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3′-ACETYL-3,5-DIIODO-L-THYRONINE: A NOVEL HIGHLY ACTIVE THYROMIMETIC WITH LOW RECEPTOR AFFINITY

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Abstract—The 4′-phenolic hydroxyl group of thyroid hormones plays an important role in receptor binding, and it has been suggested that the interaction of this hydroxyl group with the receptor involves hydrogen bonding via donation of the acidic hydrogen in a *trans* disposition to the 3′-substituent of the hormones. In order to test this hypothesis we have synthesised, and measured the hepatic receptor affinity and thyromimetic activity of 3′-acetyl-3,5-diiodo-L-thyronine (3′-Ac-T_2), a compound in which the formation of such a receptor–phenol hydrogen bond is precluded by the presence of a strong intramolecular hydrogen bond between the 3′-acetyl- and 4′-hydroxyl groups. In confirmation of the hypothesis, 3′-Ac-T_2 has a low affinity (0.5% of that of 3,5,3′-triiodo-L-thyronine, T_3) for the T_3-receptor in isolated rat hepatic nuclei. By contrast the thyromimetic activity (assessed by its ability to induce rat hepatic glycerol-3-phosphate dehydrogenase and increase the qO_2 of liver slices) was roughly equal to that of T_3. This apparent discrepancy was resolved when it was found that the capacity of 3′-Ac-T_2 to occupy hepatic receptors after *in vivo* administration, was about 100 times greater than predicted from its *in vitro* affinity. The reason for this difference between *in vivo* and *in vitro* nuclear binding is unknown at the present time.

In recent years convincing evidence has emerged to demonstrate that thyroid hormone action is initiated by the binding of the hormone to specific receptors located in the nuclei of responsive cells [1–4]. An important part of this evidence is the good correlation observed between the thyromimetic effects of 3,5,3′-triiodo-L-thyronine (T_3) analogues *in vivo* and their affinities for this nuclear binding site *in vitro*, providing account is taken of the known or likely effects of metabolism and/or distribution of some of the compounds [4].

These correlations between nuclear binding and *in vivo* activity generally utilise data obtained from different organs or tissues with the risk that receptors may not be identical in the activity and binding assays. In addition, the correlation of binding with activity, and analysis of the chemical properties influencing receptor affinity rely heavily on the results obtained from studies with close structural analogues of T_3, particularly 3′- and 3′,5′-alkyl- and halo-substituted thyronines, in which substituent lipophilicity and size are covariables [5].

These and earlier studies have identified the crucial importance of the 4′-phenolic hydroxyl group for high receptor affinity, and regression analyses [6] and theoretical calculations [7] have suggested a model of hormone binding and activity in which the interaction of the 4′-hydroxyl with the receptor involves hydrogen bonding via donation of the acidic hydrogen in a *trans* disposition to the 3′-substituent in T_3 and related compounds. The evidence for the required orientation of the 4′-hydroxyl group is largely derived from the observation of low affinity and activity found for 3′-nitro-3,5-diiodo-L-thyronine (3′-NO_2-T_2), in which the presence of a strong intramolecular hydrogen bond between the 4′-hydroxyl and the 3′-nitro groups could preclude formation of the postulated hydrogen bond with the receptor. However, since ionisation of the 4′-hydroxyl group in 3′-NO_2-T_2 could also influence both affinity and activity and further complicate the analysis, we considered it is essential to test other T_2 derivatives in which a less acidic hydroxyl group forms a strong intramolecular hydrogen bond with the 3′-substituent.

One such analogue is 3′-acetyl-3,5-diiodo-L-thyronine (3′-Ac-T_2, Fig. 1) in which the 3′-acetyl and 4′-hydroxyl groups form a strong intramolecular hydrogen bond. We have determined the affinity of 3′-Ac-T_2 for the hepatic T_3 receptor, both *in vitro* and *in vivo* and also its thyromimetic activity as measured by the capacity to induce hepatic mitochondrial glycerol-3-phosphate dehydrogenase (EC 1.1.99.5, GPDH) and increase hepatic qO_2. We have chosen these assays in order to avoid the problem, inherent in earlier work, of comparing receptor affinity and overall activity in different organs [4].

MATERIALS AND METHODS

Materials. All commercial chemicals were of the highest grade of purity obtainable. ^{127}I-T_3 was purchased from Sigma (London) Chemical Co. and L-[3′-^{125}I]T_3 (specific activity greater than 1.2 Ci/mg) from the Radiochemical Centre, Amersham.

Methods. In the experiments reported, surgically thyro-parathyroidectomised, male, Wistar rats have

been used in order to avoid complications due to the presence of an intact thyroid–pituitary axis.

