INHIBITION OF IODOTHYRONINE DEIODINASE BY PHENOLPHTHALEIN DYES

Structure–activity relationship

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1. Introduction

It has been found that several iodine-containing radiographic contrast agents are potent inhibitors of the 5′-deiodination of thyroxine (T_4) to 3,3′,5-triiodothyronine (T_3) in peripheral tissues in vivo [1] as well as in vitro [2,3]. In rat liver microsomes the 5′-deiodination of 3,3′,5′-triiodothyronine (rT_3) to 3,3′-diiodothyronine (3,3′-T_2) and of 3′,5′-diiodothyronine to 3′-monoiodothyronine as well as the 5-deiodination of T_3 to 3,3′-T_2 are also inhibited by these radiographic agents [4,5]. It is still uncertain whether a single enzyme mediates both types of deiodination or that two separate enzymes are involved, i.e., iodothyronine 5- and 5′-deiodinase [5,6].

During attempts to purify the enzyme(s) from rat liver by electrophoresis, it was noted that the tracking dye bromophenol blue strongly inhibited deiodination. This compound is structurally related to iodothyronines and several X-ray contrast agents in that it also contains two halogen substituents in the ortho positions to an electron-donating group (OH or NH_2). It was therefore thought of interest to study the structure-activity relationship of phenolphthalein derivatives as inhibitors of iodothyronine 5′-deiodination. Another point of consideration for this study was the wide use of these compounds as acid-base indicators. It was found that bromophenol blue is a very strong competitive inhibitor of iodothyronine 5′-deiodinase activity. At the same time sulfobromophthalein (BSP) and bilirubin, both compounds known to displace T_4 and T_3 from cytosol binding proteins [7], were found to inhibit the 5′-deiodination of rT_3.

2. Materials and methods

2.1. *Materials*

Phenolphthalein, chlorophenol red, thymol blue, cresol red, bromocresol purple, 2-bromophenol, 2-iodophenol and BSP were purchased from Merck, Darmstadt; bromophenol blue, bromothymol blue and bromocresol green from BDH Chemicals, Poole; bilirubin from Fluka AG, Buchs; 2,4,6-tribromophenol and 2,4,6-triiodophenol from ICN Pharmaceuticals Inc., Plainview, NY; phenol red from Serva, Heidelberg; 2,6-dibromophenol from Eastman Kodak Co., Rochester, NY; rT_3 and 3,3′-T_2 from Henning GmbH, Berlin.

2.2. *Methods*

Deiodination of rT_3 to 3,3′-T_2 by rat liver microsomal fraction was studied essentially as in [8]. In short, 0.1 μM rT_3 was reacted with 2-5 μg microsomal protein and various substances to be tested in 0.25 ml 0.06 M phosphate, 3 mM EDTA and 1 mM dithiothreitol (pH 6.5). After incubation for 10-20 min at 37°C, the reaction was stopped by the addition of 1 ml 0.06 M barbitone buffer containing 0.1% bovine serum albumin and 0.1% SDS (pH 8.6). The amount of 3,3′-T_2 produced was measured with a specific radioimmunoassay in 50 μl of the extract [9]. All values were corrected for non-enzymatically formed 3,3′-T_2 by incorporating appropriate controls, as previously described [8]. Under all conditions tested, less than 5% of added 3,3′-T_2 was degraded during the incubation period. Moreover, it was found that in all experiments the amount of 3,3′-T_2 formed is equivalent to the amount of rT_3 disappearing from the system. Consequently, nonenzymatic deiodination was not promoted by the various phenolphthalein dyes tested.

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For the determination of K_i values, conversion studies were done at pH 7.2 in the presence of 3 mM dithiothreitol and the straight lines of double-reciprocal (Lineweaver-Burk) plots were drawn by the method of least squares applied to unweighted means. Estimation of the K_i value was done by using the equation: apparent $K_m = K_m (1 + [I]/K_i)$, where the apparent K_m is -1/intercept on the abscissa in the Lineweaver-Burk plot and [I] the concentration of the inhibitor.

