The Nature of Thyroxine and Related Compounds as Electron Donors*

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ABSTRACT: Solutions containing the methochloride salt of pyridine, quinoline, or nicotinamide and thyroxine or 3,5,3′-triiodothyronine show a new absorption band in the visible region, which has the characteristics of a charge-transfer band. The association constants of 3-iodotyrosine, 3,5,3′-triiodothyronine, 3,5-diiodo-*p*-cresol, 3,5-diiodotyrosine, and thyroxine with nicotinamide methochloride, as obtained from the change in apparent color intensity with concentration of the components, are similar ($K \simeq 4$ l. mol^{-1}). The charge-transfer complexes are formed only with the substituted phenolate ions. The possible correlation between biological activity and electron donor properties of a number of thyroxine analogs is discussed.

Many biological molecules are known to form charge-transfer complexes with various electron donors or electron acceptors (Pullman and Pullman, 1966; Bullock, 1967). Although the significance of such complexes in biological processes is not yet well understood, they are assumed to exist in biological systems (Pullman and Pullman, 1966; Bullock, 1967; Kosower, 1960). One of the most important consequences resulting from the formation of a charge-transfer complex is the change in the chemical nature of the bound species due to the perturbation of their electronic clouds (Kosower, 1965). This change may play an important role in the action mechanism of biological molecules such as hormones.

In this study we show that thyroxine and 3,5,3′-triiodothyronine, when ionized, are strong electron donors. The *o*-iodinated phenol ring of these hormones is responsible for this ability to form charge-transfer complexes as suggested by others (Cilento and Berenholc, 1965). The biological significance of this finding is discussed.

Materials and Methods

Diiodo-*p*-cresol was prepared according to Datta and Prosand (1917) and crystallized from ethanol–water. The other substituted phenols were commercially available: 3-iodotyrosine, thyroxine (Calbiochem); 3,5-diiodotyrosine (Cyclo Chemical Corp.); and 2,4-dimethylphenol, 2,4,6-trimethylphenol, and α-naphthol (Eastman Organic Chemicals). 3,5,3′-Triiodothyronine was a gift from Dr. J. Gross.

The methylation of pyridine, nicotinamide, and quinoline was carried out in ethanol with methyl iodide. The methiodide was converted into the methochloride with excess of silver chloride in water. The methochloride salt of nicotinamide was recrystallized from ethanol.

Absorption spectra and optical densities were measured with a Zeiss instrument (PMQ II).

Results

Charge-Transfer Complexes with Iodinated Phenols. When the methochloride salts of pyridine, nicotinamide, or quinoline are added to a solution of thyroxine (pH 10, dimethylformamide 10%) a yellow color appears instantaneously. The color intensity was found to weaken reversibly by warming, and did not obey the Beer–Lambert law. A similar color, exhibiting the same characteristics, appeared by addition of one of the above salts to solutions containing 3,5-diiodotyrosine, 3,5-diiodo-*p*-cresol, as well as 3-monoiodotyrosine, 3,5,3′-triiodothyronine, or *o*-iodophenol at pH 8–10. The absorption spectra of some mixtures of nicotinamide methochloride and *ortho*-iodinated phenols are shown in Figures 1 and 2.

TABLE I: Association Constants K and Molar Extinction Coefficients ϵ of Complexes between Nicotinamide Methochloride and Various Iodinated Phenols.[a]

Donor	K (l. mol^{-1})	λ (mμ)	ϵ
3-Monoiodotyrosine	4[c,d]	400	190[b]
3,5,3′-Triiodothyronine	4[d]	400	285[d]
3,5-Diiodo-*p*-cresol	6[c]	460	218[c]
3,5-Diiodotyrosine	4[b]	410	264[c,d]
Thyroxine	3.6[d]	410	312[d]

[a] These values are obtained by the Benesi–Hildebrand plot in excess of acceptor (0.1–0.5 M). The donor concentration is 2×10^{-3} M. [b] Solution in Tris, pH 8.6, 0.05 M. [c] Solution in Tris (pH 8.6, 0.05 M)–ethanol (15%). [d] Sodium carbonate (pH 10)–dimethylformamide (10%).

