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Note

Behavior of iodothyronines in an isoelectric focusing system

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Isoelectric focusing studies of kidney and liver cytosol proteins that bind thyroid hormone[1,2] required the definition of the behavior of unliganded thyroxine (T4) and triiodothyronine (T3) in the LKB (Rockville, Md., U.S.A.) Ampholine system. Results of these measurements of isoelectric points (p*I*) of T4 and T3 are reported here, together with determinations in Ampholine solutions of p*K* of the phenolic hydroxyl group of the unliganded iodothyronines.

METHODS

Radioactive iodothyronines

[^{125}I]T4 and [^{125}I]T3 or [^{131}I]T3 were obtained from Abbott Laboratories (North Chicago, Ill., U.S.A.). Their purity was verified in a silica gel TLC system (1-butanol–acetone–methanol–2 *N* ammonia, 3:5:1:1) that satisfactorily resolves iodide, T3 and T4. Labeled hormones were chromatographed with carrier. Iodide and iodothyronines were identified by characteristic R_F values after radioautography of the TLC sheets. The [^{125}I]T4 and [^{125}I]T3 contained less than 1% radioiodide. There was 4% radioiodide in the [^{131}I]T3. The T3 preparations contained no radioautographically demonstrable T4.

Isoelectric focusing system

Samples of radioactive hormones were subjected to focusing in an LKB 8101 analytic column at 5° for 72 h at 4 W. The ampholyte–sucrose gradient solutions were constructed as described by the manufacturer. Hormone ($3–5 \cdot 10^{-10}$ moles) was placed in either gradient fraction 12 or 18 prior to focusing. At the end of focusing, 2.5-ml fractions were collected. Radioactivity in each fraction was quantitated in a well-type scintillation counter. Measurement of pH was made with a Beckman combination electrode and Orion meter. In selected experiments, unlabeled L-T4 (Calbiochem, Los Angeles, Calif., U.S.A.) was added to tracer in gradient fraction 12 prior to focusing. At the end of focusing, eluted radioactivity peaks were analyzed by TLC

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and radioautography for content of T4, T3 and iodide. Each fractionation was carried out at least twice.

pK values of iodothyronine hydroxyl groups in ampholyte: molar extinction coefficients

Using the method of Gemmill[3], we measured molar extinction coefficients, ε, of T4 and T3 when the latter were solubilized in previously focused ampholyte. Calculation of ε was made from the formula,

$$\varepsilon = \frac{A \cdot m}{l \cdot g}$$

where A = absorbance (nm), m = molecular weight of the iodothyronine, l = length of spectrophotometric cell (cm) and g = concentration of the iodothyronine (g/l). In these studies, unlabeled L-T4 or unlabeled L-T3 (Sigma, St. Louis, Mo., U.S.A.) was added to aliquots of previously focused ampholyte of pH 1.2 and 11.8, as well as intermediate pHs (4.0–9.0 range). The concentration of L-T4 in ampholyte solution was 1.5 mg/10 ml and that of T3 was 2.0 mg/10 ml. Absorbance was measured in a Beckman DB G spectrophotometer. Focused ampholyte at each pH to which no hormone had been added served as blank.

Calculated pI values for iodothyronines

Estimates of the isoelectric points of T4 and T3 were also calculated from the formula,

$$pI = (pK_1 + pK_2)/2 \quad \text{(see ref. 4).}$$

In the case of the iodotyrosines and iodothyronines, pK_1 refers to the carboxyl group, and pK_2 represents the phenolic hydroxyl group. Values for the respective dissociation constants of the ionizable groups of iodothyronines have been published by Gemmill[3] and others[5]. For T4 and T3, $pK_1 = 2$; pK_2 values are 6.7 (T4) and 8.4 (T3).

RESULTS

Isoelectric focusing of labeled T4 and T3

As shown in Fig. 1, radioactive T4 and T3 focus at pH 3.8–4.3 (peak, 4.3) and 4.6–5.3 (peak, 5.1), respectively. The point of introduction of iodothyronines into the column did not influence these results. Radioactivity eluted after focusing was shown chromatographically to have the R_F of the respective iodothyronine applied to the column. Addition of unlabeled T4 to focusing experiments in which [^{125}I]T4 was tracer resulted in the emergence of peak radioactivity again at pH 3.8–4.3.

