(7) Baker, B. R., "Design of Active-Site-Directed Irreversible Enzyme Inhibitors. The Organic Chemistry of the Enzymic Active-Site," John Wiley & Sons, Inc., New York, N.Y., 1967, chap. IV.
(8) Baker, B. R., *J. Pharm. Sci.*, **53**, 347(1964).
(9) *Reference 7*, chap. I, VIII–XII.
(10) Fujita, T., Iwasa, J., and Hansch, C., *J. Am. Chem. Soc.*, **86**, 5175(1964).
(11) Baker, B. R., and Rzeszotarski, W., *J. Med. Chem.*, **10**, November(1967).
(12) *Reference 7*, chap. VIII.
(13) Baker, B. R., Lee, W. W., and Tong, E., *J. Theoret. Biol.*, **3**, 459(1962).
(14) Baker, B. R., Santi, D. V., Coward, J. K., Shapiro, H. S., and Jordaan, J. H., *J. Heterocyclic Chem.*, **3**, 425(1966).
(15) Baker, B. R., *J. Med. Chem.*, **10**, 297(1967).

Method to Estimate Small Amounts of L-Triiodothyronine in D-Triiodothyronine

By IRVING B. EISDORFER and ALEX POST

One of the requirements for the use of D-triiodothyronine as a serum cholesterol-lowering agent is that it be essentially free of its optical isomer, L-triiodothyronine. A method for estimating small amounts of L-triiodothyronine in D-triiodothyronine is described. After removing any free triiodothyroacetic acid that may be present, the sample is reacted with stereospecific L-amino oxidase (from snake venom) to oxidize the L-triiodothyronine to triiodothyropyruvic acid. The triiodothyropyruvic acid is degraded to triiodothyroacetic acid, extracted, and chromatographed on paper to separate it from the unoxidized D-triiodothyronine. The final quantitative estimation is made by comparing the size and intensity of the colored spot, as visualized by an appropriate spray reagent, with those obtained from known mixtures of L- and D-triiodothyronine carried through the entire procedure. As little as 0.05 per cent L-triiodothyronine could be estimated in the various samples evaluated.

L-TRIIODOTHYRONINE (L-T3), the most potent of the naturally occurring thyroid hormones, is both a powerful calorigenic agent and an active serum cholesterol-lowering agent. Its use for the latter purpose is rather limited, however, since it cannot be used safely in patients suffering from angina pectoris and related diseases. In contrast, its optical antipode, D-triiodothyronine (D-T3), is potentially a very valuable agent for this purpose since it retains much of the serum cholesterol-lowering properties of the natural isomer without the liability of the latter's hypermetabolic activity.

Since L-T3 is such a potent calorigenic agent, its presence in any appreciable amount in lots of the D-T3 destined for clinical trials as a serum cholesterol-lowering agent would badly confound the results. For this reason, heroic efforts were made to limit the amount of L-T3 present in test lots of D-T3 to very small values (of the order of less than 0.2%). In this connection a procedure was required that would be capable of estimating the L-T3 content of samples of D-T3 in the 0–1% range. Since the analytical procedure was designed primarily for acceptance or rejection purposes, sensitivity was deemed more important than great accuracy, and values accurate to one or at most two significant figures were considered satisfactory.

Direct measurement of optical rotation as a means of determining the amount of L-T3 in samples of D-T3 proved to be rather too insensitive for this purpose. With a specific rotation of $-24.5°$ for D-T3, it was not found possible to reliably distinguish samples that contained 0.5% L-T3 from those that contained less than 0.1%. In any case, it was not possible by this procedure to detect 0.2% of L-T3 in samples of D-T3.

The most widely used procedure for determining small amounts of an L-amino acid in the presence of a large amount of its enantiomorph is by stereospecific enzymatic degradation with micromanometric measurement of gas taken up or released (1–5). While this procedure is reported to be capable of estimating as little as 0.1% of an L-amino acid in the presence of its D-isomer, the authors were not able to reliably detect less than 2% of L-T3 in samples of D-T3 manometrically. This may be due to the poor aqueous solubility of the triiodothyronines which prevented the use of an adequate size sample.