The rats were thyroidectomised when 3 weeks old and used 7 weeks later when their mean weight was 158 g. Normal male rats in our colony weigh about 300 g at this age. Only those rats growing at substantially reduced rates (less than 5 g a week) were used in these experiments. Complete thyroidectomy in rats with this low growth rate is confirmed by the observation that these rats have low GPDH levels (similar to those produced by prolonged feeding with the goitrogen, methimazole) and a mean metabolic rate (563 ± 54 (42)ml/hr/kg$^{0.75}$) 60% of that of intact rats (944 ± 86 (10)ml/hr/kg$^{0.75}$).

In those experiments where GPDH, qO_2 and metabolic rate were measured each rat received an intramuscular injection (1 ml/kg) of vehicle (0.15 M NaCl–0.01 M NaOH), T_3 or 3′-Ac-T_2. T_3 and 3′-Ac-T_2 were dissolved in the minimum volume of N NaOH and further diluted with vehicle. Forty-eight hours after this injection the rats were killed and a piece of liver weighing about 0.5 g homogenised in 19 vol of 0.25 M sucrose. The activity of GPDH was measured at 37° by the method of Fried *et al.* [8]. In this the GPDH in whole homogenates was used to catalyse the reduction, by *sn*-glycerol-3-phosphate, of 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (INT) to the corresponding formazan. The formazan was extracted with ethyl acetate and its absorbance read at 490 mμ. The activity was measured at two dilutions and corrected for non-specific reduction of INT by subtracting the change in absorbance caused by homogenate in the absence of added *sn*-glycerol-3-phosphate. Each determination was performed in duplicate. Activity is expressed as μmoles formazan produced/hr/g wet weight, assuming that the formazan has a molecular extinction coefficient of 20.1×10^3 in ethyl acetate at 490 mμ [9].

Metabolic rate was measured by a calibrated, pressure-activated device which delivered small, known volumes of oxygen to a rat in a closed chamber containing soda-lime to absorb expired CO_2. The temperature was maintained at 29 ± 0.3°. The change in metabolic rate is reported as the difference between the initial and final readings for each individual animal, expressed in ml oxygen used, at STP/hr/kg$^{0.75}$ body weight. This method of expression is independent of the body weight [10].

Liver slices were cut free-hand [11] and their qO_2 determined using 0.01 M sodium lactate as substrate [12]. The affinities of T_3 and 3′-Ac-T_2 for isolated hepatic nuclei, and the capacity of these compounds to displace L-[3′-^{125}I]-T_3 from hepatic nuclei after *in vivo* administration were measured using the methods of Samuels and Tsai [13] and Oppenheimer *et al* [14] respectively. In the latter experiments L-[3′-^{125}I]-T_3 (8 μCi/kg) was given intravenously and T_3 or 3′-Ac-T_2 given intramuscularly (1 ml/kg) 1 hr before rats were killed and their livers frozen. The hepatic nuclei were later isolated and their ^{125}I and DNA content determined.

The results are expressed as a mean ± S.D. with the number of observations in parenthesis. The statistical significance of any differences are evaluated by means of Students *t*-test. When comparing pre-treatment and terminal metabolic rates the Paired *t*-test was used.

Synthesis. The route used to synthesise 3′-Ac-T_2 is shown in Fig. 1. *o*-Methoxyacetophenone (I), on treatment with iodine tristrifluoroacetate [15], gave the iodonium salt (II), which was coupled with the protected L-diiodotyrosine (III) in the presence of copper bronze and triethylamine [16, 17], yielding the protected thyronine (IV). The 4′-methoxy protecting group in IV was cleaved by treatment with boron trichloride [18], giving the phenol (V). Sodium hydroxide hydrolysis of the alanine side chain protecting groups in V then gave 3′-Ac-T_2. Both 4′-methoxy and side chain alanine protecting groups could be removed from IV by treatment with refluxing hydrobromic and acetic acids, but this gave 3′-Ac-T_2 of inferior purity, as adjudged by HPLC.

3,3′-Diacetyl-4,4′-dimethoxydiphenyl iodonium bromide (II). To a stirred and cooled (−10°) solution of iodine tristrifluoroacetate (prepared [15] from 30.0 g, 0.118 mole, of iodine) in acetic anhydride (100 ml) was added, dropwise, a solution of *o*-methoxyacetophenone (80.0 g, 0.532 mole) in acetic anhydride (70 ml) and trifluoroacetic acid (70 ml). During the addition, the temperature was maintained at −4 ± 2°. The mixture was stirred at room temperature overnight (approx. 17 hr), then poured into a stirred solution of sodium bromide (300 g) in water (100 ml). After 1.5 hr the aqueous layer was decanted and the residual gum treated with diethyl ether, then washed with water, to give II as a fine yellow powder, yield 26.0 g (22%). A sample recrystallised from DMF/diethyl ether gave a cream solid, m.p. 191–192° (dec.); IR (nujol): ν max. 1672 cm^{-1} (–CO–CH_3); NMR (DMSO-d_6): δ2.54 (*s*, 3H, C*H*$_3$–CO), 3.94 (*s*, 3*H*, CH_3O–), 7.27 (*d*, 1H, 5-*H*), ~8.30 (*m*, 2H, 2,6-*H*), low level impurities also observed; NMR (^{13}C, DMSO-d_6): δ109.47 (*C*-I$^+$).

