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Synthesis and characterization of N-bromoacetyl-3,3′,5-triiodo-L-thyronine

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ABSTRACT

N-Bromoacetyl-3,3′,5-triiodo-L-thyronine and carrier-free [3′-^{125}I]-N-bromoacetyl-3,3′,5-triiodo-L-thyronine, to be used for affinity labeling of thyroid hormone receptors, were synthesized using a one-step procedure: a solution of the thyroid hormone 3,3′,5-triiodo-L-thyronine and bromoacetyl bromide in ethyl acetate was refluxed for an optimal period of time which depends on the amount of hormone processed. The bromoacetylated hormone thus obtained was then fractionated by high-speed counter-current chromatography which yielded N-bromoacetyl-3,3′,5-triiodo-L-thyronine that was pure by the criteria of high-performance liquid chromatography and thin-layer chromatography with different solvent systems. The pure product was well separated from all contaminants including one which in high-performance liquid chromatography was not easily separated from N-bromoacetyl-3,3′,5-triiodo-L-thyronine. The latter was characterized by ^{1}H nuclear magnetic resonance, plasma desorption mass spectrometry, thin-layer chromatography, high-performance liquid chromatography, UV spectrophotometry, and melting point. Amounts of 3,3′,5-triiodo-L-thyronine ranging from picograms, including carrier-free ^{125}I-labeled triiodothyronine, to 200 to 300 mg can be processed with the equipment used in the present investigation.

INTRODUCTION

Labeled N-bromoacetyl-3,3′,5-triiodo-L-thyronine (BrAcT3) has been widely used as an affinity label for the study of thyroid hormone binding to a variety of receptor sites. This affinity label as well as its congener N-bromoacetyl-L-thyroxine (BrAcT4) has usually been prepared by a two-step reaction: coupling of bromoacetic acid with N-hydroxysuccinimide by means of dicyclohexylcarbodiimide and reaction of the N-hydroxysuccinimide bromoacetate formed with ^{14}C- or ^{125}I-labeled 3,3′,5-triiodo-L-thyronine (T_3) or L-thyroxine (T_4) [1–8]. For the synthesis of unlabeled BrAcT3, the second step was carried out with unlabeled T3. However, no physicochemical data have been published to prove unequivocally the identity of the synthesized products and several labeled or unlabeled bands or peaks are always obtained when the crude reaction product is being analyzed by thin-layer chromato-

graphy (TLC) or by high-performance liquid chromatography (HPLC). It is not clear which one of these bands or peaks represents BrAcT3 or BrAcT4. We now report a simple one-step synthesis of BrAcT3 and of carrier-free BrAc[^{125}I]T3. (One-step syntheses of BrAcT4 and of carrier-free BrAc[^{125}I]T4 which require a different methodology will be reported later).

The synthesis of BrAcT3 is based on the reaction of bromoacetyl bromide (BrAcBr) with T3 or [^{125}I]T3. The crude reaction produced is fractionated by means of high-speed counter-current chromatography (HSCCC) [9]. The most prominent absorption or radioactivity peak obtained in this fractionation is shown below (Experimental) by plasma desorption mass spectrometry (PD-MS) and ^{1}H NMR to represent unlabeled or labeled BrAcT3.

EXPERIMENTAL

Materials

The following materials were used: T3, 99% pure (Aldrich); [3′-^{125}I]T3, carrier-free (2200 Ci/mmol) and [3′,5′-^{125}I]T4, carrier-free (4400 Ci/mmol) (DuPont/NEN 110X and 111X, respectively); BrAcBr, 98+% (Aldrich); methanol, hexane, acetonitrile (HPLC grade); acetic acid, ammonium acetate, reagent grade; ethyl acetate, (reagent grade for bromoacetylation or HPLC grade for HSCCC); water, Milli-Q (Millipore); boiling chips (amphoteric alundum granules) (Hengar).

Synthesis of BrAcT3

A round-bottom flask equipped with a short reflux condenser was used for the processing of more than 0.01 mmol T3. Thus, 0.1 mmol T3 was mixed with 25 ml of ethyl acetate and 500 μl BrAcBr were added which causes dissolution of T3. The mixture, after addition of a very small boiling chip, was refluxed for 10 min. The top of the condenser was connected via an empty safety bottle to a wash bottle containing 1 *M* NaOH in 50% ethanol to absorb any escaping BrAcBr or HBr. The reaction mixture was cooled in ice water, then 500 μl of methanol were added to destroy excess BrAcBr.

