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SOLUBILIZATION OF A PHOSPHOLIPID-REQUIRING ENZYME, IODOTHYRONINE 5'-DEIODINASE, FROM RAT KIDNEY MEMBRANES

JACK L LEONARD * and ISADORE N ROSENBERG

Department of Medicine, Boston University School of Medicine, Boston, MA 02218 and Department of Medicine, Framingham Union Hospital, Framingham, MA 01701 (U S A)

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Summary

Enzymic activities catalyzing the reductive 5'-deiodination of thyroxine and 3,3',5'-triiodothyronine were solubilized from rat kidney microsomes by treatment with 0 2% deoxycholate Deoxycholate reversibly inhibited the enzyme(s), removal of detergent restored activity and resulted in the formation of enzymatically active aggregates with a buoyant density of 1 17 g/ml resembling that of membranes Fractionation of the solubilized membrane components in the presence of 0 2% deoxycholate by either gel filtration or sucrose gradient centrifugation inactivated the enzyme(s) and activity could be restored by the addition of partially purified soybean phospholipids, this allowed some of the physical properties of the enzyme(s) to be determined 5'-Deiodinating activity of both thyroxine and 3,3',5'-triiodothyronine was associated with protein(s) with $s_{20,w}$ of 3 5 S, Stokes' radius of 32 Å, and a calculated molecular weight of 49 900 A partial specific volume of 0 74 cm^3/g was calculated from sedimentation in ^2H_2O and H_2O sucrose gradients Phospholipid reactivation of lipid-depleted enzyme preparations was concentration-dependent, with near maximal restoration when sufficient phospholipid was added to restore the phospholipid protein ratio to that of the renal microsomes The enzyme protein(s) catalyzing the 5'-deiodination of thyroxine and of 3,3',5'-triiodothyronine could not be resolved by sedimentation or molecular sieving and showed similar behavior toward deoxycholate solubilization and phospholipid reconstitution

* Present address Thyroid Unit, Peter Bent Brigham Hospital Division of Affiliated Hospital Center, and Harvard Medical School, Boston, MA 02115 U S A

Abbreviations T_4 3,5,3',5'-tetraiodothyronine, T_3, 3,5,3'-triiodothyronine, rT_3, 3,3',5'-triiodothyronine For 'tetraiodothyronine' read thyroxine

Extrathyroidal deiodination constitutes a major pathway of thyroid hormone metabolism Removal of a single iodine atom from thyroxine (T_4) results in either hormone activation (T_3 formation) or hormone inactivation (rT_3 formation) via 5′-deiodination or 5-deiodination, respectively, and provides most of the circulating T_3 and rT_3 [1—3]

Extrathyroidal T_3 formation is catalyzed by thyroxine 5′-deiodinase, a thiol dependent enzyme associated with membrane fractions obtained from liver and kidney tissue [4—9] Non-protein thiols, including reduced glutathione, have been shown to modulate the 5′-deiodination of T_4 [10—13] and studies in vitro suggested that reduced thiols participate in the reaction as a second substrate [3,5,13,14]. Thiol dependent 5′-deiodination has also been shown to be an important pathway of rT_3 metabolism [1—3]. Reported similarities in subcellular distribution [9], effects of inhibitors [7,11,14], and a mutually competitive inhibition of 5′-deiodination by either T_4 or rT_3 [6,11] led to the proposal that T_4 and rT_3 are 5′-deiodinated by the same enzyme [3,11,14], however, the data do not rule out the possibility that separate enzymes catalyze the reductive deiodination reactions

Recently, we prepared a soluble preparation of tetraiodothyronine 5′-deiodinase by treatment of renal membranes with deoxycholate [13] We have further characterized the solubilized enzyme with regard to the 5′-deiodination of both T_4 and rT_3 Gel filtration of the deoxycholate soluble enzyme resulted in co-elution of the 5′-deiodinases for both tetraiodothyronine and 3,3′,5′-triiodothyronine The solubilized enzyme in the presence of detergent has a molecular weight of 49 900, detergent removal resulted in the formation of enzymatically active aggregates with a density of 1 17 g/ml Sucrose density gradient centrifugation and gel filtration, done in the presence of detergent, resulted in large losses of deiodinating activity which could be restored by addition of a mixture of soybean phospholipids

