Effects of pH and ionic strength on the binding of L-tri-iodothyronine to the solubilized nuclear receptor

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K'_A (apparent association constant) and B_{max} (total receptor concentration) describing the interaction of tri-iodothyronine (T_3) and its solubilized rat liver nuclear receptor (R) are found to be moderately consistent in successive preparations, but both quantities diminished after a few days. To achieve comparability in the effects of ionic strength (I) and of pH on K'_A and B_{max} , appropriate measurements have been made simultaneously on single preparations. K'_A and B_{max} were found to be effectively unchanged over the range I 0.05–0.60. Both parameters have been measured over the range pH 6.4–9.0 and the values of K'_A analysed in terms of the 4'-OH ionization of T_3 and that of a cationic acidic group, shown to require $pK' = 7.6$. This group could be identified either with the terminal $\alpha-NH_3^+$ of T_3 or with a group (RH $^+$) in the receptor site. On the balance of evidence the first possibility is the more likely, in which case the variation of B_{max} with pH is ascribed to conformational changes in the receptor protein.

INTRODUCTION

In extending our investigation (Wilson *et al.*, 1984) of the factors involved in the binding of thyroid-hormone receptor protein(s) (R) to DNA, it has become necessary to understand the effect of pH and I on the receptor protein. The only systematic study (Latham *et al.*, 1976) of the effect of pH on the interaction of L-tri-iodothyronine (T_3) and L-thyroxine (T_4) with their receptor(s) led these authors to advance the hypothesis that the diminution of T_3 binding at pH values above pH 8.0 is due to the ionization of the 4'-OH group ($pK' 8.45$) and that, in consequence, it is the un-ionized form of the hormone that binds to the receptor site. Support for this conclusion has come from the structural studies of Jorgensen (1978a) on a wide range of T_3 analogues, and of Benson *et al.* (1984). By implication, although not stated explicitly (Latham *et al.*, 1976), the observed large increase of T_3 binding from pH 6.0 to the optimal pH 7.6 would be due to a change in receptor properties, either at the receptor site or in the protein conformation. Apparent confirmation of such effects was reported (Eberhardt *et al.*, 1979) by measurement of the binding parameters at pH 6.0 and 7.6, both K'_A and B_{max} increased considerably from the lower to the higher pH value. However, concurrent measurements on T_4 binding showed a decrease from a maximum at pH 6.0 to a much lower value at pH 10, and this effect was explained in terms of the increasing ionization of the 4'-OH group of T_4 ($pK' 6.7$) and to the apparent decrease of B_{max} from pH 6.0 to pH 7.6. As both these investigators and others (summarized by Apriletti *et al.*, 1983) have shown that T_3 and T_4 bind to the same receptor protein(s) at pH 7.8, it was concluded (Latham *et al.*, 1976; Eberhardt *et al.*, 1979) that the differential effect of pH on the binding of T_3 and T_4 results from the difference between the 4'-OH ionization constants and the presence of a protein component binding only T^4 . As other interpretations of the results presented by these

authors seemed possible, and as we had found some differences between the behaviour at pH 7.6 of our nuclear-extract preparations and that reported by them, we have reinvestigated some of these phenomena in an attempt to analyse and model the pH-induced alterations of thyroid-hormone–receptor interaction.

MATERIALS AND METHODS

Reagents

L-3,3',5-[^{125}I] T_3 (3380 μ Ci/ μ g) was from New England Nuclear, and unlabelled T_3 was from Sigma Chemical Co. The iodide content (< 1%) was periodically checked by elution of samples from Sephadex G-25 (fine grade) columns (See below under 'Analytical method').

