The Biological Action of Substances Related to Thyroxine

1. THE EFFECT OF *n*-ALKYL 3:5-DIIODO-4-HYDROXYBENZOATES ON OXYGEN CONSUMPTION IN MICE

By MARY M. SHEAHAN, J. H. WILKINSON and N. F. MACLAGAN
Department of Chemical Pathology, Westminster Medical School (University of London), London, S.W. 1

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Since the principle of competitive inhibition in chemotherapy was introduced by Woods (1940), several attempts have been made to prepare compounds with an inhibitory action against thyroxine. The first report of direct antagonism was that of Woolley (1946) who observed that a number of ethers of *N*-acetyldiiodotyrosine exhibited anti-thyroxine activity in amphibian metamorphosis. Harington (1948) suggested that it might be possible to distinguish between the effects of thyroxine on the pituitary and on the peripheral tissues. In an attempt to inhibit one or other of these effects he prepared the thio-ether analogue of thyroxine, but this proved to have thyroxine-like activity when tested in tadpoles. Also using tadpoles as test animals, Frieden & Winzler (1949) described the inhibitory action of a number of compounds, including the benzyl ethers of diiodotyrosine and 3:5-diiodo-4-hydroxybenzoic acid, and produced evidence to support their view that this effect was due to true competitive inhibition. Cortell (1949) has demonstrated the antithyroxine properties of 2′:6′-diiodothyronine in rats by the goitre prevention method.

The desirability of extending the methods of studying thyroxine inhibition in mammalia led us to develop a technique for determining the effects of such compounds on the rise of oxygen consumption produced by thyroxine in mice (Maclagan & Sheahan 1950). By this method one of the compounds described by Frieden & Winzler (1949), 4-benzyloxy-3:5-diiodobenzoic acid, was found to be active, and subsequently, the dimethylacetal of 3:5-diiodo-anisaldehyde (Wilkinson, 1949) also gave positive results (Maclagan, Sheahan & Wilkinson, 1949). A brief account of the effects of a series of *n*-alkyl 3:5-diiodo-4-hydroxybenzoates has already appeared (Wilkinson, Sheahan & Maclagan, 1950), and it is the purpose of the present paper to discuss more fully the results obtained and also to extend the study to a number of related substances.

Of the two methods available for the preparation of the *n*-alkyl 3:5-diiodo-4-hydroxybenzoates, that involving iodination of the *n*-alkyl *p*-hydroxybenzoates was preferred, since the alternative procedure of esterifying the diiodo- acid invariably resulted in some decomposition, and in consequence the products proved difficult to purify. Most of the required alkyl hydroxybenzoates have been described by Cavill & Vincent (1947). Several diiodophenols were prepared by Paal (1895) by treatment of the phenols with two equivalents of iodine in aqueous ethanolic solution in the presence of the calculated quantity of iodic acid. As the acid conditions of this method were liable to cause hydrolysis of the esters, we replaced the iodic acid by an equivalent quantity of potassium iodate, and, by this means, the eight *n*-alkyl esters from methyl to *n*-octyl were obtained in good yields.

EXPERIMENTAL

Chemical methods

All melting points are uncorrected. Micro-analyses were by Drs Weiler and Strauss, Oxford.

Preparation of n-*alkyl* p-*hydroxybenzoates.* The alkyl *p*-hydroxybenzoates were prepared by the method of Cavill & Vincent (1947). The *n*-octyl ester, not described by these authors, was obtained by heating a mixture of *p*-hydroxybenzoic acid (13·8 g.), *n*-octanol (45 ml.), toluene (50 ml.) and H_2SO_4 (1 g.) under reflux for 3 hr., a McIntyre still head being employed for water separation. The cooled mixture was neutralized with NaOH, acidified with acetic acid, and steam distilled. The residue was extracted with ether, dried (Na_2SO_4) and distilled. n-*Octyl* p-*hydroxybenzoate* was obtained as a pale-yellow oil, b.p. 170–172°/0·1 mm., which solidified to a colourless waxy solid, m.p. 47–48°. Yield: 15·7 g. (63 %). (Found: C, 72·6; H, 8·8. $C_{15}H_{22}O_3$ requires C, 72·6; H, 8·8 %.)