The special features of the apparent color of the above mixtures strongly suggest the formation of a charge-transfer complex between the quarternary aromatic cations, which are known to be good electron acceptors (Kosower, 1960; Mason, 1960), and the iodinated phenolate anion which acts as an electron donor. The

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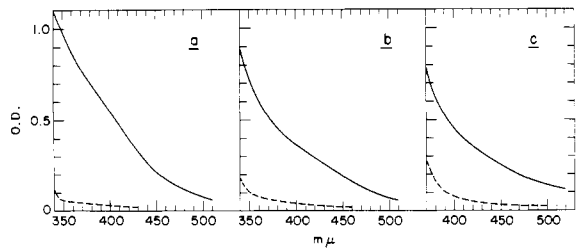

FIGURE 1: Absorption spectra in the visible region of mixtures containing nicotinamide methochloride (0.25 M) and *ortho*-monoiodinated phenols (2×10^{-3} M): *o*-iodophenol (a), 3-iodotyrosine (b), and 3,5,3′-triiodothyronine (c) (—). The solutions are in bicarbonate buffer (0.05 M, pH 10) containing 15% ethyl alcohol (a and b) or 10% dimethylformamide. For comparison the spectra of the corresponding iodinated phenol at the same concentration (2×10^{-3} M) are also given (---).

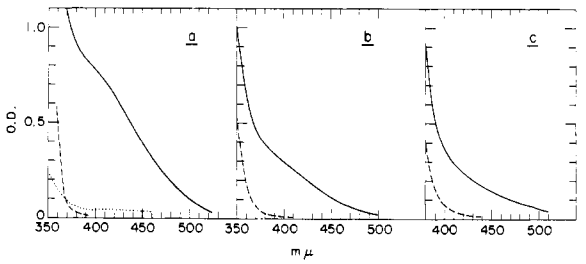

FIGURE 2: Absorption spectra in the visible region of mixtures containing nicotinamide methochloride and *ortho*-diiodinated phenols: diiodo-*p*-cresol (a), 3,5-diiodotyrosine (b), and thyroxine (c). The conditions are the same as in Figure 1. Figure 2a includes also the absorption spectrum of a mixture of nicotinamide methochloride and potassium iodide (2×10^{-3} M) (···).

association constant of complex formation, K, could be evaluated from the change in color intensity of the mixtures with the concentration of the components. For a 1:1 complex when the total concentration of component B (b) is much higher than the total concentration of component A (a), the linear relationship of eq 1 prevails (Benesi and Hildebrand, 1949), where E is

$$\frac{a}{E} = \frac{1}{K\epsilon}\frac{1}{b} + \frac{1}{\epsilon} \qquad (1)$$

is the extinction of the mixture at a wavelength where the components do not absorb light and ϵ is the molar extinction coefficient of the complex at the corresponding wavelength. The values of K and ϵ can be thus evaluated from a plot of a/E *vs.* $1/b$.

Accordingly, the optical density at 400 mμ of a series of mixtures containing an ionized iodophenol at a fixed concentration of 5×10^{-4} or 2×10^{-3} M and nicotinamide methochloride in excess at a concentration ranging from 0.1 to 0.5 M was measured. The plot of a/E *vs.* $1/b$ yielded in all cases straight lines from which the values of K and ϵ were obtained (see Table I). The association of 3,5-diiodotyrosine with pyridine methochloride and NAD^+ was also measured (Table II).