Buffers

Buffer A comprised 10 mM-Tris/HCl, 0.25 M-sucrose, 1 mM-MgCl $_2$ (for preparation of nuclei; with 2 mM-DTT and 0.4 M-NaCl for extraction of nuclei), pH 7.8 at 4°C. Buffer B comprised 10 mM-NaH $_2$ PO $_4$ /NaOH, 10% (v/v) glycerol, 0.1 mM-EDTA, 1 mM-DTT, 25 mM-NaCl, pH 7.8 at 4°C, $I = 0.05$. Buffers to give values of pH and I required contained essentially 50 mM-NaH $_2$ PO $_4$ /NaOH (pH 6–8) or 50 mM-Tris/HCl (pH 7.5–10), 10% (v/v) glycerol, 0.1 mM-EDTA, 1 mM-DTT and x M-NaCl, x being computed from standard values of pK for H $_2$ PO $_4^-$ and TrisH $^+$ at 4°C, corrected for ionic strength (I) effects by the appropriate value of $A \cdot I^{1/2}/(1 + I^{1/2})$ [the constant A (=0.5) is that defined by Debye & Hückel (1923)]. The validity of this method was checked by measurements of pH of standard solutions of NaH $_2$ PO $_4$ /Na $_2$ HPO $_4$ + NaCl and of Tris/HCl + NaCl.

Preparation of rat liver nuclear extract

This was as previously described (Wilson *et al.*, 1984), but an additional wash of the crude homogenate with buffer A was introduced before the Triton X washing; this

Abbreviations used: T_3 , L-tri-iodothyronine; T_4 , L-thyroxine; R, solubilized nuclear receptor; K'_A , apparent association constant; B_{max} , total receptor concentration; DTT, dithiothreitol.

Fig. 1. Specific and non-specific binding of T_3 to its solubilized nuclear receptor

All data reported in the present paper have been validated as in this example of binding at pH 8.20 and with total protein = 0.95 mg/ml. The line $\blacktriangle$ — $\blacktriangle$, relating non-specific binding, b , of [125 I] T_3 and equilibrium free hormone concentration F ($F = H - B$, where H = total concentration of [125 I] T_3 in the presence of a 2000-fold excess of T_3), evaluates the non-specific binding coefficient, k . The coefficient was used to calculate the specific binding concentration, $B = (B_t - kH)/(1 - k)$, and hence $F = H - B$. In order to show that there is only one set of non-interacting binding sites, B is plotted ($\odot$) against $\log F$, as recommended by Klotz (1982). The sigmoid curve and approach to saturation confirm the assumption, so that a Scatchard plot of B/F against B may be confidently used to evaluate the association constant, K'_A , and the total concentration of binding sites, $B_{\max}$. ($K'_A = 22.7 \text{ nM}^{-1}$, $B_{\max} = 0.58 \text{ nM}$). From these values the line (----) was generated by the relation $F = B/K'_A(B_{\max} - B)$.

reduces possible contamination by hormone-binding components in plasma. The crude nuclear extract was first dialysed against buffer B, with rejection of insoluble material, and further dialysed for 18 h against the buffer of appropriate pH and I at a buffer/nuclear extract ratio of 100:1. Routinely the pH values of the dialysing solutions were measured, but selected samples of dialysed nuclear extract were checked.

Analytical method

Samples (0.5 ml) of nuclear extract were charged with appropriate volumes of [125 I] T_3 or of T_3 (0.1 mg/ml) to give 2000-fold [125 I] T_3 and added before the [125 I] T_3 for samples for non-specific binding assays. After 18 h at 4°C the samples were analysed on 1.25 ml columns (20 mm $\times$ 9 mm) of Sephadex G-25 (fine grade), prewashed with the appropriate buffer. Run-off and wash by 0.75 ml of buffer recovered > 95% of applied nuclear extract. The elution pattern was checked at all values of pH and I used and found not to vary.

Non-specific binding

The non-specific-binding coefficient, k , was evaluated as described in the legend to Fig. 1, with usually six points in the range of H (total hormone concn.) = 0.5–3.0 nM

[125 I] T_3 . As Fig. 1 shows, there was no tendency to saturation even at 6 μM - T_3 + [125 I] T_3 . The value of k/mg of total protein was found to be 0.010–0.014 over 12 preparations.