Although micromanometric procedures for determining L-amino acids in the presence of the D-isomers were not sensitive enough for the purposes of this work, stereospecific oxidation with an enzyme such as L-amino acid oxidase appeared to be a promising approach to the solution of this problem, provided a sensitive procedure could be

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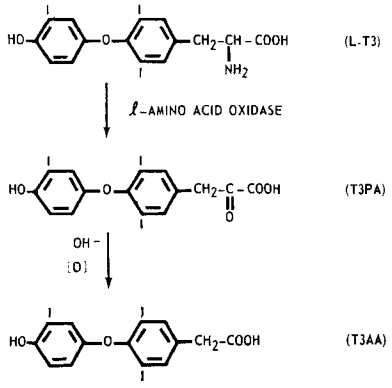
Conversion of L-T3 to T3AA



Scheme I

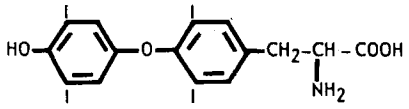
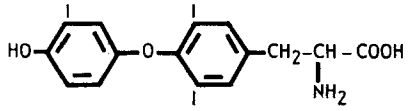
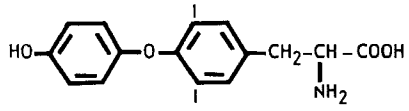
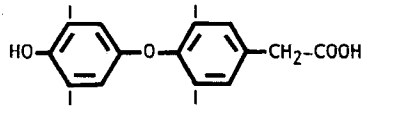
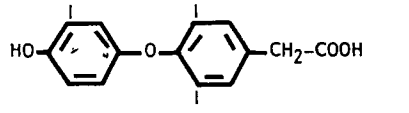
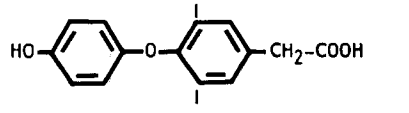
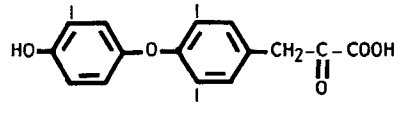
devised for estimating the very small amount of reactants that disappear or products that form. A number of workers (6–10) have reported that L-amino acid oxidase from snake venom oxidizes the L-iodothyronines to a mixture of the corresponding iodothyropyruvic and iodothyroacetic acids. The latter appears to be a degradation product of the former. Since procedures were at hand for separating and estimating small amounts of the various iodothyronines as well as their desamino derivatives by paper chromatography, this was explored as possible means for estimating the product of the oxidation reaction.

The method that was evolved and is reported here utilizes L-amino acid oxidase from snake venom to stereospecifically oxidize L-T3 in the sample of D-T3 to T3PA and T3AA. These are separated from D-T3 by solvent extraction and heated with alkali to convert the T3PA to T3AA (Scheme I). The L-T3 content of the sample is estimated from the amount of T3AA found after chromatographic separation of the digest extract. (The iodothyronines and derivatives are listed in Table I with their respective structures and abbreviations.)

Synthetic samples of D-T3 often contain small amounts of T3AA so it is essential that this material be removed before analysis for L-T3 lest the L-T3 content appear erroneously high. To this end a clean-up step has been incorporated in the procedure. The procedure as written also includes a chromatographic method for estimating the amount of T3AA present in the D-T3 sample. Since it is often valuable to know the D-T2 and D-T4 content of D-T3 samples and the same chromatographic solvent system is used as is used for the estimation of T3AA, a general procedure for this purpose is included also.