The reaction mixture was concentrated to a small volume (*ca.* 0.5 ml) in a rotating evaporator (Büchi Rotovapor R) under reduced pressure (20–30 Torr) using a water bath not exceeding 30°C.

For the processing of amounts of T3 exceeding 0.1 mmol the amounts of reagents and solvent were changed proportionately. For the processing of less than 0.01 mmol T3, including that of carrier-free [^{125}I]T3, the round-bottom flask was replaced with a glass tube fashioned from a 99447 Corning culture tube and a 19/22 joint. In that case, refluxing with a metal block heater (Multi-Blok heater No. 2090; Labline Instruments) was reduced to 5 min. In a few experiments, the rotating evaporator was replaced with a SpeedVac concentrator (Savant) after short precooling of the reaction mixture to avoid bumping. The amounts of BrAcBr, ethyl acetate and methanol were always 50 μl, 2.5 ml and 50 μl, respectively, independent of the amount of T3.

Since [^{125}I]T3 is obtained from the manufacturer as a solution in aqueous propanol, that solvent must be eliminated before bromoacetylation. This is achieved by brief lyophilization for μl amounts or by SpeedVac concentration for ml amounts.

HSCCC fractionation

A commercial model of the HSCCC centrifuge (P.C. Inc., Potomac, MD, U.S.A.) was used in this study [10]. The apparatus holds a multilayer-coil separation column and a counterweight symmetrically on the rotary frame at a distance of 10 cm from the centrifugal axis of the centrifuge. The column consists of approximately 165 m × 1.6 mm I.D. polytetrafluoroethylene (PTFE) tubing with a total capacity of approximately 330 ml. The revolution speed can be regulated up to 1000 rpm with a speed controller.

The two-phase solvent systems composed of hexane–ethyl acetate–methanol–15 m*M* ammonium acetate (pH 4.0) (1:1:1:1 and 4:5:4:5, v/v) were selected on the basis of partition coefficient values of BrAcT3 as summarized in Tables I and II. The solvent mixture was thoroughly equilibrated in a separatory funnel and the two phases were separated shortly before use.

The sample solution (*ca.* 0.5 ml, ethyl acetate) was first mixed with a proper volume of each of the other solvents (hexane, methanol and 15 m*M* ammonium acetate) to adjust the phase composition and finally brought to approximately 4 ml by adding equal volumes of the upper and lower phases used for separation.

In each centrifugation, the separation column was first entirely filled with the upper, organic (stationary) phase and the sample solution was injected through the sample port. Then, the apparatus was rotated at 800 rpm while the lower, aqueous (mobile) phase was pumped into the column at a flow-rate of 3 ml/min using a metering pump (Milton Roy minipump; LDC Analytical, Riviera Beach, FL, U.S.A.). The effluent from the outlet of the column was continuously monitored with a UV monitor (Uvicord S; LKB Instruments, Stockholm, Sweden) at 276 nm and fractionated with a fraction collector (Ultrorac, LKB Instruments) to obtain 3-ml fractions. After the desired peak (BrAcT3) was eluted, the apparatus was stopped and the column contents were fractionated by elution with methanol at a flow-rate of 6 ml/min.

Analysis of HSCCC fractions

Fractions which were of interest because they showed either high absorbance (A_{276}) or high radioactivity were analyzed by PD-MS, HPLC, or ^{1}H NMR. Depending on the concentration of solute, small aliquots of a single fraction could be used. With very little solute, as for the analysis of carrier-free preparations of BrAc[^{125}I]T3, ten or more fractions had to be combined and concentrated. As expected, unlabeled and labeled BrAcT3 (A_{278} and cpm, respectively) coeluted.

The radioactivity of fractions was assessed with a γ scintillation counter (Auto-Gamma 5000 series, Packard). For the determination of the yield of unlabeled BrAcT3, a small amount (20 μl) of [3'-^{125}I]T3 (DuPont/NEN 110X) was evaporated to dryness and mixed with unlabeled T3. The BrAc[^{125}I]T3 formed upon bromoacetylation served as an internal standard. Furthermore, small duplicate samples (2 μl) of the same [3'-^{125}I]T3 preparation were counted alongside the BrAc[^{125}I]T3 in order to determine the yield as well as mechanical losses.