Specific binding

The total binding, B_t , and the non-specific-binding coefficient, k , have been used to find the specific binding B (fmol/sample) (Fig. 1 legend), and hence F , the equilibrium free-hormone concentration. The hormone-receptor parameters K'_A and $B_{\max}$ were evaluated by using the method of Scatchard (1949) on the B and F data, with 8–12 points (see Fig. 1 legend) over an appropriate range of total hormone concentration.

Ionization constants of thyroid hormones

Apart from an unsubstantiated statement by Lardy (1955), the only reported ionization constants for the 4'-OH groups of T_3 and T_4 are from Gemmill (1955): pK' (T_3) = 8.45; pK' (T_4) = 6.73, the temperature being unspecified, but probably $\sim 20^\circ\text{C}$ and at $I = 0.04$. Correction (see 'Buffers' above) to $I = 0.6$, at which most of our measurements have been made, gives $pK' = 8.3$, but, as no temperature-coefficient data are available, even for analogous compounds, no further correction has been made. Jorgensen (1978b), discussing the data reported by Greenstein & Winitz (1961) on dibromo- and di-iodo-tyrosines, suggests that pK' of the α -amino group of T_3 and T_4 may be in the range $pK' 7.6$ –7.8.

Protein concentrations

Protein concentrations were measured by the method of Warburg & Christian (1941) by using the A_{280}/A_{260} ratio.

RESULTS AND DISCUSSION

Reproducibility of nuclear-extract parameters and the effect of aging

Subsequent to dialysis of the extract against buffer B the preparations were diluted to 1 mg of total protein/ml. Values for $B_{\max}$ of 0.6 nM ($\pm 15\%$) were found with $K'_A = 7.7$ –25 nM^{-1} (eight preparations), approx. 14 nM^{-1} (five preparations) being the most usual value. These measurements were made after 2 days from preparation; after a further 2–5 days, K'_A decreased (e.g. 12.5 $\rightarrow$ 9.0 nM^{-1}), whereas $B_{\max}$ diminished by up to 20%. Evidently comparable data could only be obtained by simultaneous measurements of K'_A and $B_{\max}$ on a single preparation under various conditions of pH or of I . It may be noted that, for a purified receptor preparation, Apriletti *et al.* (1981) have reported $K'_A = 19.0 \text{ nM}^{-1}$.

Effect of ionic strength on the binding of T_3 to nuclear receptor

As the crude nuclear extract was made at pH 7.8, $I = 0.45$, and subsequently dialysed against buffer B, pH 7.8, $I = 0.05$, with rejection of the insoluble protein (Ricketts *et al.*, 1980), it is inevitable that reduction of the pH below pH 7.8 will result in further precipitation of both total protein and receptor. In order to circumvent this situation it is necessary that the ionic strength is increased at pH < 7.8, and it was found that $I = 0.6$ would retain all material in solution at pH 6.0. At lower pH values, aggregation was apparent visibly and

markedly increased A_{280} and A_{260} values were observed. To test the effect of I on T_3 -receptor binding, samples were dialysed against phosphate buffers, pH 7.8, and with $I = 0.05, 0.10, 0.20, 0.30, 0.40, 0.50$ and 0.60 , and then charged with 0.32 nM - $[^{125}\text{I}]\text{T}_3$. Analysis for total binding, expressed as a percentage of total hormone, gave the following results: 48.1%; 50.2%; 50.6%; 52.6%; 52.3%; 53.4%, 54.8%. This small change of binding was confirmed by simultaneous evaluations of K'_A : 7.7 nM^{-1} ($I = 0.05$); 7.7 nM^{-1} ($I = 0.6$) and corresponding values of B_{max} : 0.65 and 0.69 pmol/mg of total protein. Evidently there is no large change in T_3 -receptor-binding characteristics at the higher ionic strength, so that measurement of changes in binding over a range of pH values should be valid irrespective of ionic strength. Eberhardt *et al.* (1979) reported that the total binding of $[^{125}\text{I}]\text{T}_3$ to their initial chromatin extract ($I 0.2$, pH = 7.6) from rat liver nuclei substantially decreased on increasing I . As both the values of $K'_A = 2.7 \text{ nM}^{-1}$ and B_{max} = 0.3 pmol/mg of total protein were much lower than those reported here, it is possible that the use of methods for nuclear extract preparation and buffer compositions considerably different from those used in the present work produced a modified form of receptor that is susceptible to the effect of ionic strength.