While the procedure described here was designed for estimating the L-T3 content of D-T3, it also worked equally well for the determination of L-T2 in D-T2, that is, for estimating the purity of the intermediate. There is good reason also to believe that it works satisfactorily for the determination of L-T4 in D-T4, as well as for the determination of L-T4 in D-T3, *etc.*

TABLE I—IODOTHYRONINES AND IODOTHYRONINE DERIVATIVES

Tetraiodothyronine 	T-4
Triiodothyronine 	T-3
Diiodothyronine 	T-2
Tetraiodothyroacetic Acid 	T4AA
Triiodothyroacetic Acid 	T3AA
Diiodothyroacetic Acid 	T2AA
Triiodothyropyruvic Acid 	T3PA

PROCEDURE

Chromatography—Prepare a 12 × 18-in. sheet of Whatman 3 MM paper for spotting by drawing light pencil lines connecting the diagonal opposite corners. Also draw a light line through the intersection point of the lines parallel with the long sides of the paper. Finally draw a circle with a $^3/_4$-in. radius around the intersection point of the lines. The junction points of the circle and the lines are the spotting points.

After spotting, thread a 4-in. cotton cord wick (about 2 mm. thick) through a close fitting hole in

the center of the sheet so that about 1/4 in. protrudes above and the rest hangs below. Place the sheet between the sections of a chromatographic chamber made from two 12 × 18 × 3 in. rectangular glass trays with ground rims fitted face-to-face. Allow the wick to rest in a 1 × 3-in. Petri dish placed at the center of the bottom tray.

Equilibrate a mixture of 50 ml. of isoamyl alcohol, 50 ml. of tertiary amyl alcohol, and 100 ml. of 6 *N* aqueous ammonia solution. Transfer all of the lower phase and about 20 ml. of the upper phase to the bottom of the chamber without wetting the paper or the inside of the Petri dish. Allow the paper to come to equilibrium with the solvent vapors for 2 hr., and then start development of the chromatogram by adding about 75 ml. of the upper phase of the solvent mixture to the Petri dish through a small hole in the side of the lower tray with the aid of a long pipet.

Allow the chromatogram to develop until the solvent front reaches the nearest wall of the chamber, then remove and dry in air.

Emerson Reagent (Modified)—*Solution A*—Dissolve 2 Gm. of 4-aminoantipyrine hydrochloride in 2% aqueous sodium carbonate solution. Clarify by shaking with 1% of activated charcoal, filter, and store in a dark bottle in a refrigerator until used. Discard when the solution becomes noticeably yellow.

Solution B—Two per cent aqueous potassium persulfate solution.

Spray the front and back of the chromatogram lightly with solution *A*. Dry in a stream of air at room temperature and then respray with solution *B*. Examine after drying in air at room temperature. Colors are stable.

Venom (L-Amino Acid Oxidase)—Dehydrated *Crotalus adamanteus* venom, obtained from Nutritional Biochemicals Corp., Cleveland, Ohio. Combine the contents of several bottles of venom, mix thoroughly, and standardize as described below. Transfer 10-mg. portions to individual vials, stopper tightly, and store in freezer until needed.

Venom Standardization—Pipet 5-ml. portions of a 0.10 mg./ml. solution of L-T_3 in 2% KOH into a series of beakers each of which contains 300 ml. of 0.1 *M* phosphate pH 7.2 buffer, 0.2 Gm. of KCl, and 7.5 ml. of 95% alcohol. Adjust the pH of each to 7.2 with dilute HCl or KOH. Add 10 mg. of snake venom to the first beaker, 20 mg. to the second, and 30 mg. to the third, *etc.*, then proceed as directed under *L-T3 Content*.

After extracting the product into 0.05 *N* NaOH, transfer to a 50-ml. volumetric flask, and dilute to volume. Determine the ultraviolet absorption spectrum of this solution in a 5-cm. cell over the range 300–360 mμ. Calculate the difference between the absorbance of the solution at 327.5 mμ and that of the point at the same wavelength on a straight line drawn between the points on the curve at 302.5 mμ and 350 mμ.

The smallest amount of venom that gives a maximum absorbance difference at the point chosen is the optimum weight to be used in the analysis.

Free T3AA Content—Dissolve 50 mg. of the D-T3 sample in 25 ml. of 2% KOH solution. Pipet 5 ml. of this solution into a 250-ml. separator containing 100 ml. of 0.2 *N* HCl. Extract three times with 30-ml. portions of dichloromethane (redistilled from anhydrous Na_2CO_3). Backwash the combined dichloromethane extract once with 30 ml. of 0.1 *N* HCl and once with distilled water. Retain the combined aqueous layers for determination of L-T3 content.

