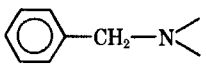
4. The IR absorption of ν_{O-H} at 3400 cm.$^{-1}$, which disappeared when the compound was ethylated, was detected.

These facts support an existence of a phenol group in the new compound. It is reasonable to consider that the signal of τ 5.51 comes from 2H, is not coupling, and is shifted differing from that of τ at about 6.7 in usual absorption due to:



Also, it can be interpreted that a benzyl skeleton having two methoxys, one phenolic hydroxy, and two hydrogens adjoined with each other is bound with piperazine at the position of one nitrogen atom, N^+, in the piperazine skeleton. The signal of τ 6.32 due to 8H was observed as a sharp singlet in the NMR spectrum of the new compound.

The chemical structure of the new compound was established also from the IR spectrum of the compound; that is, the potassium permanganate oxidation product of ethylated 1-(monohydroxydimethoxybenzyl)piperazine coincided with that of the compound 3-ethoxy-2,4-dimethoxybenzoic acid synthesized separately, and no depression in a mixed melting-point determination was observed.

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Color Reaction of Thyroxine and Its Derivatives with Nitrous Acid

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Abstract □ The 3′,5′-diiodo-4′-hydroxydiphenylether system in thyroxine was shown to be indispensable for the success of the color reaction with nitrous acid. This reaction was also successful with sodium hypochlorite and chlorine gas. The products were separated, and evidence is presented to show that this reaction involves an oxidation of the 3′- and/or 5′-position to iodoso or iodoxy derivatives while the amino acid side chain remains intact.

Keyphrases □ Thyroxine, derivatives—chemical nature of color reaction with nitrous acid □ 3′,5′-Diiodo-4′-hydroxydiphenylether system—role in color formation of thyroxine and nitrous acid

Thyroxine (I), when treated with nitrous acid, is reported to give a characteristic red color, even in a concentration of 1 in 40,000 (1). This standard procedure is described in various pharmacopeias (2) for the identification of thyroxine. Roche and Michel (3) reported on the analytical aspect of this reaction and suggested its use as a quantitative method for the determination of thyroxine. The absorption of the color formed obeyed Beer–Lambert's law within a certain concentration range, 100–300 mcg. This paper reports on the chemical nature of this reaction, which has not been investigated previously.

EXPERIMENTAL

Thyroxine (I), tetraiodothyropropionic acid (II), 3,5,3′-triiodo- and 3,5-diiodothyronine, 3,5-di- and 3-iodotyrosine, and tyrosine were used[1]. The purity of the commercially available thyroxine was checked by UV, TLC, and IR and was found acceptable. However, C, H analyses purveyed by any manufacturer were beyond the accepted limits. It was used as such without further purification. Both the D- and L-forms were used whenever available without any difference in results. Spectrophotometric readings were taken on Perkin-Elmer 202 (UV, visible) and 237 (grating IR) instruments with KBr pellets. Silica gel G was used[2].

Color Formation (1, 3)—To a solution of 5 mg. of thyroxine or its derivatives in 5 ml. of 95% acidic ethanol (enough hydrochloric acid to bring the solid in solution), a freshly prepared $NaNO_2$ (1%) solution was added dropwise until the color of the starch iodide paper became blue. The yellow color produced was intensified on boiling. When the cooled solution was made alkaline (pH 9) with ammonia, an intense red color developed, which became yellow on acidification (pH 1.5). Similarly, when the acidic solution of thyroxine was treated with NaOCl, it turned green, then yellow upon heating, and red with dilute alkali. With Cl_2 gas, the solution remained colorless until made alkaline; then it became red, and it turned yellow with acids. When the procedure was applied to Compound VI, molecular iodine and *p*-methoxyphenol were obtained (*cf.*, 4). With 3,5,3′-triiodo- and 3,5-diiodothyronine, 3,5-di- and 3-iodotyrosine, and tyrosine, the reaction mixture acquired a yellow color but did not turn red with base (*cf.*, 3).

Preparation of *N,O*-Diacetylthyroxine[3] (IV)—One gram of the sodium salt of thyroxine or its analogs was dissolved in 150 ml. of freshly distilled, cold acetic anhydride. The solution was left to stand overnight at room temperature. The solvent was evaporated to dryness under vacuum and the white amorphous residue, A, was

[1] Fluka-Buchs, Switzerland.

[2] E. Merck.

[3] This method was communicated by G. Hagen of the Veteran's Administration Hospital, St. Louis, Mo.