T_3 -receptor binding: pH profile

The variation of specific binding of T_3 to receptor with pH is shown in Fig. 2(a). Generally it resembles that reported by Latham *et al.* (1976) both in the large increase of binding beyond pH 6 and in the position of optimal binding between pH 7.1 and 7.9. The shoulder on the curve between pH 8.5 and pH 8.9, however, is not shown by the diagram in Latham *et al.* (1976), but could be the consequence of different methodologies. The reality of the phenomenon is commented on further in the following section. The variation of non-specific binding (Fig. 2b) suggests that, at lower pH values, at which the nuclear proteins are likely to be approaching their isoionic states, the effectively neutral T_3 binds strongly, perhaps by dispersion forces. Increase of pH leading to a more negative character of proteins and T_3 brings about a marked decrease in binding due to electrostatic repulsion. Although it is known, from the K'_A and B_{max} values reported in the foregoing sections, that charging of receptor with 3 nM - $[^{125}\text{I}]\text{T}_3$ will give 97% occupancy at the pH of the optimal binding, at lower or higher pH values, with the possibility of changes in both K'_A and B_{max} , a similarly high occupancy may not be effected and the fall-off of binding towards pH 6 or pH 10 (Fig. 2a) may be exaggerated. For these reasons, and because of the variation of K'_A and B_{max} from one preparation to another and with aging in a single preparation, K'_A and B_{max} at different pH values have been measured simultaneously for a single preparation.

Variation of K'_A and B_{max} with pH

The first two columns of Table 1 show the experimental data from which it appears that both B_{max} and the association constant, K'_A , are dependent upon pH. If the actual species of hormone and receptor which combine to form the complex (R^0T^0) are designated as T^0 and R^0 , then $K_A = [\text{R}^0\text{T}^0]/[\text{R}^0][\text{T}^0]$ is defined by the standard free energy change: $\Delta G^0 = -RT \ln K_A$. The pH-independence of ΔG^0 necessitates a similar independence of K_A . The experimentally determined $K'_A = [\text{R}^0\text{T}^0]/$

Fig. 2. Specific (a) and non-specific (b) binding of $[^{125}\text{I}]\text{T}_3$ to solubilized nuclear receptor preparations as a function of pH

Appropriate volumes of three nuclear extract preparations (0.74, 0.74 and 1.0 mg of protein/ml) were dialysed against 100-fold volumes of 50 mM-phosphate or Tris buffers having $I 0.6$ (see the Materials and methods section). Duplicate samples were charged with 3 nM - $[^{125}\text{I}]\text{T}_3$ for measurement of total binding or with 3 nM - $[^{125}\text{I}]\text{T}_3 + 6 \mu\text{M}$ - T_3 for non-specific binding, subsequent analysis (see the Materials and methods section), allowing evaluation of the specific binding by direct subtraction; duplicates were within 3% of each other. As the maximum specific binding differed for the three preparations (0.48, 0.50 and 0.78 nM), the specific binding at the various pH values is expressed as a percentage of the maximum binding to render the data consonant. For the same reason, the non-specific-binding data are presented as k/mg of protein (for definition of k , see the legend to Fig. 1). The ringed points (⊙) indicate coincidence of duplicate values.