Iodination of n-*alkyl* p-*hydroxybenzoates.* The alkyl hydroxybenzoate (0·1 g.mol.) was dissolved in the corresponding alcohol (100 ml.) and treated with I_2 (25·4 g.). A solution of KIO_3 (7·1 g.) in water (80 ml.) was gradually added to the boiling mixture which was then heated on a steam bath for 2 hr. The alcohol was removed by steam distillation, traces of unreacted I_2 were destroyed with $Na_2S_2O_3$, and the product collected by filtration. By this means the following compounds were prepared in yields from 80 to 95 %.

Methyl 3:5-diiodo-4-hydroxybenzoate crystallized from the reaction mixture, and, in this case, steam distillation proved unnecessary. Recrystallization from methanol gave colourless needles, m.p. 165°, identical with a sample prepared by esterification of 3:5-diiodo-4-hydroxybenzoic acid. Wheeler & Liddle (1910) report m.p. 165°.

Ethyl 3:5-*diiodo*-4-*hydroxybenzoate*. Colourless needles from ethanol, m.p. 124°. (Found: C, 26·2; H, 2·2; I, 61·4. $C_9H_8O_3I_2$ requires C, 25·8; H, 1·9; I, 60·8%.)

n-*Propyl ester*. Colourless needles from benzene, m.p. 123°. (Found: C, 27·2; H, 2·6; I, 59·2. $C_{10}H_{10}O_3I_2$ requires C, 27·8; H, 2·3; I, 58·8%.)

n-*Butyl ester*. Colourless prisms, from 90% methanol, m.p. 90°. (Found: C, 29·6; H, 2·5; I, 56·7. $C_{11}H_{12}O_3I_2$ requires C, 29·6; H, 2·7; I, 57·0%.)

Ethanol was employed as solvent for the iodination of the following esters:

n-*Amyl ester*. Colourless needles from benzene-light petroleum (b.p. 60–80°), m.p. 78°. (Found: C, 31·0; H, 2·8; I, 57·5. $C_{12}H_{14}O_3I_2$ requires C, 31·3; H, 3·05; I, 55·2%.)

n-*Hexyl ester*. Colourless waxy needles from light petroleum (b.p. 80–100°), m.p. 70–71°. (Found: C, 33·2; H, 3·4; I, 53·7. $C_{13}H_{16}O_3I_2$ requires C, 32·9; H, 3·4; I, 53·6%.)

n-*Heptyl ester*. Colourless glistening waxy plates from 90% methanol, m.p. 68°. (Found: C, 34·8; H, 3·75; I, 52·4. $C_{14}H_{18}O_3I_2$ requires C, 34·5; H, 3·7; I, 52·1%.)

n-*Octyl ester*. Colourless waxy needles from 90% methanol, m.p. 65°. (Found: C, 36·2; H, 4·3; I, 50·2. $C_{15}H_{20}O_3I_2$ requires C, 35·9; H, 4·0; I, 50·6%.)

n-*Butyl* 3:5-*diiodosalicylate* was prepared similarly from *n*-butyl salicylate. After removal of the butanol by steam distillation, the residue was extracted with ether. The extract was washed with $Na_2S_2O_3$ and water and dried (Na_2SO_4). After removal of the solvent, the residue was heated in an oil bath at 180–190°/0·1 mm. to remove a small amount of unreacted *n*-butyl salicylate. The residual oil crystallized on cooling, and recrystallization from light petroleum (b.p. 40–60°) gave the required *ester* in colourless prisms, m.p. 53–54°. (Found: C, 29·9; H, 2·7; I, 57·4. $C_{11}H_{12}O_3I_2$ requires C, 29·6; H, 2·7; I, 57·0%.)

