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EXPERIMENTAL VALUES OF THE IONIZATION CONSTANTS FOR L-3,5-DI-IODOTYROSINE AND A MODEL FOR IONIC INTERACTIONS OF THYROID HORMONE (T_3) AND ITS NUCLEAR RECEPTOR

WILLIAM L. GENT* and BRIAN D. WILSON

SAMRC Iodine Metabolism Unit, Pharmacology Department, University of Stellenbosch Medical School, Tygerberg 7505, Republic of South Africa

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Abstract—Ionization characteristics of L-3,5-di-iodotyrosine have been measured under various conditions. These data, which correct an erroneous report of pK_3 in the literature, have been used to estimate the ionization constants of the thyroid hormone L-3′,3,5-tri-iodothyronine (T_3). On this basis a reinterpretation has been made of the pH-dependent binding of the hormone to its solubilized rat liver nuclear receptor (Wilson BD and Gent WL, *Biochem J* **232**: 663–667, 1985). The interaction may depend on the ionization of the phenolic group of T_3 and an acidic group (pK′7.6) in the receptor site, leading to the formation of a hydrogen bond between the two groups. The changes of the number of binding sites with pH must then result independently from alterations in the conformation of the receptor protein.

In a previous paper [1] we confirmed that the amount of thyroid hormone L-3′,3,5′-tri-iodothyronine (T_3) bound by its solubilized rat liver nuclear receptor (R) as a function of pH has a bell-shaped profile [2]. An analysis of the apparent association constants (K_A') for T_3 and R in the range of pH 6.4–9.0 showed that the interaction could be accounted for in terms of the protonated form of an acidic group (pK' = 8.3) and the basic form of an acid having pH′ = 7.6. Because of its very small solubility [3], the only ionization constant recorded for T_3 is that of the 4′-OH (pK′ = 8.45, 27°, I = 0.04), by the use of a spectrophotometric method [4]. As such a constant would be smaller under the conditions used (4°, I = 0.6) for the measurement of K_A', the acidic group of pK' = 8.3 was identified with the 4′-OH of T_3. The assignment of pK' = 7.6 to a group within the receptor site appeared to be precluded, for it would require a large change in the total number of binding sites (B_{max}) as a function of pH, and this was not found experimentally. As early measurements [5] of the ionization constants of L-3,5-di-iodotyrosine (DIT) were found to show an abnormally low pK (α-NH_3) = 7.8, it seemed possible that a similar anomaly might exist in T_3. Consequently, it was concluded that the interaction of T_3 and R depends only on the ionization of T_3 and that the ligand form is 4′-OH, α-NH_2, α-CO_2^-. There seems, however, to be no evident structural feature of DIT which would cause pK (α-NH_3^+) to differ markedly from that for any aminoacid in which the corresponding group is not subject to proximity effect from an adjacent charged group. Accordingly we have reinvestigated the ionization characteristics of DIT, under various conditions, and report that whilst pK (4-OH) agrees with several other determinations, the values for pK (α-NH_3^+) are not anomalous, i.e. are close to well-authenticated values for the majority of a α-aminoacids. It is argued that, if for DIT the value of pK (α-NH_3^+) is close to 9.7 units (4°, I = 0.6) then a similar value must obtain for T_3. The original hypothesis [1], as outlined above, concerning the ionic component of the interaction of T_3 and R, is consequently invalidated. A new model is proposed which satisfactorily accounts for the original data [1].

MATERIALS AND METHODS

L-3,5-Di-iodotyrosine was purchased from Sigma Chemical Co. (St. Louis, MO). Triplicate samples were vacuum-dried to constant weight to give an analysis: DIT.$2H_2O$, in agreement with the reported composition [5].