A sample of the iodide, crystallised from DMF/diethyl ether, had m.p. 168–170°. Anal. Calc. for $C_{18}H_{18}I_2O_4$: C, 39.15; H, 3.28, I, 45.97. Found: C, 39.14; H, 3.30; I, 45.80.

3′-Acetyl-3,5-diiodo-N-trifluoracetyl-O-methyl-L-thyronine methyl ester (IV). Crude II (25.0 g, 0.049 mole), 3,5-diiodo-*N*-trifluoroacetyl-L-tyrosine methyl ester (III, 30.0 g, 0.055 mole), triethylamine (10 ml) and copper bronze (5.0 g) were stirred in methanol (150 ml) for 18 hr [16, 17]. The mixture was filtered, evaporated to dryness, the residue taken up in chloroform and washed successively with N HCl, 0.2 N NaOH, sat. NaCl, then dried over $MgSO_4$. The chloroform was removed and the residue (28 g) chromatographed on silica gel (Merck, kieselgel 60, 800 g). Elution with dichloromethane gave a colourless gum, yield 5.58 g (16.5%). This crystallised from diethyl ether-petroleum spirit to give IV, 2.58 g, m.p. 125–128°; IR (nujol): ν max. 3270 cm^{-1} (–N–H), 1747 cm^{-1} (–CO–O–), 1730 cm^{-1} (–CO–N–), 1658 cm^{-1} (–CO–CH_3); NMR ($CDCl_3$): δ 2.59 (*s*, 3H, C*H*$_3$CO–), 3.15 (*m*, 2H, Ar–C*H*$_2$–CH), 3.83, 3.90 (*s*, 3H each, C*H*$_3$O–), 4.85 (*m*, 1H, Ar–CH$_2$–C*H*), 7.10 (*m*, 4H, 2′-, 5′-, 6′-*H*, N*H*), 7.58 (*s*, 2H, 2-,6-*H*).

Anal. Calc. for $C_{21}H_{18}F_3I_2NO_6$: C, 36.48; H, 2.62; N, 2.03; I, 36.72. Found: C, 36.67; H, 2.73; N, 2.04; I, 36.47.