HPLC analyses were carried out with a Waters chromatograph using a 280-nm absorption detector in conjunction with an Omniscribe absorption recorder (Houston Instruments) and a FRAC-100 fraction collector (Pharmacia). Conditions were: 15-cm Nova C_{18} column; solvent A, 15 m*M* ammonium acetate, pH 4.0, prepared by adding acetic acid to an aqueous solution of 1.2 g ammonium acetate until pH 4.0 is

reached and bringing the solution to a total volume of 1 l with water; solvent B, acetonitrile; linear gradient (started 1 min after injection), 20–60% B in 40 min; flow-rate, 1 ml/min; sensitivity, 0.1 absorbance units/chart width in most instances and 0.2 or 0.5 absorbance units/chart width in some cases. Higher sensitivities (0.05 or 0.01) were required for the detection of carrier-free BrAc[^{125}I]T3.

T_4 (10^{-3} *M*) was always used as an internal standard. With a sensitivity setting of 0.1, 5 μl were injected together with the sample.

Mass spectra

PD-MS spectra were measured using a spectrometer built for the National Institutes of Health (NIH) by Professor R. Macfarlane of Texas A & M University and modified by one of us (H.M.F.) and L.K. Pannell. An accelerating voltage of 10 kV was used with a flight path of 42 cm. Samples were electrosprayed onto aluminized mylar films and spectra were allowed to accumulate for at least 1 h before processing on a Perkin-Elmer 3220 data system.

Absorption spectra

Ultraviolet spectra were determined with a Cary 219 spectrophotometer using quartz cells with a 1-cm light path.

Melting point

The melting point of BrAcT3 was taken using a Kofler hot stage microscope.

RESULTS AND DISCUSSION

A simple one-step method of synthesizing BrAcT3 consists of refluxing T3 and BrAcBr in ethyl acetate for an optimal period of time which depends on the amount of T3 processed. The reaction mixture is then fractionated by means of HSCCC (see Experimental) which results in a resolution superior to that which can be achieved with TLC or even with HPLC.

In the case of BrAcT3 the purification by HSCCC is highly dependent not only on temperature (a generally observed phenomenon), but also on solute concentration (non-linear isotherm). Table I shows the effect of temperature and concentration, using the solvent system hexane–ethyl acetate–methanol–15 m*M* aqueous ammonium acetate, pH 4.0 (1:1:1:1, v/v). The partition coefficient K (C_L/C_U) (see Tables I and II) increases with increasing temperature and with decreasing concentration. The dependence on concentration (Table I) is probably due to reversible aggregation. The table shows that the solvent system used is appropriate for a BrAcT3 concentration of 1 mg/ml, but results in excessive K values for lower concentrations. We have used this solvent system for concentrations of 1 mg/ml and greater.

For use with much lower solute concentrations, including those prevailing when carrier-free BrAc[^{125}I]T3 is to be purified, the effect of changing solvent volume ratios on K values was investigated (Table II).

The ethyl acetate and aqueous ammonium acetate buffer ratios within the total solvent system were kept constant, but the volumes of hexane and of methanol were gradually decreased. A decrease of the hexane volume results in an increase of polarity of the solvent system, while a decrease in methanol volume serves mainly to maintain

TABLE I

EFFECTS OF TEMPERATURE AND SOLUTE CONCENTRATION ON THE PARTITION COEFFICIENT (K) OF BrAcT3 IN HEXANE–ETHYL ACETATE–METHANOL–15 mM AMMONIUM ACETATE (pH 4.0) (1:1:1:1, v/v)

$K = C_L/C_U$, *i.e.*, solute concentration in the lower phase divided by that in the upper phase.

Temperature (°C)	K			
	Solute concentration (mg/ml)			
	1	0.1	0.01	<0.001
10	2.1	–	4.7	4.8
20	3.0	6.0	6.0	–
30	4.2	–	7.4	8.1

TABLE II

EFFECT OF VOLUME RATIO OF HEXANE–ETHYL ACETATE–METHANOL–15 mM AMMONIUM ACETATE (pH 4.0) ON THE PARTITION COEFFICIENT OF BrAcT3

$K = C_L/C_U$, solute concentration in the lower phase divided by that in the upper phase. Experimental conditions: temperature, 20°C; solute concentration: 0.01 mg/ml.