$[\text{R}^0][\text{T}^0 + \text{T}_1 + \dots + \text{T}_n]$, in which $[\text{T}^0], [\text{T}_1], \dots, [\text{T}_n]$ are the concentrations of the various ionic forms of the hormone determined by the appropriate pK' and pH values. Taking the simplest case, that there are no ionizable groups in the receptor site, so that $[\text{R}^0]$ is not pH-dependent, and putting $[\text{T}^0] =$ concentration of the 4'-OH form of T_3 with the amino acid terminus in a zwitterionic form: $K'_A = [\text{R}^0\text{T}^0]/[\text{R}^0][\text{T}^0 + \text{T}_1]$, and for the ionization of T^0 , $K' = [\text{T}_1][\text{H}_3\text{O}^+]/[\text{T}^0]$, $K'_A = [\text{R}^0\text{T}^0]/[\text{R}^0][\text{T}^0](1 + K'/[\text{H}_3\text{O}^+]) = K_A/(1 + K'/[\text{H}_3\text{O}^+]) = K_A/[1 + \text{antilog}(pH - pK')]$. As explained in

Table 1. Binding parameters of T_3 to its receptor as a function of pH

$B_{\max}$ and K'_A are the experimentally determined quantities. K_A^* is the apparent association constant obtained from K'_A by correcting for pH and the ionization of the 4'-OH of T_3 . K_A is the pH-independent association constant derived from K_A^* by correcting for pH and the ionization of a putative acidic group, the pK' of which was extracted from the data by the method explained in Fig. 3.

pH	$B_{\max}$ (pmol/mg of total protein)	K'_A (nM^{-1})	K_A^* (nM^{-1})	K_A (nM^{-1})
6.36	0.47	1.63	1.65	30.3
6.96	0.69	5.5	5.75	30.8
7.55	0.71	12.6	14.8	31.4
8.12	0.68	14.4	23.9	31.1
8.99	0.46	5.0	29.5	30.7

the Materials and methods section, a provisional pK' of 8.3 is assumed from which the values of K_A^* (Table 1) are calculated. Evidently K_A^* is pH-dependent and, therefore, $\Delta G^* = -RT \ln K_A^*$ likewise, indicating that a further ionization process is involved. Inspection of the data (Fig. 2a; Table 1) suggests that, because binding increases from lower to higher pH values, the generation of an aprotic form of either the hormone or receptor site could be involved. Again taking the simplest situation, it is noted (see the Materials and methods section) that the pK' of the α -amino group may be ~ 7.8 , so that the ionization of $\alpha\text{-NH}_3^+$ to $\alpha\text{-NH}_2$ could be involved: $T^0 = 4'\text{-OH}$, $\alpha\text{-NH}_2$; $T_1 = 4'\text{-O}^-$, $\alpha\text{-NH}_2$; $T_2 = 4'\text{-OH}$, $\alpha\text{-NH}_3^+$; $T_4 = 4'\text{-O}^-$, $\alpha\text{-NH}_3^+$. As the ionization constant of the $\alpha\text{-NH}_3^+$ is only supposed, then designating it as pK' and proceeding as in the foregoing arguments: $K'_A = K_A/[1 + \text{antilog}(\text{pH} - 8.3)](1 + \text{antilog}(pK' - \text{pH})) = \beta \cdot K_A$. By computing values of β for various values of pK' and plotting K'_A against β , it is shown (Fig. 3) that pK' is uniquely equal to 7.6 and, by insertion of this into the equation above, leads to a pH-independent K_A (Table 1). The average value of K_A is $30.9 \times 10^9 \text{ M}^{-1}$ and stems from $\Delta G^0 = -55.6 \text{ kJ mol}^{-1}$ at 4°C ; the maximum value of β will be at a $\text{pH} = (8.3 + 7.6)/2 = 7.95$, $K'_A = 14.7 \text{ nM}^{-1}$.

The following considerations, however, qualify these calculations. (a) The $pK' = 8.3$ for the 4'-OH ionization of T_3 is probably in error at 4°C , and a change in this value will lead to change in the pK' for the supposed ionization of the $\alpha\text{-NH}_3^+$; e.g. pK' (4'-OH) = 8.1, then pK' ($\alpha\text{-NH}_3^+$) = 7.75, the experimental data fitting equally well but giving $K_A = 42.2 \times 10^9 \text{ M}^{-1}$.