Table I—UV and Chromatographic Values of Thyroxine and Its Reaction Products

Compounds	UV Absorption in Aqueous Ethanol, nm. Acid	Neutral	Base	R_f
Thyroxine I	210, 225, 290	210, 225, 300	330	0.33
Band a	210, 225	210, 225, 280, 460	465	0.3
Band b	210, 225, 290	202, 225	225	0.23
Band c	202, 225, 290	202, 225, 290, 460	225, 460	0.2
Band d	202, 225	202, 225, 290	225	0.16
Band e	210, 225, 290	210, 225, 290	225	0.14

collected. It was shown by TLC and IR (absorptions at 1730 and 1765 cm.$^{-1}$) to be almost all IV. Impurities, as revealed by TLC, consisted only of traces of *N*-acetylthyroxine, generated probably by the alkalinity of the eluent (see *Chromatography*). Residue A did not have a definite melting point but decomposed over a range (188–210°) and could not be recrystallized. The impurity in residue A could be partly removed when it was dissolved in absolute ethanol and reprecipitated by the addition of water; yield: 0.87 g.

Preparation of *N*-Acetylthyroxine[3] (III)—Residue A (1 g.) was dissolved in about 100 ml. of 1 *N* NaOH; to this, 50 ml. of 10 *N* NaOH was added. The precipitate formed was filtered and washed with a saturated NaCl solution and dried. The amorphous powder could not be recrystallized from the common organic solvents and had no definite melting point. Its purity was checked by TLC (see *Chromatography*) and IR, where only one peak appeared in the carbonyl region at 1720 cm.$^{-1}$; yield: 0.83 g.

Chromatography—The reaction product was dried under vacuum and washed several times with distilled water and dried. The brown amorphous powder was dissolved in methanol and spotted on preparatory TLC slides (0.5 mm., silica gel G). The only eluent that gave a good separation was the organic layer of an *n*-butanol–concentrated ammonia–water mixture (200:27:23) (5). Elution was allowed for 4 hr., after which the colored bands were scraped, the silica was extracted with acidulated (HCl) methanol, and the extract was dried under vacuum and washed several times with distilled water until the washings gave a negative test for chlorides.

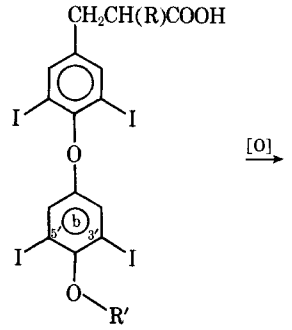
All five bands of the reaction product of I, II, or III were colored and traveled less than the starting material (R_f 0.33, 0.5, and 0.4, respectively), except the first spot which had the same R_f as the starting material but differed from it in IR and UV.

RESULTS AND DISCUSSION

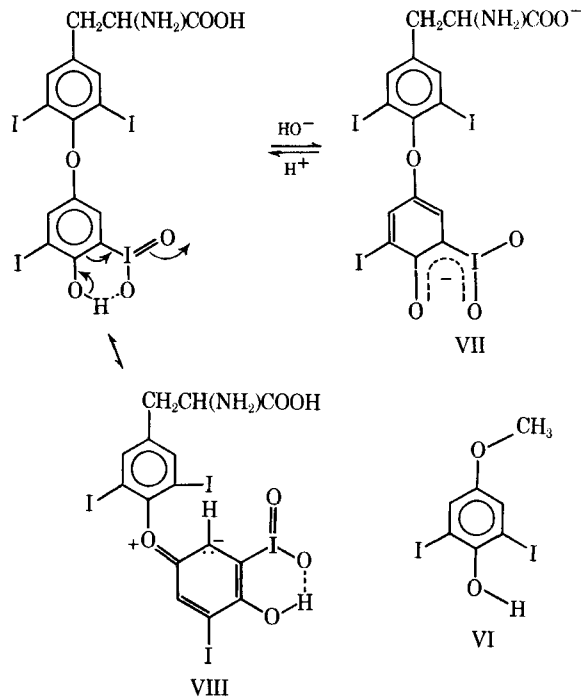
In addition to thyroxine, several of its derivatives, *e.g.*, its methyl ester (6), tetraiodothyropropionic acid (II), and *N*-acetylthyroxine (III), were found to give a positive reaction with nitrous acid, whereas *N,O*-diacetylthyroxine (IV), the 4′-*O*-methyl ether derivative of thyroxine (7) (V), 3,5,3′-tri- and 3,5-diiodothyronine, 3,5-di-[4] and 3-iodotyrosine, tyrosine, and 2,6-diiodo-4-methoxyphenol (8) (VI) gave negative results with nitrous acid.

The reaction product of I, II, and III was unextractable with lipophilic solvents (ether, ethylacetate, *etc.*) and would shift from wine-red to yellow upon changing the pH of the medium from the basic (pH 9) to the acidic side (pH 1.5) and vice versa. Besides, no iodide or elemental iodine, which may have arisen from deiodination, could be detected (9) in the medium of the reaction product[5].

