparation had a very low activity in the absence of activators, and the “thyroxine deiodinase” obtained by Lissitzky and coworkers (7, 11), none at all. Moreover, we have found that compounds described as inhibitors of the deiodination of thyroxine by the FMN-supplemented “thyroxine deiodinase” (5, 11) also inhibit the degradation of the hormone by FMN and light in the presence or absence of numerous “activating” proteins and amino acids. Both Tata (9) and Lissitzky et al. (6) calculated apparent K_m constants for their “thyroxine deiodinase” preparations assayed in the FMN-supplemented system by the method of Lineweaver and Burk (20). Using data obtained with trypsin and lysozyme, for instance (Fig. 4), and the same method, we could also calculate apparent K_m values, and it was then observed that, for a given protein, these values varied with the concentration of FMN in the assay system.

From all these results it has been concluded that the addition of FMN in the presence of light to a tissue preparation for the assay of its deiodinating capacity (7–10) is a rather dangerous procedure. The presence in the preparation of proteins or amino acids capable of activating the deiodination of thyroxine by FMN and light might yield misleading results for the endogenous deiodinating activity of the preparation. Purification of a thyroxine deiodinase merely on the basis of increasing specific activity of a given fraction in the FMN + light-supplemented assay system might well lead to the isolation of the protein or proteins in the tissue which have the highest activity as catalysts of the flavin-induced photodependent deiodination of thyroxine. Such protein(s) would not necessarily be involved in the physiological deiodinating mechanisms of the tissue. At the present moment the possibility cannot be excluded that this might be the case with the “thyroxine deiodinase” preparations isolated by others (7, 9) from skeletal muscle with an FMN + light-supplemented assay for the determination of the deiodinating activity of increasingly purified samples. Results shown in Fig. 8 suggest that this possibility is very likely. It is felt that deiodinating mechanisms of skeletal muscle ought to be reinvestigated without employing assay systems supplemented with FMN and light.

Ionization of the phenolic hydroxyl group plays an important role in the deiodination of thyroxine by FMN and light; this finding is expected from observations with other protein-free deiodinating systems (16, 21). The fact that thyroxine is deiodinated intensely by FMN and light several pH units below the pK of the phenolic hydroxyl group when the reaction is carried out in the presence of certain proteins and amino acids was unexpected, and the underlying cause has not been clarified.

FMN is anaerobically reduced by light, the FMNH₂ thus formed being reoxidized on admission of oxygen (25, 26). During these processes free hydroxyl radicals are formed which lead to the formation of hydrogen peroxide (19, 26). In the absence of certain “protecting” compounds such as DPNH and EDTA, the flavin moiety is partially degraded by light (25, 26). In the presence of these compounds, which appear to prevent or depress the photodegradation of FMN, anaerobic photoreduction of the latter may be coupled stoichiometrically to the oxidation of electron donors, DPNH for instance (19). Under aerobic conditions, photoreduction of FMN and reoxidation of the reduced flavin occur concomitantly, and electron donors, such as DPNH, reduced cytochrome c, reduced dichloroindophenol, phenols, ascorbic acid, etc. (19, 27–29), may be catalytically photoxidized at low concentrations of FMN (10⁻⁶ M). As in the case of the anaerobic process, part of the flavin moiety is degraded by light if no suitable “protecting” compounds are present.

It is suggested that the deiodination of thyroxine by FMN and light is another case of these flavin-sensitized photo-oxidations. The fact that the proteins and amino acids which are capable of activating the flavin-induced photo-oxidation (and ensuing deiodination) of thyroxine also activate the aerobic flavin-sensitized photo-oxidation of another electron donor such as DPNH lends support to this conclusion. Moreover, some inhibitors of the deiodination of thyroxine by FMN and light (reduced glutathione, ascorbate, cysteine) are known to inhibit the photoreduction of FMN and would therefore interfere with the coupled photo-oxidations (19). We believe that proteins and amino acids which we have found to catalyze the deiodination of thyroxine and the oxidation of DPNH by FMN and light act by protecting the flavin moiety from photodegradation or by stabilizing some photoactivated intermediate, or both, the end result being comparable to increasing the FMN concentration or the light absorption of the system. These conclusions are supported by the observed changes in the absorption spectrum of FMN illuminated in the absence and presence of these compounds and by the visual inspection of these solutions and their corresponding chromatograms.