The apparent color of the discussed mixtures was found to fade by lowering the pH. Spectrophotometric titrations at 400 mμ of mixtures of 3,5-diiodotyrosine or 3-iodotyrosine with nicotinamide methochloride yielded titration curves with inflection points at pH values equal to the pK of the iodotyrosine. It seems, therefore, that only the ionized form of the iodinated phenol is involved in this type of complex. It is interesting to note that the apparent color of solutions of thyroxine in dimethyl sulfoxide was found not to obey the Beer–Lambert law and to fade markedly by addition of water or by acidifying. It is thus possible that the high solubility of thyroxine in dimethyl sulfoxide is partially due to the formation of charge-transfer complexes between the solute and the solvent (Zand and Palmer, 1967).

TABLE II: Association Constants K and Molar Extinction Coefficients ϵ of Complexes between Diiodotyrosine and Three Electron Acceptors.[a]

Acceptor	*N*-Methyl-pyridinium Chloride	*N*-Methyl-nicotinamide Chloride	NAD^+
K (l. mol^{-1})	0.4	4	40
ϵ_{410} mμ	250	264	55

[a] These values are obtained by the Benesi–Hildebrand plot in excess of acceptor (0.1–0.5 M). The diiodotyrosine concentration is 2×10^{-3} M (solution in Tris buffer, 0.05 M, pH 8.6).

Charge-Transfer Complexes with Other Substituted Phenols. By addition of nicotinamide methochloride to an aqueous solution of *o*-bromophenol at pH 10 or 3,5-dibromotyrosine at pH 8.5, a yellow color which possesses charge-transfer characteristics is produced (Figure 3, curve 5). The absorption spectra of the mixtures are very similar to those found for mixtures of nicotinamide methochloride with *o*-iodophenol and 3,5-diiodotyrosine, respectively.

When *o*-bromophenol is replaced in the above mixtures by *o*-chlorophenol or 2,6-dichlorophenol, only a faint yellow color appears (Figure 3, curve 4). The absorption spectra of the mixtures containing the chlorophenols are blue shifted in comparison with the analogous mixtures containing the iodo or the bromo compounds.

The absorption spectra in the visible region of mixtures containing nicotinamide methochloride and *ortho*–*para*-methylated phenols or α-naphthol in 15% ethanol at pH 10 have also been determined (Figure 3, curves 1–3). These mixtures could not be examined when the phenols are fully ionized, since at higher pH values *N*-alkylated nicotinamide undergoes decomposition.

In contrast, phenol, *p*-cresol, or tyrosine in the presence of nicotinamide methochloride, under the above conditions, does not show any absorption at wavelengths longer than 380 mμ.

The addition of dimethylformamide (10%) or ethyl alcohol (15%) to increase the solubility of thyroxine, 3,5,3′-triiodothyronine, or methyl-substituted phenols does not change the absorption spectra of the complexes

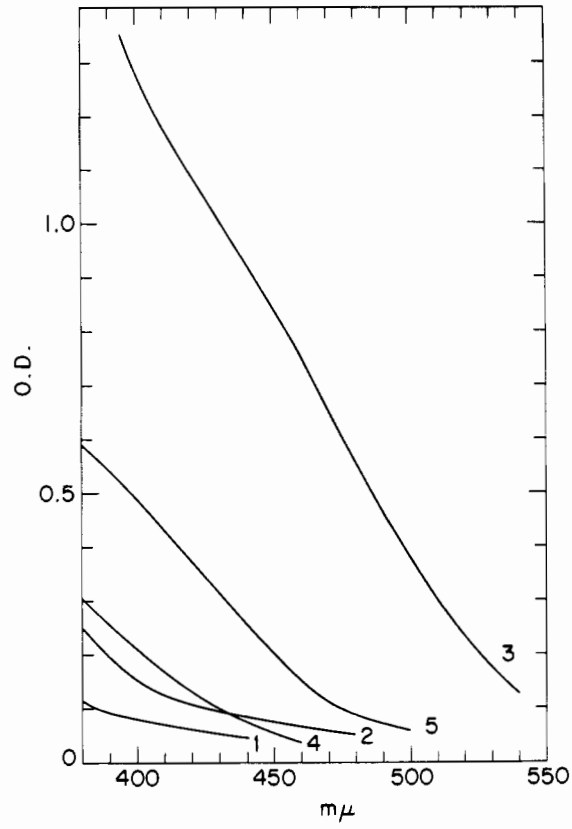

FIGURE 3: Absorption spectra in the visible region of mixtures containing nicotinamide methochloride and various substituted phenols: 2,4-dimethylphenol (1), 2,4,6-trimethylphenol (2), α-naphthol (3), *o*-dichlorophenol (4), and 3,5-dibromotyrosine (5). The conditions are the same as in Figure 1 a,b.