Materials and Methods

L-[3′- or 5′-^{125}I]T_4 (155 Ci/mmol), L-[3′- or 5′-^{125}I]rT_3 (at least 750 Ci/mmol) and [^{3}H]deoxycholic acid (4 Ci/mmol) were purchased from New England Nuclear Asolectin was purchased from Associated Concentrates Iodothyronines and ^2H_2O were purchased from Calbiochem Bovine serum albumin and catalase (bovine liver) were purchased from Sigma Sephadex G-200, Sepharose CL-6B, aldolase, ovalbumin, chymotrypsinogen, ribonuclease A and Blue Dextran were purchased from Pharmacia Rabbit antisera to T_3 and goat anti-rabbit γ-globulin serum were obtained from Antibodies, Inc. All other reagents were of the highest purity available and were obtained from commercial sources

Enzyme source

Rat kidney microsomes were prepared by differential centrifugation as described previously [5] and served as the source of iodothyronine 5′-deiodinase activity Renal microsomes were suspended at 10 mg protein/ml in 250 mM sucrose, 20 mM Tris-HCl buffer pH 7 5 containing 1 mM EDTA and 1 mM

dithiothreitol and could be kept for up to 6 months at —70° C with little or no loss of enzyme activity

Enzyme assays

Tetraiodothyronine 5'-deiodinase Enzyme activity was determined by measuring the amount of T_3 formed from L-T_4 as previously described [13] The amount of newly formed T_3 was determined by either a radiometric assay measuring [^{125}I]T_3 formation from L-[3'- or 5'-^{125}I]T_4 after paper chromatographic separation of iodothyronines [13], or by a radioimmunoassay for T_3 [8] When T_3 formation was measured by radioimmunoassay, reaction mixtures contained in a total volume of 200 µl 20 µmol potassium phosphate buffer (pH 7 0), 0 2 µmol EDTA, 0 2 µmol dithiothreitol, 2 nmol L-T_4, and enzyme The reaction, carried out at 37° C, was started by the addition of enzyme, and stopped 30 min later by addition of 400 µl of cold (4° C) ethanol and the tubes were kept at 4° C for 16 h prior to clarification by centrifugation Recovery of T_3 in the ethanol extracts was 89 ± 4% The rabbit antiserum to T_3 showed the following cross reactivities L-T_4, 0 11% and L-rT_3, 0.05% Assays were done in triplicate with tissue-free or boiled tissue blanks as controls One unit of enzyme activity represents the formation of 1 pmol T_3 from L-T_4 per min

3,3',5'-Triiodothyronine 5'-deiodinase assay 5'-deiodination of rT_3 was determined by measuring the [^{125}I]iodide liberated from L-[3'- or 5'-^{125}I]rT_3 as described previously [14] In brief, iodide and iodothyronines were separated by ion-exchange chromatography on Dowex 50W-X2 Reaction mixtures contained in a total volume of 100 µl 10 µmol potassium phosphate buffer (pH 7 0), 0 1 µmol EDTA; 0 1 µmol dithiothreitol, 100 pmol L-[3'- or 5'-^{125}I]-rT_3 (1000 cpm/pmol), and enzyme. Incubations, done at 37° C, were started by the addition of enzyme and stopped usually after 10 min by the addition of 200 µl of an ice-cold solution containing 10 µM T_4 and 10 µM 6-*n*-propylthiouracil. Reaction mixtures (200 µl aliquots) were applied to 3 ml columns of Dowex 50W (equilibrated in 1 74 M acetic acid) and iodide was eluted with two successive 5 ml additions of 1.74 M acetic acid Assays were done in triplicate with tissue-free or boiled tissue blanks as controls One unit of enzyme activity represents the release of 1 pmol I^- from rT_3 per min.