n-*Butyl* 3:5-*diiodobenzoate* was obtained by heating a mixture of 3:5-diiodobenzoic acid (2·9 g.), *n*-butanol (30 ml.), toluene (30 ml.) and H_2SO_4 (1 g.) as described for *n*-octyl *p*-hydroxybenzoate. The crude product (2·99 g.) was extracted (Soxhlet) with light petroleum (b.p. 40–60°) and the extract concentrated. The brown solid which separated crystallized from light petroleum (b.p. 40–60°) in buff-coloured needles, m.p. 50°. (Found: I, 58·6. $C_{11}H_{12}O_2I_2$ requires I, 59·1%.)

n-Butyl 3:5-dibromo-4-hydroxybenzoate was prepared as described by Cavill (1945).

n-*Butyl* 3:5-*dichloro*-4-*hydroxybenzoate*. *n*-Butyl *p*-hydroxybenzoate (9·7 g.) was dissolved in acetic acid (50 ml.) and the solution cooled to 10°. Cl_2 was passed through the solution until it was saturated, about 2 hr. were required. The temperature was maintained at 10–15° throughout this operation. Ice water (200 ml.) was added and the precipitated solid (12·7 g., 96%) collected by filtration. The product was washed free from acid with water and dried over $CaCl_2$. The required *ester* crystallized from light petroleum (b.p. 60–80°) in colourless prims, m.p. 87–88°. (Found: C, 49·9; H, 4·6; Cl, 27·3. $C_{11}H_{12}O_3Cl_2$ requires C, 50·2; H, 4·6; Cl, 27·0%.)

The ester (0·13 g.) was dissolved in 2 N-NaOH (4 ml.) and heated under reflux for 30 min. On acidification with HCl, 3:5-dichloro-4-hydroxybenzoic acid (91 mg.) m.p. 265°, was obtained. Several values, ranging from 255° to 265°, for the melting point of this acid appear in the literature (cf. Beilstein's *Handbuch*, vol. x, p. 177).

The benzoic and *p*-hydroxybenzoic acids and their *n*-butyl esters were commercial samples purified where necessary.

Measurement of oxygen consumption

The methods used for the determination of the rate of O_2 consumption were essentially those of Maclagan & Sheahan (1950), but a number of slight modifications were made. Before treatment the mice were randomized into groups of eight. Each group remained together throughout the experiment to eliminate any restlessness that might be caused by mixing strange mice for the first time on the day upon which their O_2 consumption was to be measured. The inhibitory compounds when given alone to normal mice showed no significant alteration in metabolism and we have therefore relied on the depression of O_2 consumption in mice previously treated with thyroxine to demonstrate inhibitory effects. In each experiment two groups of untreated mice were used as controls, two were given thyroxine only and two were given thyroxine plus the compound under test.

The total dose of the compounds was administered in two parts, half being given at 11 a.m. on the first day and the remainder at 11 a.m. on the second day. A single dose of the monosodium salt of DL-thyroxine was given at 4 p.m. on the first day. O_2 consumption was measured on the third day, i.e. 48 hr. after the first injection of the compound and 43 hr. after the thyroxine injection. The compounds and the hormone were given by subcutaneous injection in suspension or solution in 0·1% (w/v) aqueous Na_2CO_3. In addition, several experiments at different dose levels were performed in which *n*-butyl 3:5-diiodo-4-hydroxybenzoate was given orally.

Each group of mice was placed in a desiccator: 0·5 hr. was allowed for the atmosphere to warm up, when the first reading was taken. Six more readings were taken at half-hourly intervals. Thus there were twelve readings for each group of treated mice, and the statistical significance of these results was analysed by student's '*t*' test.