Evaporate the dichloromethane to small volume, transfer to a 50-ml. pear-shaped flask, and evaporate to dryness under vacuum. Dissolve the residue in 0.5 ml. of methanol and transfer by repeated spotting and drying under a stream of N_2 gas to a spotting point on a sheet of Whatman 3 MM paper prepared for chromatography. Rinse the flask twice with 0.25 ml. of methanol and transfer each in turn to the same spotting point.

Transfer 10, 20, and 30 mcg. of T3AA reference compound to other spotting points and chromatograph. Spray the dried chromatogram with Emerson reagent. Estimate the amount of T3AA in the sample from the relative size and intensity of the sample spot as compared to those of the corresponding standards.

L-T3 Content—*Enzymatic Oxidation*—Transfer the aqueous layer obtained under *Free T3AA Content* determination to a 600-ml. beaker. Heat the solution to about 37° and bubble air through it until the odor of the dichloromethane is no longer evident. Cool to room temperature and add 1 *N* KOH dropwise with stirring until the solution is approximately neutral to litmus paper. Add 7.5 ml. of 95% ethanol and 200 ml. of 0.1 *M* pH 7.2 phosphate buffer. Adjust to pH 7.2 ± 0.1 by dropwise addition of dilute HCl or KOH if necessary. Mix thoroughly with a mechanical stirrer for 30 min., and filter through Whatman No. 41 paper. Adjust the temperature of the filtrate to 37 ± 1°, add the required amount of L-amino acid oxidase, and bubble a very slow stream of filtered air through the gently stirred solution for 3 hr.

Transfer the solution to a separator, add 10 ml. of concentrated HCl, mix thoroughly, cool to room temperature, and extract 4 times with 40-ml. portions of redistilled dichloromethane. Collect the extracts in another separator, remove the aqueous phase, and break the slight residual emulsion by the addition of anhydrous sodium sulfate. Filter the clear dichloromethane layer into another separator and wash the residual salt mixture with two 15-ml. portions of dichloromethane. Wash the combined dichloromethane extract with 25 ml. of water and discard the aqueous phase. Finally, extract the dichloromethane solution four times with 10-ml. portions of 0.05 *N* NaOH.

Conversion of T3PA to T3AA—Heat the combined alkaline extract in a loosely stoppered vessel for 2 hr. on a steam bath. Cool to room temperature, add 1 ml. of concentrated HCl, and extract 3 times with 15-ml. portions of redistilled dichloromethane. Filter each portion of extract through a small bed of anhydrous Na_2SO_4 supported in a funnel by a small pledget of well-extracted adsorbent cotton. Evaporate the combined filtrates to dryness under vacuum in a 50-ml. pear-shaped evaporation flask.

Estimation of L-T3 Content—Dissolve the residue in 0.5 ml. of methanol and transfer, by repeated spotting and drying under a stream of N_2 gas to a spotting point on a sheet of Whatman 3 MM paper prepared for chromatography. Rinse the flask twice with 0.25-ml. portions of methanol and trans-

fer each in turn to the same spotting point. In a similar fashion transfer the residues from equivalent weight samples of pure D-T3,[1] pure D-T3 that has had 0.2%, and pure D-T3 that has had 0.4% of L-T3 added and carried through the entire procedure starting from *Free T3AA Content* to other spotting points on chromatographic sheet. Transfer to other spotting points a mixture of 10 mcg. each of L-T3 and T3AA as reference points.

Develop the chromatogram, dry thoroughly in air, and spray with Emerson reagent. Estimate the amount of L-T3 in the sample from the size and intensity of the T3AA spot as compared to that of the T3AA spot present in each of the known mixtures carried through the entire procedure.