Hexane–ethyl acetate–methanol–15 mM ammonium acetate	K
5:5:5:5	5.7
4:5:4:5	1.5
3:5:3:5	0.21

the interfacial tension between the two phases. A decrease in interfacial tension, which would occur if the methanol volume were kept constant, would result in loss of peak resolution due to a decrease of the volume of the stationary (organic, upper) phase retained in the column. For the purification of BrAcT3 present in low concentration (0.01 mg/ml or less) including carrier-free BrAc[^{125}I]T3 we have chosen a hexane–ethyl acetate–methanol–15 mM ammonium acetate volume ratio of 4:5:4:5 because Table II shows that this ratio results in a K value not too far from unity.

Fig. 1 shows absorbance (276 nm) and radioactivity profiles in HSCCC. The yield of BrAcT3 in this and similar bromoacetylations, as described above (Experimental) was about 54%. It was somewhat lower when very small amounts of T3 (<0.01 mmol) were used. Derivatization of carrier-free [3'-^{125}I]T3 (Fig. 2A) resulted in yields of 30–40%. This lower yield is presumably due to mechanical losses such as adsorption of BrAcT3 on the PTFE wall of the HSCCC column and to more pronounced side reactions when only trace amounts of T3 are being processed.

PD-MS of the byproduct eluting from the column at fractions 37–39 in Fig. 1 indicated that it consists largely of acetyl-T3 presumably formed by aminolysis of ethyl acetate. Similarly, PD-MS of the very hydrophobic material eluting in fractions 170–190 (Fig. 1B) and consisting of a mixture of two or more compounds strongly

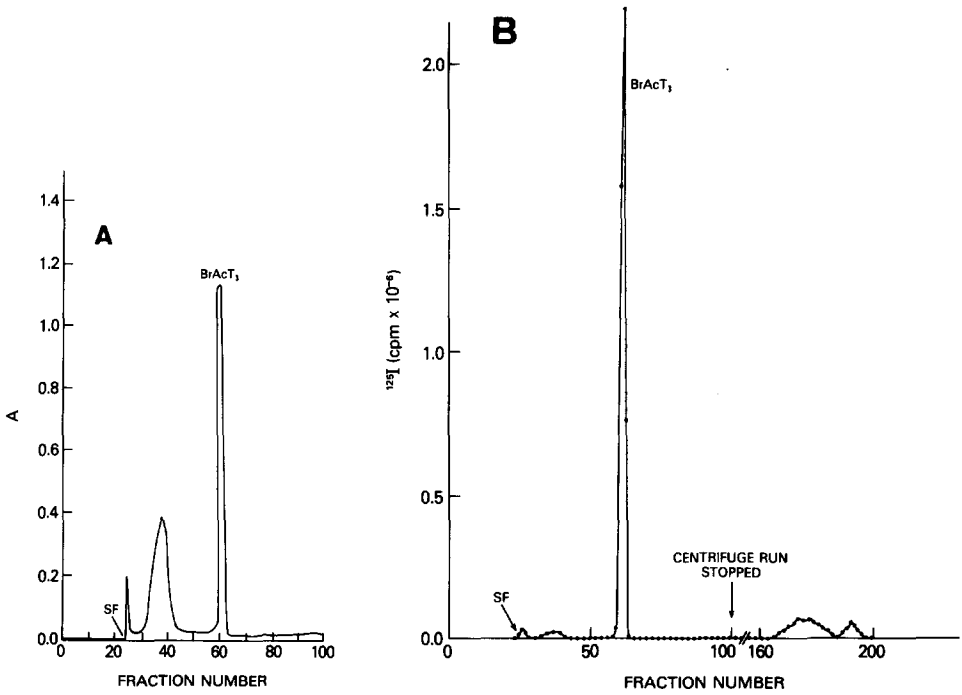

Fig. 1. Chromatograms (HSCCC) of crude BrAcT3 obtained by reacting T3 with BrAcBr. Chromatograms were monitored by UV absorbance at 276 nm (A) and by radioactivity of a ^{125}I-labeled internal marker (B). Sample No. 180A; solvent system, hexane–ethyl acetate–methanol–15 m*M* ammonium acetate, pH 4.0 (1:1:1:1); mobile phase, lower (aqueous) phase; flow-rate, 3 ml/min; 3-ml fractions; revolution, 800 rpm; retention of stationary phase, 77% of column capacity (330 ml); maximal column pressure, ·65 p.s.i. SF = solvent front.

suggests that one of these is BrAcT3 ethyl ester presumably arising from transesterification of BrAcT3 with ethyl acetate. While acetyl-T3 cannot be directly linked covalently to T3 receptors, BrAcT3 ethyl ester should be able to react with nucleophiles in T3 receptors to establish a covalent bond. The amounts of all byproducts increase with refluxing beyond the optimal period of time. However, refluxing for much shorter periods of time results in incomplete conversion of T3 to BrAcT3. The small peak at the solvent front in Fig. 1A and B is caused by unreacted T3.