RESULTS

A typical experiment showing the effect on oxygen consumption of thyroxine when given alone, and when given with *n*-butyl 3:5-diiodo-4-hydroxybenzoate, is illustrated in Fig. 1. It will be seen that the inhibitor produced no significant effect when given alone. Fig. 2 shows the minimum dose at which 3:5-diiodo-4-hydroxybenzoic acid and its *n*-alkyl esters were effective in reducing the oxygen consumption in thyroxine-treated mice. All the esters were more active than the acid, and the first four esters showed a high degree of potency. Of these the *n*-butyl ester was the most active, producing appreciable inhibition at 25 mg./kg. by the subcutaneous route, whilst the remaining four esters were much less active. Detailed results for the *n*-butyl ester are given in Table 1 which shows that, even at the highest dose level, inhibition was not complete, though there appears to be some relation between the degree of inhibition and the dosage. When administered orally this ester was active at a total dose of 50 mg./kg., but was devoid of activity at lower doses.

The acid produced a significant inhibition in mice at a molar ratio of 1000 : 1, in contrast to the

experience of Frieden & Winzler (1949) who, using tadpoles, found it inactive at the same molar ratio. It would seem that with this compound results obtained with one species may not be directly applicable to another.

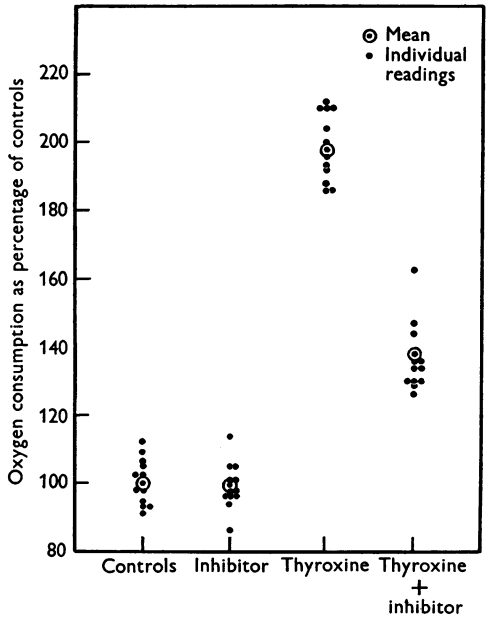


Fig. 1. Oxygen consumption of mice injected subcutaneously with thyroxine and *n*-butyl 3:5-diiodo-4-hydroxybenzoate. Measurements were taken after two doses of 25 mg./kg. of the inhibitor and 2·0 mg./kg. DL-thyroxine sodium.

Earlier work (Maclagan *et al.* 1949), in which 3:5-diiodoanisaldehyde dimethylacetal caused partial inhibition of the thyroxine effect, whilst the free aldehyde was inactive, originally led us to suppose that increase in lipophilic properties might parallel an increase in antithyroxine action. The results obtained in the course of the present work, however, indicate that the relationship between chemical constitution and biological activity is more complicated than could be accounted for by a simple partition effect between fat and water.

Replacement of the iodine atoms by other halogens or hydrogen completely abolished the activity of the *n*-butyl ester (Table 2). Replacement of the hydroxyl group by hydrogen to give *n*-butyl 3:5-diiodobenzoate also caused complete loss of activity.

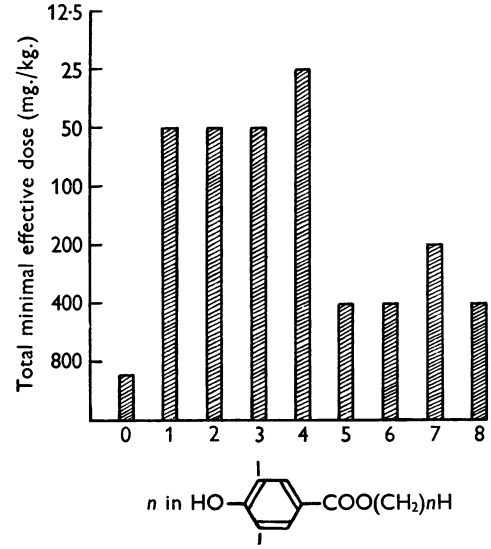


Fig. 2. The effect of variations in chain length upon antithyroxine activity of *n*-alkyl 3:5-diiodo-4-hydroxybenzoates.