T2 and T4 Content of D-T3 Samples—Transfer 10 μl. of a 10 mg./ml. solution of the sample dissolved in 2% ammonia in ethanol to one of the spotting points and 20 μl. to another. Transfer 2 μl. of a solution containing 1 mg./ml. each of T-2 and T-4 dissolved in 2% ammonia in ethanol to one of the other spotting points and 4 μl. to another. The sample spots should contain 100 and 200 mcg., respectively, and the standards should contain 2 and 4 mcg. each of T-2 and T-4. Develop the chromatogram. Spray the dried chromatogram with Emerson reagent, or alternatively immerse it in a 0.25% solution of ninhydrin in 1:100 acetic acid–acetone and allow to dry in air at room temperature. Estimate the amount of T-2 and T-4 present in the sample from the sizes and intensities of the spots as compared to those of the appropriate standards (Fig. 1).

RESULTS AND DISCUSSION

Paper Chromatography—Samples of D-T3 could contain D-T2, D-T4, T2AA, T3AA, and possibly T4AA, all of which are intermediates and side reaction products of the synthesis of D-T3. A chromatographic procedure was needed that would separate these compounds from each other. The chromatographic procedure used in this work does indeed accomplish this purpose. (Figs. 2–4.) Detection of all of these compounds at very low levels was accomplished by use of a modification of the Emerson reagent (10) for phenols. As originally described, potassium ferricyanide was used as the oxidant to bring out the color. This caused the colors to appear (red, orange, purple, *etc.*) on a yellow background. By substituting potassium persulfate for the potassium ferricyanide, it was possible to achieve equivalent color production on a white background with resultant increase in sensitivity (Table II).

Under certain circumstances, it was found useful to be able to distinguish the iodothyronines from the iodothyroacetic acids. Ninhydrin immersion proved to be a valuable means for making the thyronines visible without bringing out the desamino acids. Air drying at room temperature produced colors of equal sensitivity as those produced with the Emerson reagent. The latter, however, produced different shades of color with different degrees of iodine substitution while the former, of course, gave purple colors with all of the amino acids used. All of the compounds used in this work were visible even at very low levels under short wavelength U.V. light (256 mμ) which proved useful under certain circumstances. Table II lists the R_f values, colors, and limits of detection for each of the compounds used in this work.

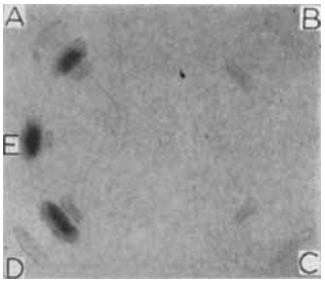


Fig. 1—Chromatogram of small amounts of T2, T4 in T3 sample. Key: A, *100 mcg. T-3 + 2 mcg. T-2 and T-4;* B, *4 mcg. T-2 and T-4;* C, *2 mcg. T-2 and T-4;* D, *100 mcg. T-3 + 4 mcg. T-2 and T-4;* E, *100 mcg. T-3.*

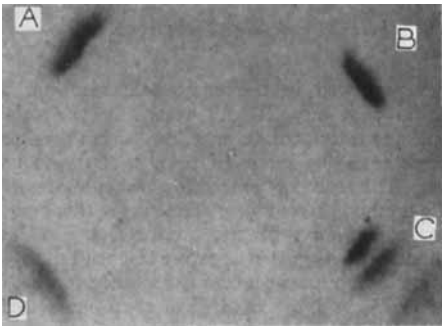


Fig. 2—Chromatogram of T2, T3, T4. Key: A, *10 mcg. T-3;* B, *10 mcg. T-4;* C, *10 mcg. T-2, T-3, T-4;* D, *10 mcg. T-2.*

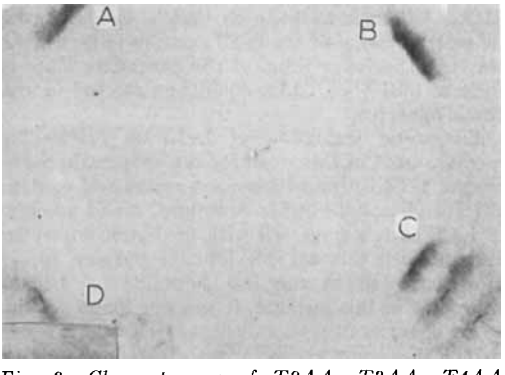


Fig. 3—Chromatogram of T2AA, T3AA, T4AA. Key: A, *10 mcg. T-3AA;* B, *10 mcg. T-4AA;* C, *10 mcg. T-2AA, T-3AA, T-4AA;* D, *10 mcg. T-2AA.*