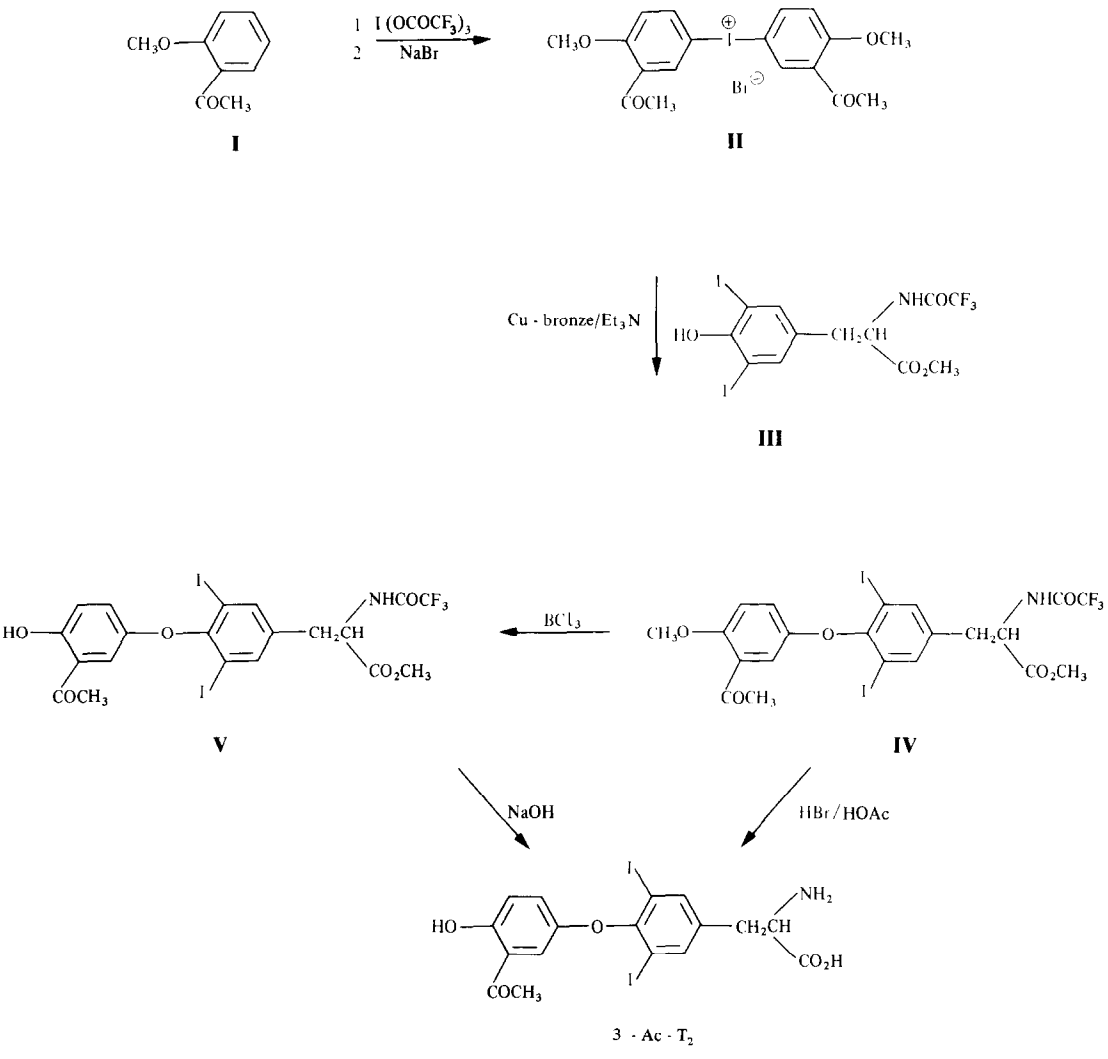

Fig. 1. Synthesis of 3′-acetyl-3,5-diiodo-L-thyronine (3′-Ac-T_2).

3′-Acetyl-3,5-diiodo-N-trifluoroacetyl-L-thyronine methyl ester (V). To a stirred solution of IV (0.50 g, 0.723 mmole) in dry dichloromethane (15 ml) at −78° was added dropwise a 1 M solution of boron trichloride [18] in dichloromethane (6.0 ml, 6.0 mmol). After 19 hr at room temperature the reaction mixture was poured onto ice (20 g), the organic layer removed, washed thoroughly with water, and dried over $MgSO_4$. The dichloromethane was removed and the residue chromatographed on silica gel (Merck, kieselgel 60, 75 g). Elution with dichloromethane, then recrystallisation from dichloromethane-petroleum spirit gave V, 0.370 g (76%), m.p. 177–178°; IR (KBr): ν max. 3820 cm^{-1} (–N–NH), 1736 cm^{-1} (–CO–O–), 1707 cm^{-1} (–CO–N–), 1648 cm^{-1} (–CO–CH_3); NMR (DMSO-d_6): δ2.54 (*s*, integral obscured, CH_3CO–), ~3.2 (*m*, integral obscured, Ar–CH_2–CH), 3.69 (*s*, 3H, CH_3O–), 4.70 (*m*, 1H, Ar–CH_2–*CH*), 6.91 (*m*, 2H, 5′-, 6′-*H*), 7.13 (*m*, 1H, 2′-*H*), 7.83 (*s*, 2H, 2-,6-*H*), 9.91 (*d*, 1H, –N*H*), 11.29 (*s*, 1H, –O*H*).

Anal. Calc. for $C_{20}H_{16}F_3I_2NO_6$: C, 35.47; H, 2.38; N, 2.07; I, 37.48. Found: C, 35.35; H, 2.58; N, 2.06; I, 37.80.

The NMR spectrum (DMSO-d_6) of 3,5-diiodo-*N*-trifluoroacetyl-L-thyronine methyl ester showed a singlet at δ9.01 for the phenolic OH [19], confirming that the chemical shift (δ = 11.29) of the phenolic OH in V is consistent with a strong intramolecular hydrogen bond.

3′-Acetyl-3,5-diiodo-L-thyronine (3′-*Ac-T_2*). V (0.330 g, 0.487 mmole) was dissolved in ethanol (25 ml) and 2 N NaOH (8 ml). After 2 hr the solution was acidified to ~pH 6 with glacial acetic acid, then concentrated *in vacuo*. The precipitate was collected, washed successively with water, ethanol and diethyl ether to give 3′-acetyl-3,5-diiodo-L-thyronine, 0.230 g (83%), m.p. 252–253° (dec.); IR (KBr): ν max. 3000–1900 cm^{-1} (–OH, $-NH_3^+$), 1652, 1635, 1620 cm^{-1} (–$COCH_3$, $-CO_2^-$, δ-NH_3^+); NMR (N NaOD): δ2.80 (*m*, 2H, Ar–CH_2–CH), 3.48 (*m*, 1H, Ar–CH_2–*CH*), 6.71 (*d*, 1H, 5′-*H*), 6.86 (*d*, 1H, 2′-H), 6.95 (*d* of *d*, 1H, 6′-*H*), 7.76 (*s*, 2H, 2-, 6-*H*); acetyl resonance not observed because of proton exchange; $[\alpha]_D^{25°}$ = +12.1° (1.01% soln. in HOAc/EtOH/N HCl, 10:9:1). HPLC, performed using a C_{18} μ-Bondapak column with, as eluent, a mixture of acetonitrile (35%) and water containing 1% acetic

acid and 1 g of camphor sulphonic acid/l (65%), indicated the product contained no significant impurities (purity > 99.9%).