Measurement of the pK values of the phenolic hydroxyl groups of T4 and T3 in prefocused Ampholine

From ε determined at 330 nm (T4) and 320 nm (T3), a plot (Fig. 2) of ε *vs.* pH was made for T4 and T3 in previously focused Ampholine of various pHs. The average of three molar extinction measurements at pH 1.2 and pH 11.8 for each iodothyronine was then applied to the ε *vs.* pH plot and the pK of the phenolic

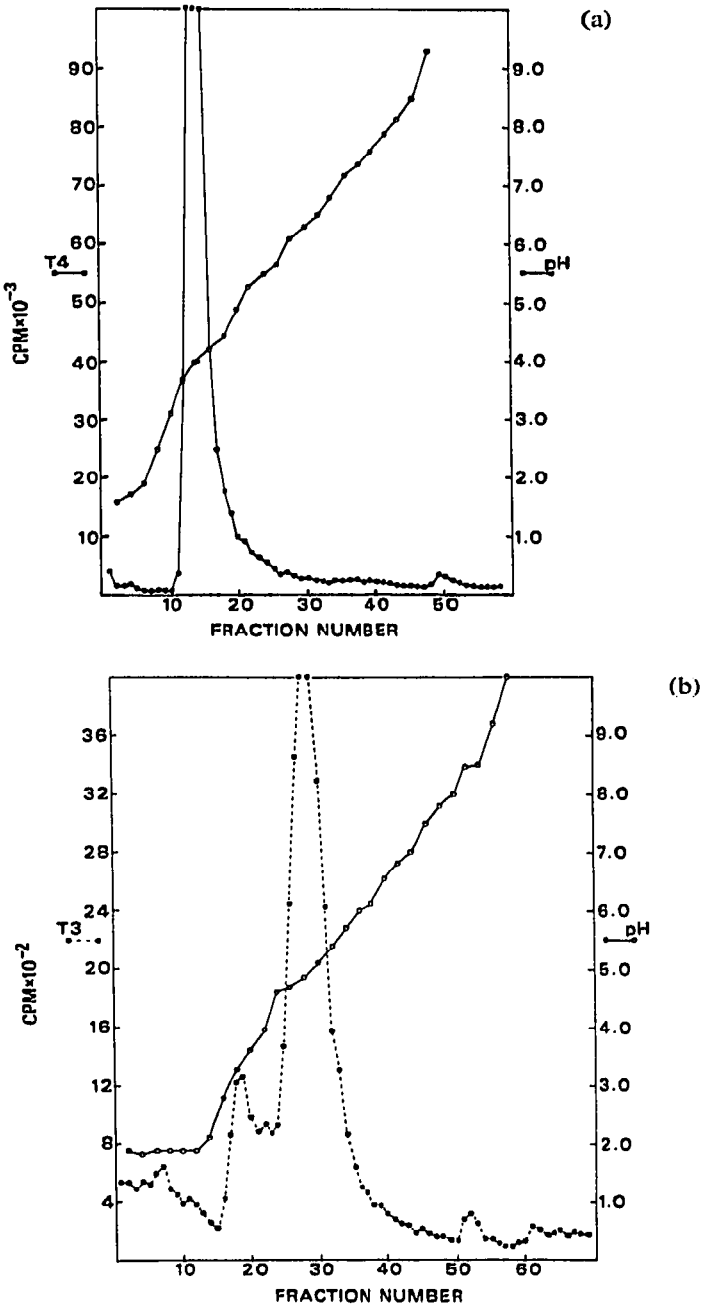

Fig. 1. (a), Isoelectric focusing of [^{125}I]T4 in the LKB Ampholine system (pH 3–10 Ampholine). [^{125}I]T4 ($3.6 \cdot 10^{-10}$ moles) was introduced into gradient fraction 12 prior to focusing of LKB 8101 column at 5° for 72 h at 4 W. T4 emerges in the pH range 3.8–4.3 (peak, 4.3). TLC of radioactivity from this peak revealed only T4. (b), Isoelectric focusing of [^{131}I]T3 in the LKB Ampholine system. Radioactive T3 ($4.3 \cdot 10^{-10}$ moles) was introduced into gradient fraction 12 prior to focusing under the same conditions as in (a). T3 emerges in the pH range 4.6–5.3 (peak, 5.1), as verified by TLC of this peak. Material emerging as a radioactivity peak in fractions 18–20 in this experiment is unidentified.