3. Results

Figure 1 shows the effect of increasing concentrations of various phenolphthalein derivatives on the conversion of rT_3 into 3,3'-T_2. The inhibitory activities and the structures of these compounds are given in table 1. Replacement of the carboxyl group in phenolphthalein by sulfonic acid, yielding phenol red, does not alter inhibitory activity. Addition of methyl (Me) groups to the 5' and 5'' positions of phenol red, yielding cresol red, does not affect inhibitory activity, whereas addition of Cl, yielding chlorophenol red, enhances activity sixfold. Addition of Br to the 3' and 3'' positions of cresol red, yielding bromocresol purple, strongly increases activity, and so does further substitution of Br for Me in bromocresol purple as well as for isopropyl (iPr) in bromothymol blue, yielding bromophenol blue and bromocresol green, respectively. Comparing the latter compounds, it is obvious that Me in the 2' and 2'' positions has a deleterious effect. Despite the presence of this Me group in thymol blue its activity is similar to chlorophenol red and tenfold higher than phenol red, demonstrating the favourable effect of iPr in the 5' and 5'' positions. (It should be noted that in the absence of 2' and 2'' substituents, positions 3' and 3'' are equivalent with 5' and 5''). The negative logarithm of the ID_{50} (pID_{50}) of the phenolphthalein derivatives was inversely correlated with their pK values ($r = -0.84; p < 0.005$). In fig.2 the effect of three bromine-containing phenolphthalein derivatives on the 5'-deiodination of rT_3 is shown. The results demonstrate that all three compounds inhibit this reaction competitively with rT_3. From the change in apparent K_m for rT_3 the K_i values were calculated (table 2). In addition, BSP and bilirubin were found to be inhibitory to the 5'-deiodination of rT_3, the type of inhibition being competitive. The K_i values for these agents are shown in table 2.

To find out whether bromine attached to a rela-

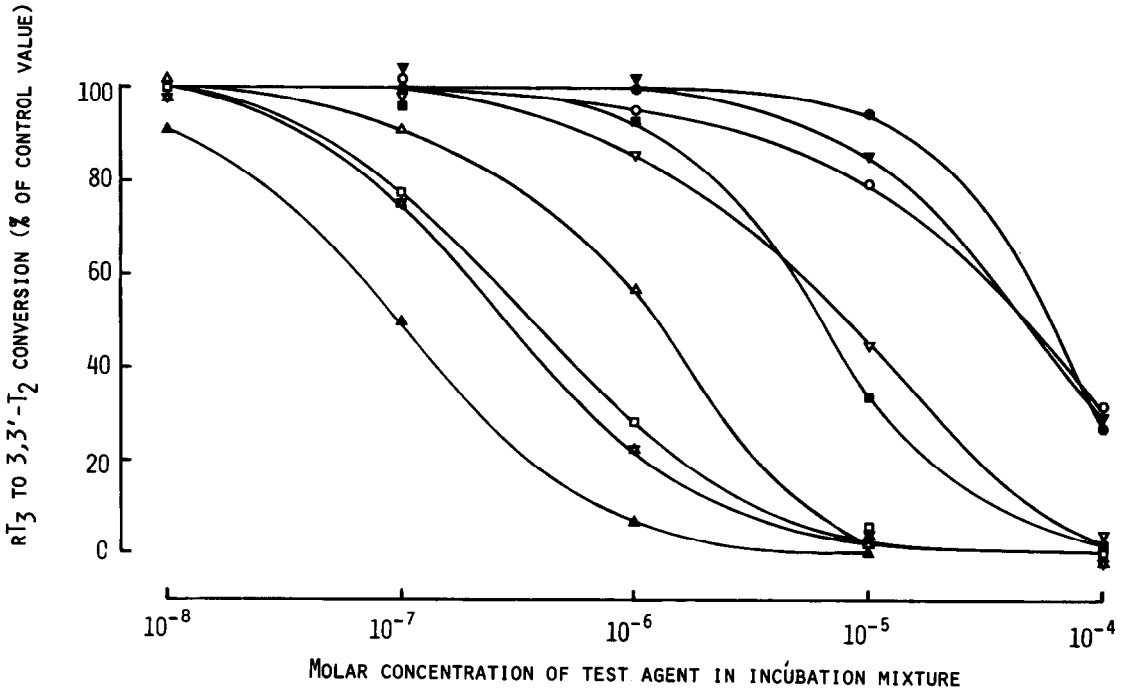
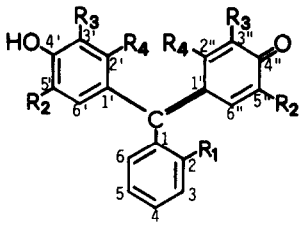


Fig.1. Inhibition of the conversion of rT_3 into 3,3'-T_2 by increasing concentrations of phenolphthalein derivatives. For details see section 2 and for explanation of symbols see table 1. Results are mean of 3 closely agreeing experiments performed in duplicate.

Table 1
Inhibitory activity of phenolphthalein derivatives



		R_1	R_2	R_3	R_4	pID_{50}	pK
○	phenolphthalein	COO^-	H	H	H	4.3	9.6
●	phenol red	SO_3^-	H	H	H	4.2	7.9
▽	chlorophenol red	SO_3^-	Cl	H	H	5.1	6.0
▼	cresol red	SO_3^-	CH_3	H	H	4.3	8.3
◻	bromocresol purple	SO_3^-	CH_3	Br	H	6.4	6.3
■	thymol blue	SO_3^-	$CH(CH_3)_2$	H	CH_3	5.2	8.9
△	bromothymol blue	SO_3^-	$CH(CH_3)_2$	Br	CH_3	5.9	7.0
▲	bromophenol blue	SO_3^-	Br	Br	H	7.0	4.0
✱	bromocresol green	SO_3^-	Br	Br	CH_3	6.5	4.7

pID_{50}: the negative logarithm of the concentration giving 50% inhibition of deiodinase activity. Symbols refer to fig.1.