Estimation of Free T3AA Content—Since final estimation of the amount of L-T3 present in the sample of D-T3 is dependent upon the amount of

[1] D-T3 and T3AA samples used as reference materials in this study were prepared at Smith Kline & French by standard procedures. D-T3 free of L-T3 was prepared by recrystallization of D-T3 samples until no T3AA spot was shown on the chromatogram after being carried through the above procedure. This indicated the presence of less than 0.05% of L-T3 in the D-T3 sample.

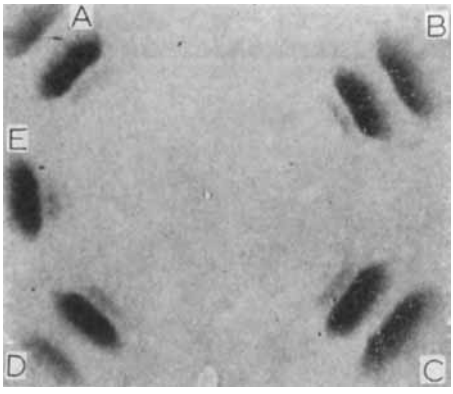


Fig. 4—Chromatogram of T3AA and T3. Key: A, *50 mcg. T-3 + 10 mcg. T-3AA;* B, *50 mcg. T-3 + 20 mcg. T-3AA;* C, *50 mcg. T-3 + 30 mcg. T-3AA;* D, *50 mcg. T-3 + 5 mcg. T-3AA;* E, *50 mcg. T-3.*

T3AA produced in the enzymatic reaction, it is essential that any T3AA present in the original sample (as intermediate or side reaction product of the synthesis) be removed prior to enzyme treatment. The solvent extraction scheme described in the method proved very efficient for this purpose. When tested on synthetic mixtures, the dichloromethane extracts gave a negative test with ninhydrin showing no significant loss of the iodothyronines into the extract, and the final dichloromethane extract gave a negative test with Emerson reagent indicating complete extraction of T3AA in the first two extractions. This was further confirmed by spectrophotometric examination of the two phases.

All of the samples of D-T3 evaluated in this study contained detectable amounts of T3AA (Table III). Although as little as 0.05% of T3AA was detected in some of the D-T3 samples (Fig. 5), this was not the lower limit of the procedure since as little as 0.01% of T3AA could be detected in synthetic mixtures.

Enzymatic Oxidation of L-T3 to T3PA—The medium used in this work for the enzymatic oxidation of L-T3 by snake venom L-amino acid oxidase, pH 7.2 phosphate buffer containing small amounts of KCl, corresponds well with media recommended for use with this enzyme for this purpose (6, 7). However, due to the low solubility of triiodothyronine in this medium, it was not found possible to get enough of the sample dissolved in the medium to achieve anywhere near the desired sensitivity. Reasoning, however, that it was only necessary to retain the L-T3 in solution and not the D-T3, a slow recrystallization step was incorporated in the procedure. This proved to be quite efficient as shown by the good sensitivity of the final procedure and the proportionate recovery of different amounts of added L-T3. The addition of a small amount of alcohol appeared to aid the enzymatic action as well as increase the solubility of the iodothyronines in the medium. The addition of too much alcohol inactivates the enzyme.

Conversion of T3PA to T3AA—Since it was reported that L-T2 was oxidized by snake venom oxidase to T2PA, and the latter partially oxidized to T2AA by peroxides formed by accompanying enzymes, it was presumed that L-T3 reacted the same way. This indeed proved to be the case, so it was not possible to accurately estimate the amount of L-T3 present in a sample until a means could be devised for converting it to one instead of two products.