A sample of N-acetyl-T3 obtained as a byproduct in HSCCC (sample No. 137A, fraction 31) was checked against the PD-MS pattern of authentic N-acetyl-T3. It showed $(M+H)^+$, $(M+Na)^+$, and $(M+2Na-H)^+$ ions at *m/z* 691, 714 and 736, *i.e.*, *ca.* 79 a.m.u. less $(-Br+H)$ than BrAcT4 (see below). Ions are also present at *m/z* 588 and 610 for the losses of HI from the sodiated species.

A sample apparently containing BrAcT3 ethyl ester (sample No. 163A, fraction 136) showed $(M+Na)^+$ and $(M+H)^+$ ions at *m/z* 824 and 801, *i.e.*, 28-29 a.m.u. higher than the corresponding ions in BrAcT4. Ion A (Fig. 3) is also present at *m/z* 606 showing that the excess mass is confined to the carboxyl group, presumably as the ethyl

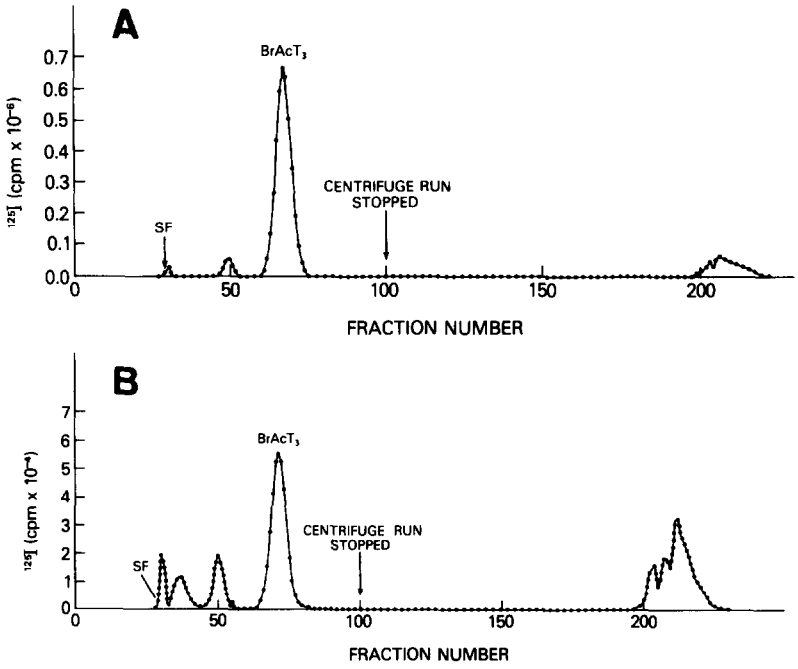

Fig. 2. Chromatograms (HSCCC) of crude ^{125}I-labeled BrAcT3 obtained by reacting carrier-free [^{125}I]T3 with BrAcBr. Chromatograms were monitored by radioactivity. Samples No. 171A, freshly prepared (A) and sample No. 175A, stored for 4.2 months in ethyl acetate at −20°C (B); solvent system, hexane–ethyl acetate–methanol–15 m*M* ammonium acetate, pH 4.0 (4:5:4:5). Other conditions as in Fig. 1.

ester. This is in agreement with its long retention by the non-polar (organic) phase in HSCCC.