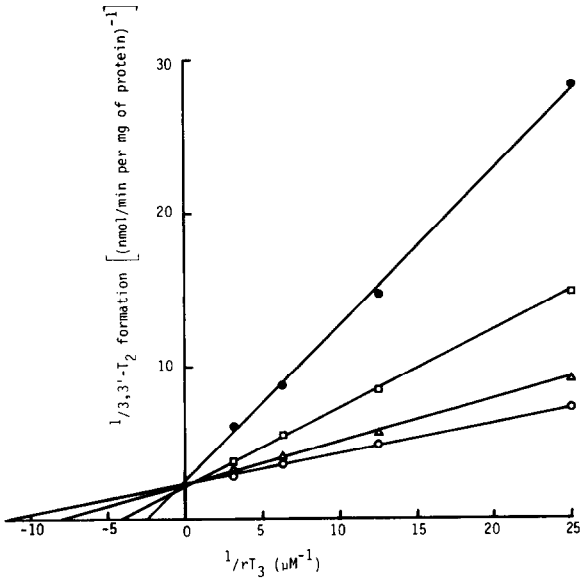


Fig.2. Lineweaver-Burk plot of the conversion of rT_3 into 3,3'-T_2 at pH 7.2 in the absence (○) and presence of 0.2 μM bromophenol blue (●), 0.4 μM bromothymol blue (△) or 0.2 μM bromocresol green (□). For details see section 2. Results are means of 3 experiments performed in duplicate.

tively simple aromatic ring is also inhibitory or that a more complicated structure is required, 2-bromophenol, 2,6-dibromophenol and 2,4,6-tribromophenol were tested. The results are shown in fig.3 and are compared with the effect of 2-iodophenol, 2,6-diisopropylphenol (unfortunately, 2,6-diiodophenol was not available) and 2,4,6-triiodophenol. This last mentioned agent appeared to be a very strong inhibitor [4].

Table 2
K_i values of bromine-containing phenolphthalein derivatives and bilirubin in the 5'-deiodination of rT_3

Inhibitor	K_i of inhibitor (μM)
bromophenol blue	0.04 ± 0.01
bromothymol blue	1.36 ± 0.49
bromocresol green	0.11 ± 0.01
BSP	1.33 ± 0.48
bilirubin	2.12 ± 0.94

The K_m of the 5'-deiodination of rT_3 was 0.10 ± 0.04 μM. The type of inhibition is competitive. Values are means ± SE (n = 4)