TABLE III—PER CENT COMPOSITION OF D-T3 SAMPLES

No.[a]	Sample	L-T3	T3AA	D-T2	D-T4
1	GJ-3-254	1	0.05	1	1.5
2	GJ-3-176B	0	0.05	1	5
3	GJ-3-250B	0.75	0.05	1.5	2
4	GJ-3-186	0	0.05	1	<0.5
5	GJ-3-153A	0.1	0.05	1	1
6	PD-262	0	0.05	0.5	1.5
7	PD-102	0.1	0.1	1.5	3
8	AQ-3-5273	1.5	0.3	...	...
9	051-10A	1.5	0.2	...	...
10	RFS-2318-144A	1.5	0.3	...	...
11	O-23W	2	0.9	...	...
12	O-1N	1.5	1.25	...	...
13	O-167	1.5	0.75	...	...

[a] Samples *1–10:* prepared by the Organic Chemistry Laboratories, Smith Kline & French Laboratories, Philadelphia, Pa. Samples *11–13:* obtained from an outside supplier.

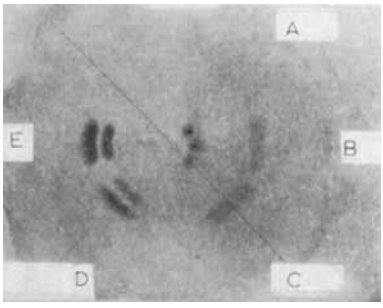


Fig. 5—Chromatogram of free T3AA from D-T3 samples. Key: A, *PD-102;* B, *PD-262;* C, *PD-263;* D, *T3 and T3AA, 10 mcg.;* E, *T3 and T3AA, 20 mcg.*

TABLE II—CHROMATOGRAPHIC CHARACTERISTICS OF IODOTHYRONINES AND DERIVATIVES

		Detection Techniques					
		Emerson Reagent		Ninhydrin Reagent		U.V. Light	
Compd.	R_f	Color	Sensitivity, mcg.	Color	Sensitivity, mcg.	Color	Sensitivity, mcg.
T2	0.47	Orange	1	Blue	1	Purple	1–2
T3	0.36	Red	1	Blue	1	Purple	1
T4	0.28	Purple	1	Blue	1	Purple	1
T2AA	0.60	Red	1	X	X	Purple	1–2
T3AA	0.50	Red	1	X	X	Purple	1
T4AA	0.41	Red	1	X	X	Purple	1

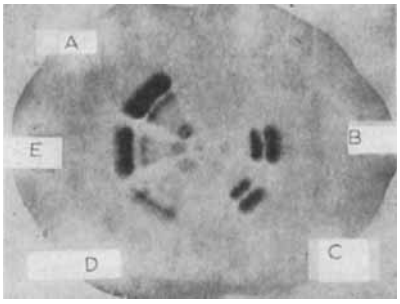


Fig. 6—Chromatogram of T3AA from L-*T3 in* D-*T3 samples, comparison with external T3AA standards. Key:* A, *O-IN;* B, *T3 + T3AA, 20 mcg.;* C, *T3 + T3AA, 10 mcg.;* D, *PD-102;* E, *0–167.*

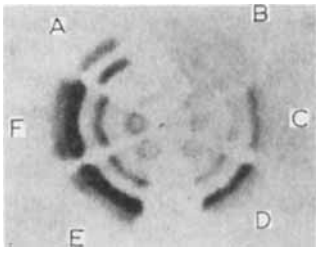


Fig. 7—Chromatogram of T3AA from L-*T3 in* D-*T3 samples, comparison with internal standards. Key:* A, *T3 + T3AA;* B, *PD-262;* C, *PD-262 + 0.25%* L-*T3;* D, *PD-262 + 0.50%* L-*T3;* E, *O-IN;* F, *O-23W(2).*

Since both T3PA and T3AA are strong absorbers in the U.V. and have maxima at slightly different wavelengths, attempts at conversion of one to the other were followed by this means. An initial attempt to prevent oxidation of the T3PA to T3AA by addition of catalase appeared promising but it failed to work reliably so it was discarded. Conversion of T3PA to T3AA by heating with dilute alkali proved to be a more reliable procedure. The appropriate conditions for this conversion were determined by following the reaction spectrophotometrically using known mixtures as well as enzyme digests.