The main HSCCC peak was clearly identified as BrAcT3 by PD-MS as well as by ^{1}H NMR. It is a very narrow peak when milligram amounts of T3 are being processed (fractions 59–63 in Fig. 1A and B) and broader when carrier-free T3 is being used (fractions 61–74 in Fig. 2A). A sample of BrAcT3 purified by HSCCC (sample No. 123A, fraction 37) produced the mass spectrum shown in Fig. 3. As is common for acidic and hydroxylic groups in PD-MS, the most abundant peak in the molecular weight region at *m/z* 818 (calculated 816.9) corresponds to the $(M+2Na-H)^+$ ion with smaller peaks for the $(M+H)^+$ and $(M+Na)^+$ ions at *m/z* 772 and 796. Losses from all three of these species of HBr and HI are evident while the $(M+H)^+$ ion also loses formic acid and bromoacetamide to give the corresponding styrene ions. Benzylic cleavage (ion A, Fig. 3) and formation of the imine ion B, Fig. 3, are also prominent. At lower masses the only important ions are at *m/z* 173 and 219 due to $(Na_2I)^+$ and the phenolic ring, respectively. Ions at *m/z* 322, 363 and 413 were variable in intensity and may represent impurities.

The NMR spectrum of a second sample of the same retention volume (sample No. 135A, fraction 50) in [$^2H_4$]methanol showed a quartet at δ 3.83 ($J=11.2$) from geminal coupling of the hydrogens of the CH_2 of the N-bromoacetyl group, the asymmetry arising from the proximity of the α-amido acid carbon. Likewise, the two

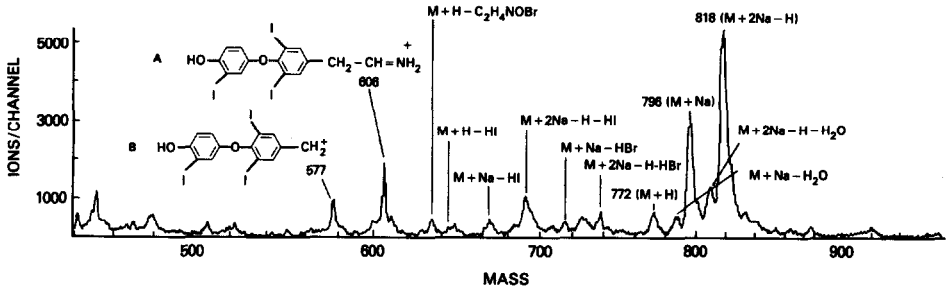

Fig. 3. Mass spectrum (PD-MS) of BrAcT3. Sample No. 123A, HSCCC fraction 37.

benzylic protons were seen at δ 2.95 and 3.24 ($J = 12.8$) coupled further to the α proton at δ *ca.* 4.65 ($J = 8.8$ and 4.8) as shown by decoupling.

When a preparation of crude carrier-free BrAcT3 (prior to HSCCC) was kept in ethyl acetate in a well-sealed container at −20°C for over 4 months, partial decomposition of BrAcT3 took place as a comparison of Fig. 2A and B shows, but even after this period of time pure BrAcT3 can still be obtained in somewhat reduced yield by HSCCC. Satisfactory conditions for long-term storage of BrAcT3 are now being investigated.

For the resolution of crude BrAcT3 (or of crude BrAcT4), HSCCC is far superior to TLC and also to HPLC. For example, a main disadvantage of TLC is that several consecutive runs are required to obtain a fairly pure BrAcT3 preparation and this causes considerable losses due to irreversible adsorption to the silica gel. It is difficult to obtain reasonable amounts of unlabeled BrAcT3 as required for competition studies and as reference material.

When crude preparations of BrAcT3 were analyzed by HPLC, the BrAcT3 peak was always accompanied by a satellite peak eluting immediately before the main peak. The two peaks were not resolved when preparative HPLC was used. The HSCCC peak material eluted in fractions 37–39 in Fig. 1A and B (eluted long before BrAcT3) produced an HPLC peak having a retention time identical to that of the satellite peak (not shown). Crude BrAcT3 preparations obtained by the two-step method mentioned in the Introduction also showed in HPLC a satellite peak with identical retention time. Therefore, the satellite material presumably consists at least in part of acetyl-T3 (see above).

HPLC elution patterns of crude BrAcT3 (sample No. 180A, prior to HSCCC) and of pure BrAcT3 (sample No. 180A, after HSCCC, fraction 61) are shown in Fig. 4A and B. Pure BrAcT3 eluted very reproducibly 2.5 min after T4 (4B). The 35.90-min peak in Fig. 4A and the smaller peak with still longer retention time presumably correspond to two components of the very hydrophobic HSCCC double peak (Fig. 1B, fractions 166–196). T3 eluted before T4 (not shown), not only under our experimental conditions but also under those used by Mol *et al.* [11] who report that their BrAcT3 (prepared by treatment of unlabeled T3 + [^{125}I]T3 with N-bromoacetyl chloride, followed by Sephadex LH-20 liquid chromatography) eluted before T3. We have no explanation for the discrepancy between their results and ours.