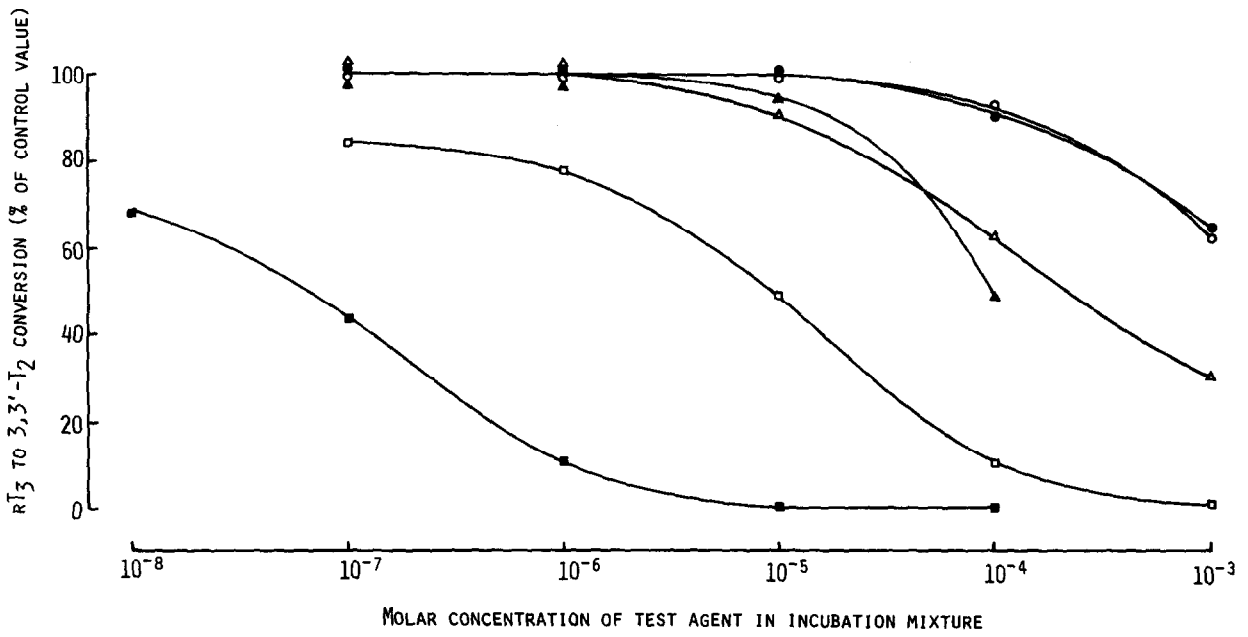


Fig.3. Inhibition of the conversion of rT_3 into 3,3'-T_2 by increasing concentrations of 2-bromophenol (○), 2-iodophenol (●), 2,6-dibromophenol (△), 2,6-diisopropylphenol (▲), 2,4,6-tribromophenol (□) and 2,4,6-triiodophenol (■). For details see section 2. Results are mean of 2 closely agreeing experiments performed in duplicate.

4. Discussion

The present study indicates that 5'-monodeiodination of rT_3 is strongly inhibited by phenolphthalein derivatives. Inhibitory potency of the various dyes is enhanced, in this order, by Me, Cl, iPr and Br substituents in the ortho positions of either phenolic ring. The most active compound tested is the tetra-bromo derivative bromophenol blue. The presence of a methyl group in the meta position has a deleterious effect. All bromine-containing phenolphthalein derivatives are competitive inhibitors of the rT_3 5'-deiodination (fig.2, table 2).

The reason why bromophenol blue is such a strong competitive inhibitor of iodothyronine 5'-deiodination is unclear. Part of the structure of this dye is similar to that of T_4 and rT_3, viz. the 3,5-dihalogen-4-hydroxyphenol group. However, comparing the activity of 2,4,6-tribromophenol with that of 2,4,6-triiodophenol, it is clear that for a proper fit on the enzyme I is a better substituent than Br. This is in agreement with the finding that iodothyronines are better substrates for the deiodinase than bromoiodothyronines [10]. Yet, the K_i for bromophenol blue (0.04 μM) is less than the K_m for rT_3 (0.1 μM). The structure shown in table 1 for phenolphthalein derivatives is that present in solution below the pK of these compounds. Above the pK the rings equilibrate between the quinoid and phenolate resonance structures. Our finding of a negative relationship between the dissociation and inhibition constants for these compounds suggests that the phenolate anion is the preferred form for interaction with the enzyme. This may also explain the increased activity of 2,4,6-tribromophenol compared with 2,6-dibromophenol considering the higher acidity of the OH in the former. An important implication of these findings is that one should be careful with the use of these dyes in deiodination studies. A well known example is the application of bromophenol blue as a marker in protein separation studies. The concentration used in these experiments often amounts to 0.1 mM, which lies far beyond the K_i value (0.04 μM) of this dye. One should also keep in mind the use of phenol red as an indicator in various growth media. This latter compound is mostly present in a concentration of about 0.3 mM, which amount results in more than 80% inhibition of deiodinase activity as can be gathered from fig.1.

BSP, which is used for liver function test and bilirubin, an intermediate in the degradation of hemoglo-

bin, were also tested. It has been shown that BSP and bilirubin can displace both T_4 and T_3 from the cytosol binding proteins Y (ligandin) and Z [7]. However, the affinity of these binding proteins for T_4 and T_3 was much ($\sim 10^4$-times) higher than for BSP. Furthermore, BSP and bilirubin are able to inhibit uptake of T_4 and T_3 by rat liver slices [11] and BSP can displace T_4 from the liver in vivo [12]. BSP is a bromine-containing phenolphthalein derivative with only one bromine-containing phenyl ring, which is substituted with four bromine atoms, while the two remaining positions in this ring are also occupied. Nevertheless, we found BSP to be a relatively strong competitive inhibitor (K_i 1.33 μM) of the 5′-deiodination of rT_3. Bilirubin also inhibited this reaction, though less effectively than BSP (table 2). A reason for the inhibitory activity of the seemingly structurally unrelated bilirubin cannot be given at the moment. The finding that bilirubin inhibits the 5′-deiodination of rT_3 could be of clinical importance. If we take the line that one enzyme is responsible for the 5′-deiodination of all iodothyronines, bilirubin would also be inhibitory to the 5′-monodeiodination of T_4 [4,6]. In hepatic cirrhosis serum T_3 is unusually low relative to T_4, which is probably due to a reduction in peripheral conversion of T_4 to T_3 [3]. This inhibition of the 5′-monodeiodination of T_4 may be caused in part by the increased bilirubin levels.

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