Anal. Calc. for $C_{17}H_{15}I_2NO_5$: C, 36.00, H, 2.66; N, 2.46; I, 44.75. Found: C, 35.94; H, 2.73; N, 2.47; I, 44.81.

RESULTS

Overall activity. 3′-Ac-T_2 was found to be as active as T_3 in the induction of hepatic GPDH. Figure 2 shows dose–response curves to T_3 and 3′-Ac-T_2. Each point represents the average hepatic GPDH activity of twelve rats given T_3 or two rats given 3′-Ac-T_2 48 hr previously. This data was fitted by an unweighted, non-linear curve-fitting procedure to the equation:

$$r = \frac{R_M}{1 + (ED_{50}/\text{dose})} + R_0$$

where r is the measured activity, R_M is the maximum increase in activity achievable, R_0 is the calculated activity in the untreated animal and the ED_{50} is that dose of thyromimetic which caused an increase in GPDH activity of 0.5 R_M. As can be seen from the graph this hyperbolic equation describes the dose–response relationship reasonably well. The calculated R_M for T_3 is 131 ± 6 GPDH units, R_0 is 28 ± 3 GPDH units and the ED_{50} 92 ± 15 nmoles/kg. The calculated R_M for 3′-Ac-T_2 is 101 ± 8 GPDH units, R_0 26 ± 5 GPDH units and ED_{50} 116 ± 0 nmoles/kg. The similarities of the ED_{50} values show that these two compounds are essentially equipotent. The slightly lower value of the R_M for 3′-Ac-T_2 is probably a consequence of the day to day variation in the sensitivity of the liver to thyromimetics rather than a real reduction in R_M since the increase in GPDH activity caused by T_3 at 2 mg/kg (a maximally effective dose) in the same experiment was also 101 units.

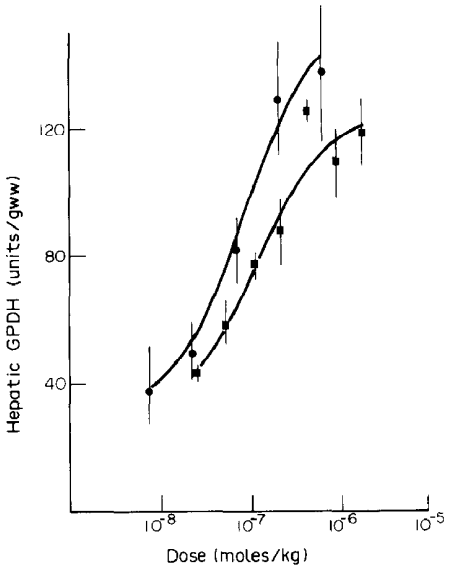

Fig. 2. The effect of T_3 or 3′-Ac-T_2 on hepatic GPDH. Male, thyroparathyroidectomised rats were given various doses of T_3 (●; N = 12) or 3′-Ac-T_2 (■; N = 2) by intramuscular injection of 48 hr before hepatic GPDH was determined. Points are shown as mean ± S.D. and solid lines represent the best fit of the data to an hyperbolic curve.

3′-Ac-T_2 was also a very active thyromimetic when its effects on hepatic and whole body oxygen consumption of thyroidectomised rats were measured. In the experiment shown in Table 1 doses of 3′-Ac-T_2 were chosen to give an intermediate response (0.1 mg/kg: 0.17 μmoles/kg) or a maximum response (2 mg/kg: 3.5 μmoles/kg) in the liver. In the same experiment the effects of T_3 at 0.1 mg/kg (0.15 μmoles/kg) or 2 mg/kg (3.1 μmoles/kg) were also determined.

Table 1. Effect of T_3 and 3′-Ac-T_2 on metabolic rate, hepatic GPDH and qO_2

Treatment	Hepatic GPDH (Units-gww)	qO_2 (μl O_2 at STP/hr/mg dry wt)	Increase in metabolic rate (ml O_2 at STP/hr/kg)
Vehicle	22 ± 5(6)	7.1 ± 1.1(6)	31 ± 52(6)
T_3 154/nMoles/kg (0.1 mg/kg)	122 ± 25(6)*	12.1 ± 1.2(6)*	202 ± 55(6)*
T_3 3077/nMoles/kg (2 mg/kg)	161 ± 35(6)*	14.7 ± 1.7(6)*	235 ± 40(6)*
3′-Ac-T_2 176/nMoles/kg (0.1 mg/kg)	90 ± 20(6)*	10.5 ± 1.4(6)*	89 ± 64(6)
3′-Ac-T_2 3527/nMoles/kg (2 mg/kg)	142 ± 21(6)*	12.6 ± 2.2(6)*	164 ± 36(6)*

* Significantly greater than control group ($P < 0.01$).