In earlier work [1–6] TLC (silica gel plates, acetic acid–ethyl acetate, 1:9, v/v) was used, while HPLC was used in some later work [7,8,11]. Before switching to HSCCC in

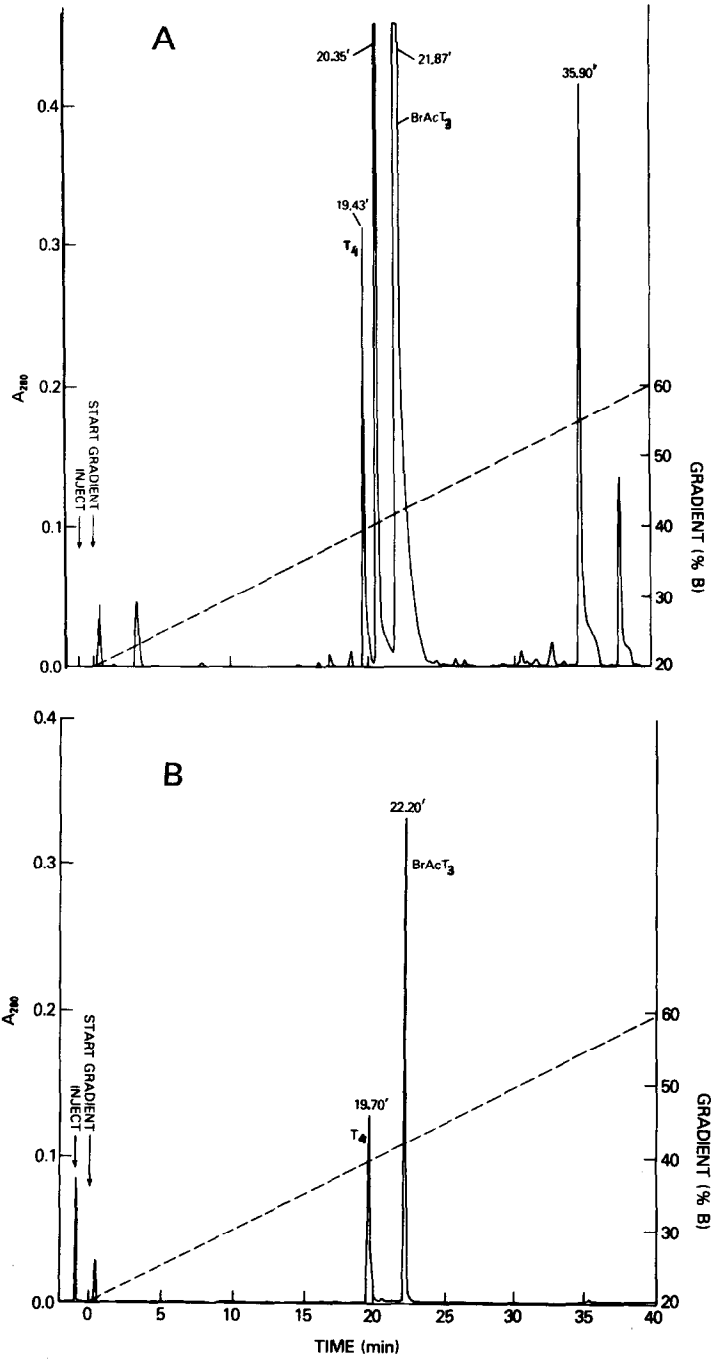

Fig. 4. HPLC of crude BrAcT3 (prior to HSCCC), sample No. 180A (A) and of purified BrAcT3, sample No. 180A, HSCCC fraction 61 (B). Waters chromatograph; 15-cm C_{18} Nova column (Waters); flow-rate, 1 ml/min; sensitivity, 0.5 absorbance units/chart width; solvents, 15 m*M* ammonium acetate, pH 4.0 (solvent A) and acetonitrile (solvent B); linear gradient (started 1 min after injection), 20–60% B in 40 min; internal standard, 20 μl of 10^{-3} *M* T4. An internal standard (10 μl of 10^{-3} *M* T4) was injected together with 2 μl of the sample.