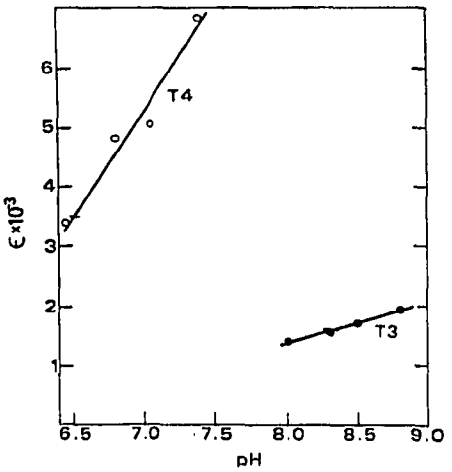

Fig. 2. Determination of phenolic hydroxyl p*K*s of T4 and T3 in focused ampholyte, using ε. Determination of ε is described in the text. For T4, absorbance was measured at 330 nm, for T3, at 320 nm. Lines that cross the curves represent average of acidic and basic molar extinction measurements; at these points pH = p*K*. Determined under these conditions, the p*K* of T4 is 6.6 and that of T3 is 8.4.

hydroxyl group (pK_2) estimated. Results of these experiments are shown in Table I. Measurements of ε and pK_2 for T4 in the present study are in close agreement with those reported by Gemmill[3]. However, the ε for T3 was considerably below that reported by Gemmill (Table I); the ε *vs.* pH slope was systematically flattened (Fig. 2), compared with results obtained in phosphate or borate buffers[3], but the estimate of pK_2 for T3 made in Ampholine agreed with that of Gemmill.

TABLE I

PHENOLIC HYDROXYL p*K* (pK_2) AND ε OF THYROXINE AND TRIIODOTHYRONINE

Measurements of ε in the present study were made in prefocused Ampholine of various pHs (see text); determinations of ε in the study of Gemmill were conducted in phosphate or borate buffer.

	pK_2		ε	
	Present study	*Gemmill*[3]	*Present study*	*Gemmill*[3]
Thyroxine	6.6	6.73	3723	3799
Triiodothyronine	8.4	8.45	1608	2705

DISCUSSION

The behavior of small charged molecules in the LKB Ampholine system —a system intended for fractionation of large (protein) molecules— has not been extensively studied. Using the LKB system, we have measured the isoelectric points of T3 and T4 to be 5.1 and 4.2, respectively. These direct measurements of p*I* for T3 and T4 are in agreement with values (5.1 and 4.4) calculated from the dissociation constants of the carboxyl and phenolic hydroxyl groups of these iodothyronines. Thus, assessments of p*I* for these low-molecular-weight ampholytic hormones can be made in the LKB Ampholine system, suggesting a lack of interaction of carrier

ampholyte with the charged groups on T3 and T4 (ref. 4). Support for this conclusion was developed in dissociation studies in which we found that Ampholine ampholytes do not affect significantly the ionization of the phenolic hydroxyl group (pK_2) as determined in absorption spectra analyses. This functional group and the iodothyronine carboxyl group are primary determinants of p*I*[4,5].

The p*I* value for T4 is comparable with that reported for diiodotyrosine (4.29)[5]. Thus, there is a wide $pI - pK_1$ difference* for T4 as well as T3, suggesting unsuitability of such compounds, themselves, as carrier ampholytes[6].

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* The pK_1 of both iodotyrosines and iodothyronines is *ca.* 2 (ref. 4), so that the $pI - pK_1$ differences are greater than 2.