Upon chromatography, five colored spots of small and very close R_f values (Table I) were obtained with the reaction products of I, II, and III with an *n*-butanol–ammonia system on silica plates. These spots ranged in color from yellow to violet and had the same R_f value whether prepared from I or III; the ones prepared from II remained close together but invariably traveled faster, presumably due to the absence of the aliphatic amino group. When these bands were isolated and extracted from preparatory TLC, they: (*a*) gave a positive ninhydrin test; (*b*) contained nitrogen (as determined by elemental analysis), except those isolated from thyropropionic acid (II); (*c*) had no absorption around 1500 (nitroso) or 1523–1544 cm.$^{-1}$



I: R = NH_2, R′ = H
II: R = H, R′ = H
III: R = $NHCOCH_3$, R′ = H
IV: R = $NHCOCH_3$, R′ = $COCH_3$
V: R = NH_2, R′ = CH_3



Scheme I

(nitro) (10); (*d*) had superimposable IR spectra through the whole range; (*e*) had different $\lambda_{max.}$ and common peaks at 225 and 290 nm. (Table I); (*f*) had no melting points; and (*g*) were insoluble in the common organic solvents and changed in color as the medium was shifted from alkaline to acidic.

Such evidence as described in the preceding paragraphs allowed for the following conclusions:

1. The integrity of the 3′,5′-diiodo-4′-hydroxydiphenylether moiety is a necessary and sufficient requirement for the success of this reaction[6].

[4] Roche and Michel (3) reported that this compound gave a positive reaction with nitrous acid, but the compound did not develop a red color in the present study.

[5] G. M. De Escobar, P. Llorente Rodriguez, T. Jolin, and F. Escobar del Rey, *Biochem. J.*, **88**, 526(1963), reported on the deiodination of thyroxine.

[6] It was reported to be specific for *o*-diiodophenols (3).

2. The R—CH(NH_2)COOH of the thyroxine side chain is unnecessary and remains intact in the product (1150 and 2800 $cm.^{-1}$ when in the zwitterion form).

3. There is no displacement of the aromatic-bound iodine (4).

Furthermore, when one of the pure bands isolated from the reaction product of I, II, or III was respotted a few hours after its isolation, it gave two to three spots. These new spots had R_f values similar to one or more of the bands present in the original reaction mixture[7]. This strongly implies the presence in the reaction product of iodoso derivatives, which are known (11) to disproportionate on standing or on TLC to the iodoxy and the original iodo compound.

Further spectral studies, such as mass spectrum, electron spin resonance, and NMR, could not be used in the elucidation of the structure, mainly because of a lack of volatility and insolubility problems. Although it is attractive in the case of thyroxine to speculate that the red color in the product is due to a stable free radical (12), limited electron spin resonance experiments did not substantiate this proposition.

Other agents, *e.g.*, chlorine and sodium hypochlorite, gave results similar to the ones obtained with nitrous acid.

Diphenyl ethers are reported to be stable systems (13). However, autoxidation of thyroxine with oxygen in dimethyl sulfoxide, followed ultimately by rupture of the diphenyl ether linkage, was shown (14) very recently to give rise to quinones[8], while nitrous acid gave rise to quinones with 1,2,3-trimethoxybenzene (15).

In this study, however, the facts that: (*a*) the use of different oxidizing agents such as nitrous acid, sodium hypochlorite, and chlorine gave the same products as determined spectrophotometrically and by TLC; (*b*) the fractions isolated from any of these oxidations were identical to one another in IR, different in UV, and gave a positive ninhydrin test, except those isolated from II; (*c*) the physical behavior of all fractions (ease of decomposition, color, lack of solubility, *etc.*) are characteristic of polyvalent iodine compounds (11); (*d*) II and III gave rise to products similar to the ones obtained from thyroxine; and (*e*) there was the indicatorlike behavior of all fractions towards acid and bases (Scheme I), can best be reconciled by visualizing an oxidation of the iodine at the 3′- and/or 5′-positions of thyroxine to the iodoso or more probably to the iodoxy compound (or both). At this point, it is proposed that the *N,O*-diacetylthyroxine (IV) and the 4′-*O*-methylether derivative (V) of thyroxine were not readily oxidized because a substituent on the oxygen atom at C-4′ interferes sterically or mesomerically with the formation of Structure VII.

The hindrance to free rotation around the ether oxygen in the diphenyl ether system was studied (16). Moreover, in the case of thyroxine, the bulk of the iodine at positions 3 and 5 (17), plus the greater participation of the *p*-electrons of the ether oxygen into ring b (4) of thyroxine (see Structure VIII), makes the 2′ and 6′- as well as the 3′- and 5′-positions, nonequivalent[9] (18). This may be used to explain the number of fractions appearing on TLC by suggesting that an oxidation at 3′ by nitrous acid, sodium hypochlorite, or chlorine gas does not give rise to the same compound obtained by oxidation at 5′.

[7] Two-dimensional chromatography was performed with no further separation.

[8] Which, contrary to a popular theory, may not be involved in the metabolism of thyroxine in humans, *cf.*, C. S. Pittman, V. H. Read, and J. B. Chambers, *J. Clin. Invest.*, **49**, 373(1970); E. Gruenstein and J. Wynn, *J. Theoret. Biol.*, **26**, 343(1970).

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