Titrimetry. Because of the low solubility of DIT (1.4 mM, 25°) at its isoelectric pH = 4.4 [5], samples of the compound were solubilised by 2 eq of NaOH and made up to 7.5 mM (25°) or 3.5 mM (4°), at which concentrations and ionic strengths it was possible to titrate by HCl into the isoelectric range without precipitation. For measurements at I = 0.6 appropriate quantities of NaCl were included. Quadruplicate 20 ml aliquots of the solutions were titrated by 0.15 M HCl (25°) or 0.075 M HCl (4°), pH being measured after each successive 50 μl addition of acid. Data were analysed, for determination of pK_2 (4-OH) and pK_3 (α-NH_3^+), essentially by the method of Van Slyke [6], i.e. from the intersection of the linear regression equations for $1/(pH_i - pH_j)$ on $(pH_i + pH_j)/2$, seven points being available on each shoulder. For all pK values (Table 1) SE = ±0.02 units.

RESULTS

The values of pK_2 and pK_3 obtained under the

* To whom correspondence should be addressed.

Table 1. Ionization characteristics of L-3,5-di-iodotyrosine

$t°$	I	pK'	pK'
4	0.007*	6.52	10.10
	0.60	6.24	9.74
25	0.15*	6.42	9.51
	0.60	6.15	9.21
$\Delta H'$kJmol^{-1}†		+7	+42

* For the low ionic strength determinations I was taken as equal to the concentration of NaCl formed at the isoelectric pH.

† The apparent heat of ionization $\Delta H'$ was calculated by the van't Hoff equation in the form: $\Delta H' = 75.4\,[pK'(25°) - pK'(4°)]$ kJmol^{-1}, pK' being taken as the mean of values at the two ionic strengths.

various sets of conditions are shown in Table 1 and considered as follows:

(a) pK_2'

Various reported values (corrected to $I = 0$) and at 25° are as follows:

6.51 Dalton *et al.* [5], solubility method;
6.52 Crammer and Neuberger [8], spectrophotometric method;
6.46 Gemmill [4], spectrophotometric method;
6.48 Table 1.

The small temperature coefficient (Table 1) is similar to that found by the solubility method [5] and, in the same laboratory [9] for the dichloro- and dibromo-analogues.

(b) pK_3'

The magnitude of this constant, its temperature coefficient and the derived value of $\Delta H'$ are entirely in line with corresponding data for a wide range of aminoacids [10]. An early report [5], however, gave much lower values, viz. pK_3' ($I = 0.002$) = 8.31; 7.82; 7.45 at, respectively, $t = 0°$; 25°; 40°, with somewhat similar magnitudes for the dibromo- and dichloro-homologues [9]. Consideration of the technique employed suggests that errors in pH measurement were responsible for anomalous values of the ionization constants for the α-NH_3^+ group of DIT.

(c) *Possible ionization constants of* T_3

Gemmill's value [4] for pK_2' of T_3 has been corrected by the same decrement as is shown by DIT (Table 1), to give pK_2' = 8.3, $t = 4°$, $I = 0.6$. The more remote situation of the 4'-O^- group in T_3 may well result in pK_3' being lower than in DIT by a few tenths of a unit but for the present purpose it is sufficient to ignore this effect and to assume that the constant is the same as for DIT (Table 1), viz. pK_3' = 9.7, $t = 4°$, $I = 0.6$. Pending a spectrophotometric investigation of the ionization of T_3, similar to that developed by Edsall and his collaborators [7, 11] in the case of tyrosine, it is not possible to evaluate exactly the actual ionizations of T_3 but trial calculations (not shown) indicate that errors of only a few per cent arise if the ionization of the 4'-OH alone is considered over the pH range 6–9 units.