It would appear, therefore, that the activation by numerous proteins and amino acids (“thyroxine deiodinase” probably included) of the deiodination of thyroxine by FMN and light is a rather nonspecific phenomenon, both from the point of view of the large number of compounds with this property and from that of the nonspecificity of the oxidizable substrate.

The general tentative mechanism suggested by Vernon (19) to explain reactions catalyzed by FMN in the presence of light might be proposed for the deiodination of thyroxine by such a system. The present experiments do not, however, permit conclusions as to the molecular species actually responsible for the oxidation and deiodination of thyroxine. This might be either photoactivated FMN or the free hydroxyl radicals produced during the oxidation of water. The fact that thyroxine was not deiodinated during the dark aerobic reoxidation of photochemically reduced FMN, during which reaction free hydroxyl radicals also appear to be formed (26), but only during the light-induced phase of the reaction, would suggest that photoactivated FMN is more likely to be the molecular species involved in thyroxine deiodination, thus making the reaction less interesting from the point of view of possible physiological deiodinating mechanisms occurring in the absence of light.

The present results also stress the great number of artifacts which may arise during reactions involving flavins in the presence of light if interactions with proteins and amino acids of the type described here are not taken into consideration.

Oxidation of thyroxine leads to the deiodination of the hormone, as may be easily shown by adding labeled thyroxine in the anodic and the cathodic chambers of an electrolytic cell; deiodination only occurs at the anodic side, the hormone being very stable at the cathode.
SUMMARY

1. Using \( ^{131}\text{I} \)-labeled \( l \)-thyroxine, we have shown that this hormone is degraded by riboflavin monophosphate (FMN) + light, giving iodine as main radioactive reaction product. Addition to this reaction mixture of any one of a large number of proteins or of some amino acids chosen at random alters the reaction rate and the nature of the degradation products. In the presence of most proteins the chief iodinated reaction products are iodide and "origin material," apparently an iodinated protein. Many of these proteins and some of the amino acids tested at random intensely accelerate the rate of the deiodination of thyroxine by FMN + light.

2. In the presence of FMN and light, the "deiodinating activity" of these proteins is proportional to their concentration; none of the proteins or amino acids tested so far has any deiodinating activity in the absence of FMN, light, or both.

3. We have found that the behavior of the "thyroxine deiodinase" preparations described by others (7, 9), which were isolated from skeletal muscle in an FMN plus light assay system both for their isolation and for the study of their properties, may be entirely mimicked, qualitatively and quantitatively, by use of FMN plus light and any one of a large number of proteins.

4. Proteins and amino acids found to activate the deiodination of thyroxine by FMN plus light, "thyroxine deiodinase" included, also accelerate the rate of another flavin-sensitized photochemical reaction, such as the oxidation of reduced diphosphopyridine nucleotide by FMN plus light (19).

5. The ability of some amino acids and many proteins, including "thyroxine deiodinase," to catalyze both the deiodination of thyroxine and the oxidation of DPNH by FMN plus light appears to be correlated with typical alterations of the absorption spectrum of the flavin moiety during illumination of FMN in solutions containing these compounds. We have tentatively interpreted this and other findings described in the present paper as indicating that these proteins and amino acids protect FMN from photodegradation, or stabilize some photoactivated derivative thereof, or both, thus resulting in an enhancement of the rate of flavin-sensitized photoreactions.

6. Isolation of a "thyroxine deiodinase" from a given tissue with an FMN plus light-supplemented assay system for the determination of thyroxine-deiodinating activities might well lead to the purification of compounds which actively catalyze a flavin-dependent photo-oxidation and which are not necessarily involved in the physiological endogenous deiodinating mechanisms of a tissue. It therefore appears that the problem of thyroxine-deiodinating systems of skeletal muscle ought to be re-investigated, avoiding systems supplemented with FMN and assayed in the presence of light.

REFERENCES

Activation of the Flavin Photodeiodination of Thyroxine by "Thyroxine Deiodinase" and Other Proteins
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