between nicotinamide methochloride and water-soluble iodo compounds.

Discussion

N-Alkylated cations of heteroaromatic compounds such as the *N*-methylnicotinamide ion are known to be good electron acceptors (Kosower, 1960; Mason, 1960). Ion pairs between such cations and iodide, which is a good electron donor, possess a characteristic charge-transfer band part of which falls in the visible region (Kosower, 1960; Mason, 1960). The special characteristics of the apparent absorption bands of mixtures containing nicotinamide methochloride and ionized *ortho*-iodinated phenols indicate the existence of ion pairs of the charge-transfer type in these systems, as has been previously shown by Cilento and Berenholc (1965).

The excitation energy of a charge-transfer complex is approximately given by eq 2 where I is the ionization

$$h\nu_{CT} = I - E - \Delta \qquad (2)$$

potential of the donor, E is the electron affinity of the acceptor, and Δ is the difference of the sum of all intermolecular energies of the excited and the ground states (Pullman and Pullman, 1966). The maximum absorption of the charge-transfer complex occurs at a wavelength dependent on both I and E. In the present example, as in the similar case of indole–nicotinamide complexes (Shifrin, 1964), this maximum falls in the region where one of the components, here thyroxine, absorbs light.

Assuming that Δ is the same for all complexes with the nicotinamide ion, then the best donors (I is small) will show charge-transfer bands at longer wavelength than poor donors. Thus 3,5-diiodotyrosine, thyroxine, and 3,5,3′-triiodothyronine when ionized are considerably stronger electron donors than iodide (see Figures 1 and 2). In addition, mono- and di-*ortho*-iodinated phenolate ions have very similar ionization potentials as they show similar absorption spectra in the presence of nicotinamide ion, while the *o*-bromophenolates are as good electron donors as the analogous iodo compounds but better than the chloro compounds (compare Figures 1–3).

The electron-withdrawing effect of iodine atoms at the *ortho* position of phenols not only lowers the pK_a of the phenol but also decreases markedly the ionization potential of its ionized state. At physiological conditions (pH 7.4) 3,5-diiodotyrosine and thyroxine are almost completely ionized (pK_a = 6.5) and may act as strong electron donors.

It is generally admitted as a working hypothesis that the action of hormones is initiated at the molecular level by a specific interaction of the hormone with a macromolecular target. The steric correspondence between the receptor site and the hormone allows some electronic interaction to take place. The specificity is therefore of two different forms: (1) steric and (2) electronic.

The study of the biological activity of a number of thyroxine analogs outlined the steric and electronic requirements for hormonal activity (Kharasch and Saha, 1958; Jorgensen, 1964; Barker *et al.*, 1965). Regarding the upper cycle of the thyroxine molecule, the following conditions are required to maintain the biological activity. (a) The hydroxyl group in the 4′ position must be free. (b) The presence of a single iodine atom (*e.g.*, 3,5,3′-triiodothyronine) confers to the derivative a greater activity. (c) The iodine atoms in 3′ and 5′ positions can be replaced by electron-releasing groups (methyl, isopropyl, and *t*-butyl). (d) The upper ring can be replaced by an α-naphthyl group.

From these studies Jorgensen suggested that the upper ring should be an electron donor which interacts with a hypothetical "functional receptor" (Jorgensen, 1964; Jorgensen *et al.*, 1962).