Male, thyroparathyroidectomised rats were given a single intramuscular injection of vehicle, T_3 or 3′-Ac-T_2 48 hr before hepatic GPDH and qO_2 were determined. Metabolic rate was measured before treatment and immediately before death. All values for treated rats are significantly greater than for control animals ($P < 0.01$) except for the increase in metabolic rate in rats given the low dose of 3′-Ac-T_2. The hepatic GPDH and qO_2 in these rats were significantly less than in rats given the low dose of T_3 ($P < 0.05$). The increase in metabolic rate was significantly different from zero in all except the control group.

The low dose of 3′-Ac-T_2 caused a large, highly significant ($P < 0.01$) increase in hepatic GPDH activity which was slightly less than that induced by the corresponding dose of T_3. The high dose of both agents produced larger responses which were not significantly different from each other. The effects of these compounds on the qO_2 of surviving liver slices were entirely analogous. The low dose of 3′-Ac-T_2 caused a significant, 50% increase in qO_2 ($P < 0.01$) which was less than that due to the low dose of T_3. The responses to the high doses were not significantly different from each other. Both doses of 3′-Ac-T_2 increased the metabolic rate of thyroidectomised rats although these changes were significantly smaller than those caused by the corresponding doses of T_3 ($P < 0.01$).

Receptor binding. In contrast to the high level of overall thyromimetic activity shown by 3′-Ac-T_2 its affinity for the T_3-receptor in isolated hepatic nuclei was very low. In the experiment shown in Fig. 3 increasing concentrations of T_3 and 3′-Ac-T_2 diminished the binding, by hepatic nuclei, of a tracer concentration of ^{125}I-T_3. The EC_{50} for T_3 in this experiment was about 0.78×10^{-9} M; the corresponding value for 3′-Ac-T_2 was about 0.15×10^{-6} M, giving an affinity for 3′-Ac-T_2 of about 0.5% T_3.

In order to investigate the disparity between receptor affinity and overall activity we also determined the doses of T_3 and 3′-Ac-T_2 required to displace 50% of the ^{125}I-T_3 binding to hepatic nuclei in thyroidectomised rats (Fig. 4). In this experiment increasing doses of non-radioactive T_3 or 3′-Ac-T_2, given intramuscularly, decreased the radioactivity of nuclei isolated one hour after an intravenous injection of ^{125}I-T_3. The dose of T_3 which decreased binding by 50% was about 2.9×10^{-8} moles/kg; the corresponding dose for 3′-Ac-T_2 was 5.7×10^{-8} moles/kg.

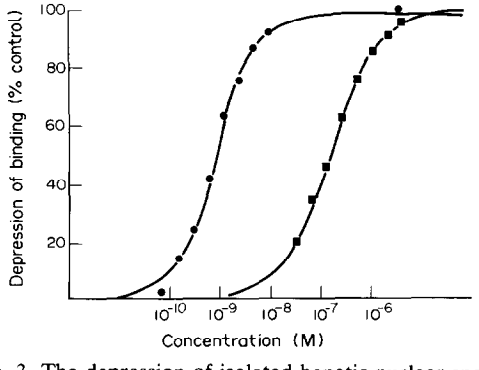

Fig. 3. The depression of isolated hepatic nuclear specific L-[3′-^{125}I]T_3 binding by T_3 (●) and 3′-Ac-T_2 (■). Hepatic nuclei from male, thyroparathyroidectomised rats were incubated with approx. 0.075 nM L-[3′-^{125}I]T_3 (s.a. > 1200 μCi/μg) alone or with increasing concentrations of non-radioactive T_3 or 3′-Ac-T_2 for 45 min at 37°. The % depression of specific binding (specific binding = total nuclear counts − nuclear counts in the presence of a 50,000-fold molar excess of unlabelled T_3) was determined in triplicate for each analogue concentration. The means are plotted against analogue concentration on the horizontal axis. The solid lines represent the best fit of the data to an hyperbolic curve.