(d) *Reconsideration of the ionic interaction between* T_3 *and its nuclear receptor*

It was shown previously [1] that the apparent association constant K_A', at a particular pH for the combination of T_3 and its solubilized nuclear receptor R, can be expressed as the product of a pH-independent association constant K_A and the quantities F_a (8.3), the fraction of an acid of pH = 8.3 present in the acidic form at the particular pH, and F_b (7.6), the fraction of an acid of pK = 7.6 present as the conjugate base under the same condition, i.e.

$$K_A' = K_A \cdot F_a\,(8.3) \cdot F_b\,(7.6) \qquad (1)$$

The acid of pK = 8.3 can reasonably be identified with the 4'-OH of T_3 but, from the further evidence considered under Results (c), the acid of pK = 7.6 is unlikely to be the α-NH_3^+ of T_3. Furthermore, it is not possible to propose that the probable pK' of α-NH_3^+ is lowered to a value of 7.6 units on formation of the complex $R \cdot T_3$, for in this case F_b (7.6) would appear as the multiplier of K_A' in Eqn (1). Consequently the acid of pK' = 7.6 must relate to a group within the receptor site but, if this were the case, the experimentally determined maximum concentration of receptor sites (B_{max}) would change with pH: $B_{max} = B^*_{max}\,F_b\,(7.6)$, B^*_{max} being the limiting value of B_{max} at high pH. It was shown experimentally [1] that this is not so and that B_{max} changes with pH to only a small extent and in a quite different way from that required by this equation.

The conflict of evidence can be resolved by noting that:

$$F_a(8.3) \cdot F_b(7.6) \cdot K(8.3) \qquad (2)$$

$$= F_b(8.3) \cdot F_a(7.6) \cdot K(7.6)$$

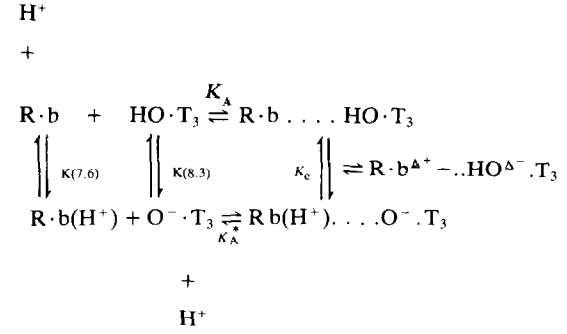
in which $K(7.6)$ and $K(8.3)$ are the dissociation constant of the acids of pK(7.6) and pK(8.3), so that the experimentally determined values of K_A' previously reported [1], can just as well be described by the relationship:

$$K_A' = K_A^* \cdot F_b(8.3) \cdot F_a(7.6) \qquad (3)$$

as by Eqn (1); from Eqn (1) and (2) it follows:

$$K_A^* = K_A \cdot K(7.6)/K(8.3) = 5.01\,K_A \qquad (4)$$

Denoting the acidic form of the receptor site as $R \cdot b(H^+)$, with conjugate base $R \cdot b$ and postulating that the geometry of the hormone–receptor complex is such that direct interaction can occur between the ionizable groups on each of the components, the possibilities of interaction are then either $R \cdot b \ldots HO \cdot T_3$ or $R \cdot b(H^+) \ldots O^- \cdot T_3$. Because, however, $K(7.6) > K(8.3)$ an intramolecular proton transfer would take place in the second of these configurations and the overall reaction of T_3 and R could be described by the scheme:



The equilibrium mixture of the two forms of the hormone–receptor complex on the right-hand side of the equation may be regarded as a single hydrogen-bonded species, e.g. if $R \cdot b(H^+)$ is a cationic acid then the complex may be represented as $R \cdot b^{\Delta+} -. .H-O^{\Delta-}.T_3$, and which can be formed by, or dissociate into either pair of components. The equilibrium constant K_e then represents the contribution of each of the two forms of the complex to the hydrogen-bonded structure. Defining K_e

$$K_e = [R \cdot b(H^+). . . . O^-.T_3]/R.b. . . . HO \cdot T_3]$$

then K_e may be evaluated from Eqn (4)

$$K_e = K_A^*/K_A = K(7.6)/K(8.3) = 5.01 \qquad (6)$$

and hence, the charge separation Δ in the hydrogen-bonded structure will be: $\Delta = 1/(1 + K_e) = 0.17$. The true association constant of R and T_3, $K(R,T_3) = [R \cdot T_3]/[R][T_3]$, in which $R \cdot T_3$ is the polar hydrogen-bonded species of Eq (5) with [R] and $[T_3]$ as the *total* concentrations of both ionic forms of each of the molecules R and T_3 respectively, will be independent of pH and may be evaluated:

$$K(R,T_3) = K_A \cdot K_e/(1 + K_e) = K_A^*/(1 + K_e) \qquad (7)$$

From the value of K_A previously reported [1] as $30.9 * 10^9\,M^{-1}$ and K_e from Eq (6)

$$K(R,T_3) = 25.8 * 10^9\,M^{-1} \text{ at } 4° \text{ and } I = 0.6$$

It further follows that because in this model both forms of the receptor, viz $R.b(H^+)$ and R.b are involved in the interaction with the hormone, then $B_{max} = [R\ b(H^+)] + [R.b]$ and B_{max} will not change with pH. The small changes of B_{max} with pH found experimentally (2) may then be ascribed to the effect of pH on the receptor protein, changes in conformation of which lead to major changes in the structure of the receptor site.

DISCUSSION

The ionization constants for di-iodotyrosine reported here confirm several [4, 5, 8] determinations of pK(4-OH) whilst correcting erroneous values [5] found for p$K(\alpha\text{-}NH_3^+)$. Although Jorgensen [12] had queried the apparent anomaly, indicated by the early work, no other report has been located. If, indeed, p$K(\alpha\text{-}NH_3^+)$ of DIT is similar to that for many aminoacids not having a charged group in the proximity of the α-aminoacid zwitterion, then it is reasonable to suppose that in T_3 also pK $(\alpha\text{-}NH_3^+) \approx 10$ at low ionic strength.

On this basis the original hypothesis proposed by us [1], concerning the role of ionic interactions between T_3 and its solubilized nuclear receptor, becomes untenable as this required that pK $(\alpha\text{-}NH_3^+) \approx 7.6$. Because the data [1], on the variation of the apparent association constant for T_3 and R with pH require two acidic ionizations, one of p$K' = 8.3$ and the other of p$K' = 7.6$, for their interpretation, then inevitably the group of p$K' = 7.6$ must be ascribed to the receptor site itself, if p$K' = 8.3$ is identified with that of the 4′-OH group in T_3. Such an assumption, as noted originally would require a large change in the number of binding sites as a function of pH and this was not observed experimentally. By postulating, however, that binding sites having the acidic form $R \cdot b(H^+)$ and the basic form R b can interact with the forms of T_3 having 4′-O^- and 4′-OH, respectively, then variation of the number of binding sites with pH is shown not to occur and the small variation of the number of T_3 binding sites with pH will be the result of changes in protein conformation.

The two kinds of ionic interaction between T_3 and R are shown to lead to the same hydrogen-bonded structure. As such a bond might be expected to make a substantial contribution to the total binding energy of R and T_3, our evidence supports the long-sustained assertion by Jorgensen, as finalized by Bolger and Jorgensen [13], that hydrogen-bond formation by T_3 is essential to its function.

Samuels *et al.* [14] have shown that the low capacity, high-affinity binding sites for T_3 found in liver *in vivo* have essentially the same affinity characteristics as those in isolated intact nuclei and in solubilized receptor preparations. Consequently, the marked dependence on pH shown by T_3 binding to solubilized receptor previously reported [1] may well reflect the possibility of such changes *in vivo*. It is now shown that the alteration in T_3 binding with pH may be quantitavely accounted for in terms of the ionization of the 4′-OH group of T_3 and of an acidic group in the receptor binding site. In view of the magnitude of the effect, which amounts to a reduction of 50% of maximum binding for a decrease of pH from 7.6 to 7.1 units, the possibility of intracellular pH changes consequent upon metabolic activity in the liver, reviewed by Haüssinger and Gerok [15], being an important modulating factor in the operation of the nuclear receptor, revealed by the work of Oppenheimer and co-workers [16], may need to be considered.

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