L-T3 in D-T3—When samples of D-T3 containing known added amounts of L-T3 in the range of interest were taken through the procedure, the T3AA spots found on the chromatogram were quite adequate in sensitivity; however, comparison of their size and intensity with those of known amounts of T3AA spotted on the same sheet, showed rather less than complete recovery (Fig. 6). Whether these loses were due to incomplete recovery of L-T3 in the recrystallization step, incomplete oxidation by the enzyme, or to glass surface adsorption, *etc.*, is not known. Attempts to increase recovery by increasing the enzyme ratio proved of no avail. It was found, however, that there was a remarkable proportionality between the size and intensity of the T3AA spots found on the chromatograms and the L-T3 content of D-T3 samples that contained known added amounts of L-T3. Since it was not possible to use absolute reference standards, relative standards were used. That is, D-T3 samples containing known amounts (added) of L-T3 were run through the procedure with the unknown samples using the same enzyme, reaction times, *etc.*, and also spotting the samples and knowns on the same chromatogram, *etc.* Results obtained by this relative standardization procedure proved highly satisfactory if a bit tedious (Fig. 7).

TABLE IV—L-T2 CONTENT OF D-T2 SAMPLES

Sample	% L-T2
051-67B	Trace
051-73A	0.5
051-73B	1.5
051-8A	0.6
PD-39-281	0
051-25A	2
051-25B	0.25

L-T2 in D-T2—The procedure described for estimation of L-T3 in D-T3 also proved satisfactory for the estimation of L-T2 in D-T2, the immediate precursor of D-T3 in the synthetic process (Table IV).

Table III is a list of the amounts of T2, T4, T3AA, and L-T3 found in representative lots of D-T3 prepared at Smith Kline & French and elsewhere. It can be seen that it is possible to produce and analyze D-T3 of very low L-T3 content.

SUMMARY

A procedure has been developed for estimating as little as 0.05% of L-triiodothyronine in samples of D-triiodothyronine. It is also applicable to the determination of equally small amounts of L-diiodothyronine in samples of D-diiodothyronine and very probably for the determination of small amounts of L-tetraiodothyronine (thyroxine) in D-tetraiodothyronine.

A chromatographic procedure has also been described for separating and estimating diiodo-, triiodo-, and tetraiodothyronines in the presence of each other as well as in the presence of diiodo-, triiodo-, and tetraiodothyroacetic acids.

These procedures have been used to estimate the L-triiodothyronine and the D-diiodo and tetraiodothyronine contents of a number of lots of D-triiodothyronine derived from different sources.

REFERENCES

(1) Meister, A., Levintow, L., Kingsley, R. B., and Greenstein, J. P., *J. Biol. Chem.*, **192,** 535(1951).
(2) Parikha, J. R., Greenstein, J. P., Winitz, M., and Birnbaum, S. M., *J. Am. Chem. Soc.*, **80,** 953(1958).
(3) Greenstein, J. P., Birnbaum, S. M., and Otey, M. C., *J. Biol. Chem.*, **204,** 307(1953).
(4) Gilbert, J. B., Price, V. E., and Greenstein, J. P., *ibid.*, **180,** 473(1949).
(5) Zeller, E. A., "Advances in Enzymology," vol. 8, Nord, F. F., ed., Interscience Publishers, Inc., New York, N. Y., 1948, p. 459.
(6) Nakano, M., Danowski, T. S., and Utsumi, A., *Endocrinology*, **65,** 242(1959).
(7) Nakano, M., *ibid.*, **67,** 880(1960).
(8) Tong, W., Taurog, A., and Chaikoff, I. L., *J. Biol. Chem.*, **207,** 59(1954).
(9) Albright, E. C., Larson, F. C., Tomita, K., and Lardy, H. A., *Endocrinology*, **59,** 252(1956).
(10) Emerson, E., *J. Org. Chem.*, **8,** 417(1943).