A hydroxyl group in the 2-position, however, restored the antithyroxine activity to some extent, for *n*-butyl 3:5-diiodosalicylate produced appreciable inhibition. *n*-Butyl benzoate was without significant action. Neither benzoic acid nor *p*-hydroxybenzoic acid had any effect on oxygen consumption, though Keeser (1949) has reported that benzoic acid inhibited the action of thyroxine on carbohydrate metabolism in rats, and also on iso-

Table 1. *The effect of* n-*butyl* 3:5-*diiodo*-4-*hydroxybenzoate on the oxygen consumption of thyroxine-treated mice*

Dosage (mg./kg.) Inhibitor (*I*)	Dosage (mg./kg.) DL-Thyroxine (*T*)*	Molar ratio (*I*/*T*)	Oxygen consumption as percentage of controls (Mean ± S.E.) +Thyroxine (*a*)	Oxygen consumption as percentage of controls (Mean ± S.E.) +Thyroxine +inhibitor (*b*)	Percentage inhibition $\left(\frac{a-b}{a}\times 100\right)$	Significance (Student's *t* test)
1000	1·5	1150	187±5·1	128±5·5	68	*P* 0·001
400	1·5	460	183±8·5	115±3·3	82	*P*<0·001
200	1·5	230	187±3·3	122±4·0	75	*P*<0·001
100	2·0	87	161±3·5	117±2·9	72	*P*<0·001
50	2·0	44	199±2·9	137±3·0	63	*P*<0·001
25	2·0	22	161±2·8	137±6·1	56	*P*<0·001
12	1·0	20	194±3·2	190±3·5	4	Not significant

* The dose of thyroxine was adjusted to produce a standard response, i.e. 60–90% increase in oxygen consumption.

Table 2. *Effect of analogues of* n-*butyl* 3:5-*diiodo*-4-*hydroxybenzoate on thyroxine-treated mice*

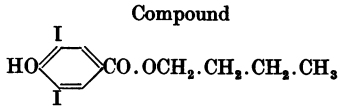
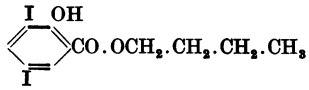
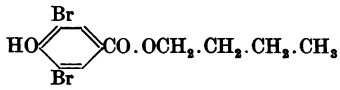
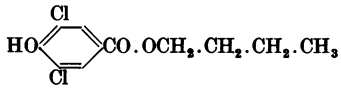
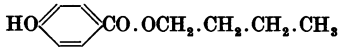
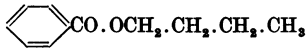
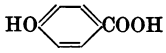
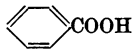
Compound	Activity
	Inhibitory at 25 mg./kg.
	Inhibitory at 1000 mg./kg.
	Inactive at 400 mg./kg.
	Inactive at 400 mg./kg.
	Inactive at 400 mg./kg.
	Inactive at 400 mg./kg.
	Inactive at 400 mg./kg.
	Inactive at 1000 mg./kg.
	Inactive at 400 mg./kg.
NaI	Inactive at 700 mg./kg.

Table 3 *Toxic and effective doses of* n-*butyl* 3:5-*diiodo*-4-*hydroxybenzoate*

	Minimum* effective dose (mg./kg.)	LD_{50} (mg./kg.)	Ratio LD_{50}/M.E.D.
Oral route	50	1500	30
Subcutaneous route	25	3500	140

* The minimum effective dose is the smallest dose which produced a statistically significant diminution of the O_2 consumption of thyroxinized mice.

lated enzyme systems. Sodium iodide in amounts equivalent to the highest tolerated doses of inhibitors was also without effect.