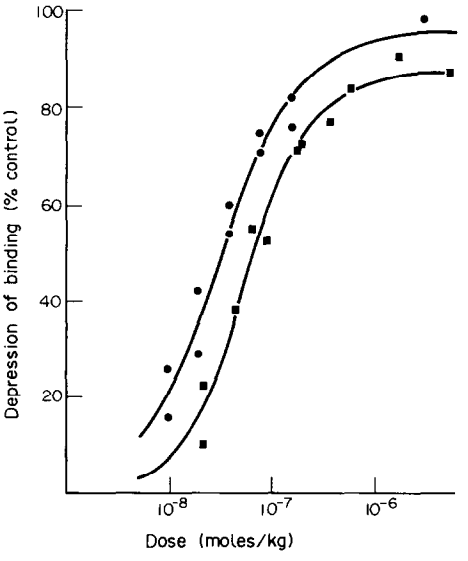

Fig. 4. Displacement of L-[3′-^{125}I]T_3 from hepatic nuclei *in vivo*. Male, thyroparathyroidectomised rats were given L-[3′-^{125}I]T_3 (8 μCi/kg) intravenously and non-radioactive T_3 (●) or 3′-Ac-T_2 (■) intramuscularly. The % depression of nuclear ^{125}I (cpm/mg DNA), after the various doses of cold ligand, shown on the horizontal axis, was determined 1 hr later. Each point is the mean of two separate determinations. Data from two similar experiments has been included. The solid lines represent the best fit of this data to an hyperbolic curve.

The "affinity" of 3′-Ac-T_2 determined *in vivo*, therefore, was 51% of that of T_3, in marked contrast to their relative affinities for isolated nuclei.

DISCUSSION

Jorgensen and his colleagues have proposed a specific mode of binding of the 3′-substituent and 4′-hydroxyl group of 3′-substituted-3,5-diiodo-thyronines to the T_3-receptor based on results obtained from an assay using a solubilised extract of rat liver nuclei [6, 7]. This working model, which is based on a QSAR analysis of group contributions to receptor affinity and on theoretical considerations, proposes that:

(1) The unionised 4′-hydroxyl group interacts with the receptor by hydrogen bonding to an acceptor group located near the 5′-position of the 3,5-diiodothyronine (i.e. 4′-hydroxyl and 3′-substituent are in a *trans* conformation).

(2) A lipophilic 3′-substituent is essential for high receptor affinity and is presumed to bind in a size-limited hydrophobic pocket in the receptor.

(3) A second lipophilic substituent adjacent to the 4′-hydroxyl group in the 5′-position reduces affinity in direct relationship to its size, consistent with a steric interaction of the 5′-substituent inhibiting formation of the 4′-hydroxyl-acceptor hydrogen bond.

(4) Receptor affinity is enhanced by electron-withdrawing 3′- and 5′-substituents.

This proposed receptor interaction of the 4′-hydroxyl in 3,5-diiodothyronines is fully consistent with the intermolecular interactions observed for

ortho-substituted phenols in the solid state [20, 21]. Thus, the majority of monosubstituted phenols form strong, intermolecular hydrogen bonds in which the hydroxyl protons are positioned *trans* to the ortho-substituent and bond to the oxygen atom of another molecule. A second ortho-substituent reduces this tendency to form intermolecular hydrogen bonds due to non-bonded steric interactions.

The model proposed by Jorgensen *et al.* relies on the analysis of the affinities of analogues of T_2 with halogen and alkyl groups at the 3′- and 5′-positions and 3′-NO_2-T_2. No alternative polar 3′-substituents, capable of forming strong intramolecular hydrogen bonds with the 4′-hydroxyl group, have been considered, with the result that the regression equation supporting a *trans* conformation for the 4′-hydroxyl and the 3′-substituent is solely dependent on inclusion of the 3′-nitro derivative. Since the measured pK_a of the 4′-OH in 3′-NO_2-T_2 is 6.85 at 37° [22], approximately 91% of this compound will be ionised at the pH (7.85) of the liver nuclear binding assay and, therefore, the nature of the species binding to the receptor cannot be predicted with confidence. Thus if 3′-NO_2-T_2 is included in a regression equation a correction for ionisation is necessary.

o-Acetylphenol is known to exist almost totally in the *cis* conformation due to the presence of a strong intramolecular hydrogen bond [23–25]. The NMR spectrum of *o*-acetylphenol in DMSO-d_6 exhibits a sharp hydroxyl peak at 11.97 ppm, compared with a corresponding absorption at 9.23 ppm for phenol and 10.28 ppm for *p*-acetylphenol [23]. It was argued that this large downfield shift for *o*-acetylphenol is consistent with the presence of an intramolecular hydrogen bond which overcomes any tendency to hydrogen bond to DMSO, and therefore the strength of this intramolecular hydrogen bond must be greater than that (6.5 kcal/mole) found between phenol and DMSO [26]. In our own studies in DMSO a similar large difference (2.28 ppm) between the hydroxyl chemical shifts in the alanine protected derivatives of 3′-Ac-T_2 and T_2 is observed. This confirms that a strong intramolecular hydrogen bond is also present in thyronine analogues possessing a 3′-acetyl substituent (see Materials and Methods).