The preceding results show that the two naturally occurring hormones, thyroxine and triiodothyronine, are good electron donors when ionized and that only the upper cycle is responsible for this property. This is in good agreement with Jorgensen's and Kharasch's suggestions (Kharasch and Saha, 1958; Jorgensen *et al.*, 1962).

Since the pK of thyroxine is 6.5 the circulating hormone is ionized, and therefore acts as an electron donor. In contrast the pK of 3,5,3′-triiodothyronine is higher (8.45) (Gemill, 1955); nevertheless in the receptor

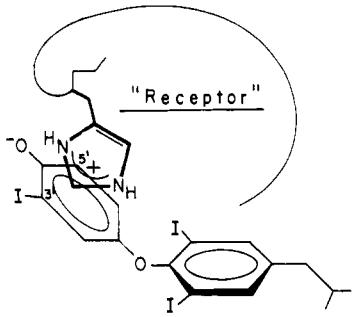

FIGURE 4: Schematic representation of a charge-transfer-type interaction between thyroid hormones and a hypothetical proteinic receptor.

site the microenvironment can be such that the bound hormone may have a lower apparent pK value than in the free form. Moreover, the formation of a hydrogen bond between the phenolic hydrogen and a carboxylate residue of the receptor molecule may be equivalent to ionization. For biological activity the hydroxyl group must be unsubstituted. Binding through this group is therefore likely and in this condition, despite its high pK, triiodothyronine could act as an electron donor.

The presence of a single iodine atom is sufficient to confer strong electron donor properties to the phenol ring. The substitution of a second atom does not increase the affinity for a given acceptor. In this respect the structure of 3,5,3′-triiodothyronine is optimal. The decrease in biological activity upon substitution of a second iodine atom in the 5′ position can be explained tentatively by a steric hindrance due to this additional substituent (Jorgensen, 1964). The studies of analogs support this explanation since in methyl, isopropyl, or *t*-butyl analogs the disubstitution dramatically decreased the thyromimetic activity (Greenberg *et al.*, 1963; Buess *et al.*, 1965; Pittman *et al.*, 1961).

The ionized naphthol ring, which can replace the upper ring of thyroxine (Jorgensen *et al.*, 1962), is a better electron donor than iodinated phenols. Considering this analog the correspondence between electron donor properties and thyromimetic activity seems good. In contrast with the other substituted phenols tested in this study, the correlation between the two properties is less evident. The bromo derivatives are as good donors as the iodo compounds, but have low thyromimetic activity (Michel and Pitt-Rivers, 1957; Jorgensen and Reid, 1965). The *o*-methyl-substituted phenol is a weak electron donor but the corresponding analog of triiodothyronine is 2.5 times more active than thyroxine (Buess *et al.*, 1965; Pittman *et al.*, 1961). The dimethyl-substituted phenol is a better donor than the monomethylated compound but is less active than thyroxine. Finally the dichlorophenol is at least as good an electron donor as the methyl phenols but the 3′,5′-dichloro analog is only 15% as active as thyroxine (Jorgensen and Reid, 1965). There is thus no simple relation between electron donor properties of various substituted phenols and thyromimetic activity of the corresponding analogs of thyroxine. This may be expected since physiological activity depends upon additional factors such as transport and metabolism.

The finding that the naturally occurring hormones are good electron donors under physiological conditions seems good circumstantial evidence for a complementary electron acceptor constituent of the receptor. The only residue, in proteins, known to act as an electron acceptor is the protonated imidazole ring of histidine (Shinitzky and Katchalski, 1968). Figure 4 gives a scheme of the hormone molecule bound to a hypothetical proteinic receptor. Another possibility could be that thyroxine might interact with a low molecular weight acceptor like NAD^+ (Wolff and Wolff, 1957), quinones (Cilento and Berenholc, 1965), or FMN (Cilento and Berenholc, 1965). The action of thyroxine at the NAD enzyme level of the respiratory chain, recently reported (Hoch, 1968), is pertinent here.

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