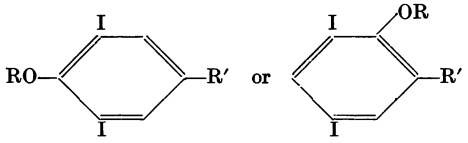
Table 3 shows the relationship between the minimal effective dose (M.E.D.) and the toxicity (LD_{50}) for *n*-butyl 3:5-diiodo-4-hydroxybenzoate by oral and subcutaneous routes. The ratio, LD_{50}/M.E.D. appears to be sufficiently high to suggest that this compound might be suitable for clinical trial. It may be noted here that the ethyl ester was substantially more toxic, producing four deaths in eighteen mice at a dosage of 1000 mg./kg. subcutaneously.

DISCUSSION

The work described above was carried out with the object of finding compounds which would antagonize the various actions of thyroxine by a process of competitive inhibition. We consider that the evidence presented, although incomplete, strongly suggests that the active compounds tested are, in fact, producing their effects in this way. The principal points in favour of this hypothesis are as follows: (1) The effect of the inhibitors is greatest at the time of maximum thyroxine effect, i.e. 2 days after the injection of thyroxine. (2) The dose of the hormone

given in our experiments is so large that a mere inhibition of endogenous thyroxine production would probably have little effect on the results. The mode of action has not, however, been completely defined by our present method of testing, which was adopted as a screening method in order to select suitable substances for further biological study. The fact that the compounds when given alone are without effect on oxygen consumption is difficult to explain, but it is well known that such a depression of metabolism is not easy to achieve in mice even with drugs of the thiouracil type.

Whatever the mechanism of the effect observed, it appears to require a fairly high degree of specificity in molecular structure, and the structures of successful compounds bear at least a superficial resemblance to that of thyroxine. Thus activity in this series of compounds is confined to those substances which contain either the group



and it is possible that the diiodophenol part of the esters discussed may compete with the same part of the thyroxine molecule for a position on some receptor surface.

We have been privileged to read the manuscript of a paper by Barker, Kiely, Dirks, Klitgaard, Wang & Wawzonek (1950) which is in general agreement with this hypothesis. These workers describe the antithyroxine effect of a series of iodophenoxyacetic acids in normal and thyroidectomized mice.

SUMMARY

1. The effect of a series of *n*-alkyl esters of 3:5-diiodo-4-hydroxybenzoic acid on the oxygen consumption of thyroxine-treated mice has been studied.

2. The methyl, ethyl, propyl and butyl esters exhibited a strong antithyroxine effect, the butyl compound being the most active. The activity was almost as great by mouth as by the subcutaneous route. The higher esters were less potent, and the acid itself showed only weak activity.

3. Variations in halogen substitution or elimination of the 4-hydroxyl group in the *n*-butyl ester completely abolished the thyroxine-inhibitory effect. *n*-Butyl 3:5-diiodosalicylate showed slight antithyroxine activity. Sodium iodide was inactive.

4. Evidence is presented in support of the view that a specific competitive inhibition of thyroxine is involved.

It is a pleasure to acknowledge the continued assistance given to this research by Glaxo Laboratories Ltd. The toxicity tests on *n*-butyl 3:5-diiodo-4-hydroxybenzoate were carried out by them.

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Metabolism of Derivatives of Toluene

6. TOLUNITRILES, BENZONITRILE AND SOME RELATED COMPOUNDS

By H. G. BRAY, Z. HYBS and W. V. THORPE
Department of Physiology, Medical School, University of Birmingham

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We have suggested previously (see Thorpe, 1950) that methyl and carbamyl groups in aromatic compounds may be termed 'potential centres for conjugation', since under suitable conditions they are converted by the animal to carboxyl and the resulting acids conjugated with glycine or glucuronic acid. We are investigating the behaviour in the rabbit of other potential centres which might be converted to carboxyl, e.g. —CH_2OH, —CHO, —CN, and this present report is concerned with the nitrile group as it occurs in benzonitrile and the tolunitriles. This group might be hydrolysed to carbamyl or to carboxyl. No previous investigation of the fate of the tolunitriles appears to have been carried out.