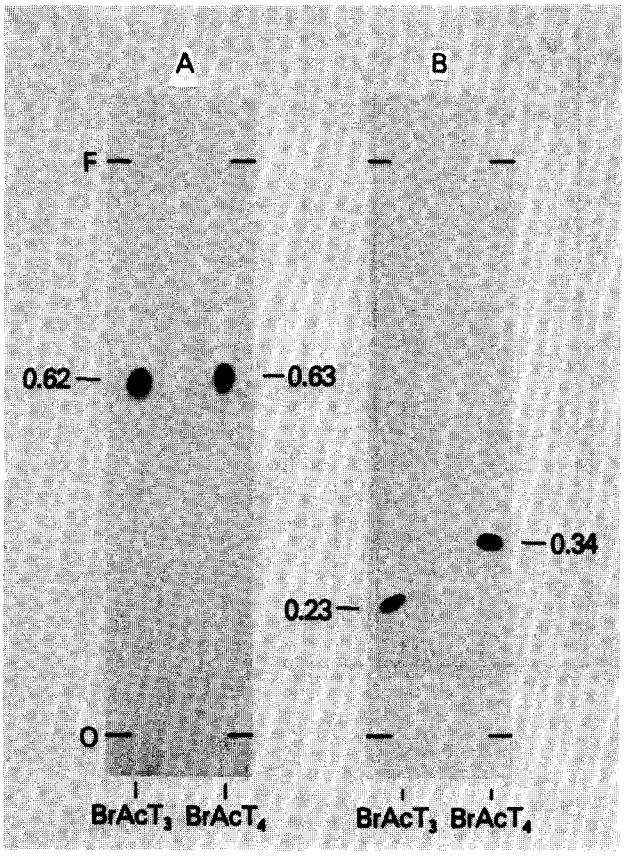

Fig. 5. TLC of BrAcT3 and of BrAcT4 in two solvent systems: acetic acid–ethyl acetate (1:9, v/v) (A) and acetic acid–methanol–chloroform (1:4:45), freshly prepared (B). Sample No. 180A, HSCCC fraction 61; Whatman K6F silica gel plates, 20 × 5 cm. O = Origin; F = front.

conjunction with HPLC, we also used the solvent system shown in the legend to Fig. 5B because it has a superior resolving power. Fig. 5 shows the chromatograms of HSCCC-purified BrAcT3 and BrAcT4 in the two solvent systems. The R_F values shown in Fig. 5 are reproducible only with the same type of TLC plates (Whatman K6F, 250 μm layer); R_F values obtained with preparative plates (Whatman PLK5F, 1000 μm layer) are considerably lower. Also the presence of a large amount of impurity changes R_F values.

Pure BrAcT3 has a melting point of 217.5–218.5°C with apparent decomposition since the melt is brownish, while the BrAcT3 used was a white solid material. It was obtained by concentrating HSCCC sample No. 173A, fraction 67 to one half of its volume in a SpeedVac concentrator (Savant) and then precipitating BrAcT3 by adding water. The precipitated material was collected by filtration, dried in a vacuum desiccator, and stored at room temperature in an amber container.

The UV absorption spectrum of this 94.4% pure BrAcT3 (HPLC) in 0.01 *M* NaOH containing 0.5% ethanol, showed the typical two peaks of 3′-iodo-4′-

hydroxydiphenyl ethers having a side chain in position 1. One has λ_{max} at 320 nm (molar absorption coefficient, $\varepsilon = 5500$ l mol^{-1} cm^{-1}), the other one at 225 nm ($\varepsilon = 49\,300$ l mol^{-1} cm^{-1}). In an acidic solvent (lower phase of HSCCC solvent 1:1:1:1) and in 1-butanol the higher-wavelength peaks were at 297 and 299 nm, respectively. Values for T3, T4 and other iodoamino acids have been summarized by one of us (H.J.C.) [12]. 3,3',5'-Triiodo-L-thyronine ("reverse T3") has its higher-wavelength peak at 322 nm ($\varepsilon = 6150$ l mol^{-1} cm^{-1}) in 0.01 *M* NaOH (unpublished data).

It can be concluded from our investigations that HSCCC is the method of choice for the purification of crude BrAcT3, conveniently prepared by a simple one-step bromoacetylation of T3.

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