The aqueous solubility of 3′-Ac-T_2 was found to be 2.8×10^{-6} mole/l at pH 7.4, 37° [22]. This low solubility prevented the measurement of the pK_a of 3′-Ac-T_2 due to precipitation; however, since the reported [24] pK_a (10.27) for *o*-acetylphenol is higher than that reported [27] for phenol (9.99), it must follow that the pK_a of 3′-Ac-T_2 will be higher than 9.3, the value reported [28] for T_2. Therefore, the neutral unionised form will predominate in the pH range 7–8. The greater lipophilicity of *o*-acetylphenol ($\log P_{oct} = 1.95$) than phenol ($\log P_{oct} = 1.46$) reflects the predominance of this intramolecularly hydrogen-bonded neutral form [22].

The special properties of *o*-acetyl-substituted phenols led us to conclude that the affinity of 3′-Ac-T_2 for the thyroid hormone receptor may provide additional, valuable evidence to assess the role of the 4′-OH group in receptor binding. 3′-Ac-T_2 was found to have an affinity of 0.5% of that of T_3, close to the published (0.3%) [6] and our own value (0.1%) for T_2. Thus, the 3′-acetyl group in 3′-Ac-T_2 makes no overall contribution to binding, despite a greater lipophilicity of 3′-Ac-T_2 compared with T_2. It is likely therefore, that the relatively weak affinity of 3′-Ac-T_2 is most probably due to the inability of the receptor to form an intermolecular hydrogen bond with the 4′-hydroxyl group, a conclusion which is consistent with the model proposed by Jorgensen *et al.* for the receptor interaction of T_2 derivatives in which the 4′-hydroxyl group acts as a proton donor to the receptor [6, 7].

In contrast to its low receptor affinity the activity of 3′-Ac-T_2 (GPDH and qO_2) was indistinguishable from that of T_3. This 200-fold discrepancy between the relative receptor affinity (*in vitro*) and relative activity (*in vivo*), measured in the same organ, was resolved by the observation that the relative ED_{50} for liver nuclear binding measured *in vivo*, is only twice that of T_3, i.e. *in vivo* activity is fully consistent with the nuclear occupation found for 3′-Ac-T_2 *in vivo*.

A similar biological profile to 3′-Ac-T_2 has been reported for 4′-deoxy-T_3 [4] and for L- and DL-3,5-dimethyl-3′-isopropyl thyronine (L- and DL-DIMIT) [29]. Koerner *et al.* [4] found that 4′-deoxy-T_3 had 10% of the affinity of T_3 for the receptor when measured *in vivo* in the rat, in good agreement with a biological activity of 19% T_3 in the rat antigoitre assay, but only 0.2% T_3 in the *in vitro* nuclear binding assay. It was suggested, in this case, that the discrepancy between *in vitro* and *in vivo* receptor affinities could be explained by metabolic 4′-hydroxylation to T_3 in the *in vivo* nuclear binding experiment, since compounds with blocked 4′-hydroxyl positions are inactive and aromatic hydroxylation is a common metabolic pathway.

The reasons for the large ratios of *in vivo/in vitro* affinities in rat liver for 3′-Ac-T_2 (ratio = 102) and DIMIT (ratio = 43 – unpublished results) are not obvious. In addition to metabolic activation, other variables such as tissue distribution, intracellular concentrating mechanisms and protein binding could lead to a discrepancy between *in vivo* and *in vitro* nuclear binding. Significantly, Schwartz *et al.* [30] have reported recently that the higher potency, in rat heart and liver, of L-T_3 compared with D-T_3 is not due to differences in receptor affinity, metabolism, or overall cellular distribution but rather to preferential accumulation of L-T_3 by hepatic and cardiac nuclei. We must conclude, therefore, that a comprehensive mechanistic study is likely to be required in order to explain fully discrepancies of the type reported here for 3′-Ac-T_2. In particular, the results reported by Schwartz *et al.* suggest that the factors governing nuclear accumulation in tissues need to be elucidated as part of this overall analysis.

In 1975 Ahmad *et al.* [31], predicted the high thyromimetic activity of 3′Ac-T_2 in a QSAR analysis of 3′-substituted-3,5-diiodothyronines. These authors showed that substituent parachor (P_r) values could be used effectively in regression equations to replace π values and predicted that a 3′-acetyl substituent with a parachor ($P_r = 107$), close to the optimum value of 120, would 'be a highly potent thyromimetic'. This interesting prediction of high activity for 3′-Ac-T_2 may indicate that 3′-substituent P_r could be a useful parameter in assessing activity for 3′-substituted T_2 analogues. However, several

biological factors will determine overall activity and these will be affected differently by substituent changes. Consequently, a more diverse range of structural types should now be studied to test the validity of this apparent correlation between activity and parachor (P_r).

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