(b) The explanations and calculations advanced here relate only to K_A and therefore the loss of sites, evident from the data on $B_{\max}$ (Table 1) at lower and higher pH values would arise from change in receptor site configuration as the outcome of local or overall changes in the receptor protein conformation [but see (c) below]. In particular, the consonance of the K'_A value at pH 8.99 (Table 1) with the data at lower pH values suggests that the shoulder in the hormone binding/pH profile [Fig. 2(a)] is due to a sudden deletion of sites from pH 8.0 to 8.5. Measurements on another preparation at pH 8.2

Fig. 3. Evaluation of the putative ionization constant pK' of an ionizable group in the T_3 -receptor site region

The values of K'_A in Table 1 are used. The pH-dependent coefficient, β (see the text) of the intrinsic association constant, K_A , has been evaluated for the range of pK' values shown at the top of the diagram and with the pH values (corresponding to the K'_A values) shown to the right of the diagram. To avoid clutter, the reflexion of the plots, from the maxima of $K'_A = 14.41$ ($\text{pH} = 8.12$) towards the set of values for $K'_A = 5.00$ ($\text{pH} 9.00$), is indicated by the arrows pointing to the β -values in the range $\beta = 0.15$ for $pK' = 8.0$ to $\beta = 0.17$ for $pK' = 7.2$. Only $pK' = 7.6$ allows the required relationship $K'_A = \beta \cdot K_A$, the reflexion from $K'_A = 14.41$ to $K'_A = 5.00$ being co-linear. The slope yields $K_A = 30.9 \times 10^9 \text{ M}^{-1}$ uniquely as the pH-independent association constant.

and 8.4 both gave similar K'_A values, but with a 40% decrease in $B_{\max}$.

(c) The interpretation that has been made of the data of Table 1 may not be unequivocal, as the following considerations show: should the acidic group, which is identified as the α -amino group of T_3 , be located in the receptor site, then the concentration of unoccupied receptor will become pH-dependent, i.e. the term $[R^0]$ in the denominator of the expression for K'_A requires correction for the unobserved non-binding (acidic) form. Denoting the ionization constant as pK' , then the equation for K'_A becomes identical with that derived for the double ionization of T_3 . There is, however, a subtle difference between the two models: the experimental value for equilibrium unoccupied receptor concentration = $[R^0 \cdot T^0]_{\max} - [R^0 \cdot T^0]$, so that if both are multiplied by $[1 + \text{antilog}(pK' - \text{pH})]$, then $B_{\max} = [R^0 \cdot T^0]_{\max}$ will be a function of pH. Because pK' will necessarily appear as 7.6 for this model, as for that based on the double ionization of T_3 , then if B^* = absolute concentration of receptors: $B_{\max}(\text{pH } 6.36) = 0.054 B^*$, $B_{\max}(\text{pH } 7.55) = 0.471 B^*$, i.e. there should be about 8.7-fold increase of $B_{\max}$. The data (Table 1) show, however, that it is only about 1.5-fold; furthermore, at pH 7.6, only half of the sites would be available and $B_{\max}$ should increase continuously, whereas, in fact, it decreases (Table 1).

(d) Bolger & Jorgensen (1980) report that the affinity of 3,3',5-tri-iodothyropropionate for solubilized receptor is some 2.3-fold greater than for T_3 , and conclude that the T_3 α - NH_3^+ (assumed to have the same $pK' = 9.1$ as in tyrosine) is inhibitory to the formation of an electrostatic linkage of the α - CO_2^- to a cationic group in the receptor site. The probable effect of such a cationic group would be to enhance the ionization of α - NH_3^+ , i.e. to lower the pK' . This would seem a more likely explanation of the value $pK' = 7.6$ arising from the calculations here, than that the lowered pK' is a result of the composition and structure of T_3 itself (see the Materials and methods section).

In conclusion, it seems possible that the variation of K'_A with pH is the result of dual ionization of T_3 , the hormone binding in the 4'-OH, α - NH_2 , α - CO_2^- form. The changes in B_{max} are due to the effect of pH modification of the receptor protein conformation, changing the proportion of effective receptor sites amongst all possible sites.

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