Solubilization A standard method for solubilizing iodothyronine 5'-deiodinase was adopted A 5% solution of sodium deoxycholate (three-times recrystallized from 80% acetone/H_2O) was added (final concentration 0 2%) to renal microsomes suspended at 5 mg protein/ml in 250 mM sucrose 20 mM Tris-HCl 1 mM EDTA 1 mM dithiothreitol (pH 7.5). After 20 min at 4° C the suspension was centrifuged at 250 000 × g_{max} for 30 min in a Beckman type 65 rotor The clear supernatant, after centrifugation, was referred to as the 'deoxycholate extract' [13]. When appropriate, the deoxycholate extract was concentrated by ultrafiltration on a PM-10 ultrafilter (Amicon).

Sucrose gradient centrifugation

Sedimentation was done on 5—20% linear sucrose gradients containing 20 mM Tris-HCl 1 mM EDTA 1 mM dithiothreitol 0 2% deoxycholate (pH 7.5 at 4° C) as described by Martin and Ames [15]. Gradients were centrifuged at 175 000 × g_{max} for 16 h at 4° C in a Beckman SW 50 rotor, and 30 fractions

(20 drops each) were collected, bottom to top Fractions obtained from gradients of the 'deoxycholate extract' were supplemented with 50 μg of soybean phospholipid and treated with Dowex 1 resin prior to enzyme assay (see below) Sedimentation coefficients were determined by the method of Clarke [16] Partial specific volumes ($\bar{v}$) were estimated by comparison of sedimentation on 5—20% linear sucrose gradients prepared in H_2O and 90% ^2H_2O as described by Clarke and others [16—18] Values of $s_{20,w}$ and $\bar{v}$ for standard proteins were obtained from the literature [19,20]

Isopycnic banding experiments were done on linear 10—60% (w/v) sucrose gradients containing 20 mM Tris-HCl 1 mM EDTA 1 mM dithiothreitol (pH 7 5) Centrifugation was done at 175 000 × g_{max} for 24 h at 4° C and 20 fractions were collected, bottom to top, as described above Enzyme assays were done without removing sucrose from the fractions

Gel filtration

Sephadex G-200 and Sepharose CL-6B were equilibrated in 20 mM Tris-HCl 1 mM EDTA (pH 7 5) and 0 9 × 58 cm (Sephadex G-200) and 1 5 × 85 cm (Sepharose CL-6B) columns prepared at 4° C Column void volumes and total column volumes were determined with blue dextran and ^3H_2O, respectively The columns were re-equilibrated with buffer containing 1 mM dithiothreitol and 0 2% deoxycholate prior to gel filtration of the deoxycholate extract Iodothyronine 5′-deiodinase activity was assayed after supplementing column fractions with 200 μg soybean phospholipid and removing deoxycholate by Dowex 1 treatment (see results) The Stokes' radius, R_s, was determined graphically from a plot of $K_d^{1/3}$ vs R_s as proposed by Porath [21], catalase, aldolase, ovalbumin, bovine serum albumin, chymotrypsinogen and ribonuclease A served as reference proteins

Analytical methods

Protein determinations were done by the method of Lowry et al [22] with crystalline bovine serum albumin as the protein standard Samples containing deoxycholate were first treated with Dowex 1-X2 to remove detergent prior to protein estimation. The dye-binding method of Bradford [23] was used to quantitate protein in fractions obtained after sucrose gradient centrifugation The phospholipid content was estimated from total phosphate determinations done by the method of Fiske and SubbaRow [24] Greater than 89% of the phosphate present in the microsomal membranes was extracted by chloroform/methanol (2 1, v/v) treatment of the membrane preparations

Results

Solubilization with deoxycholate

We previously reported that treatment of renal microsomes with 0 2% deoxycholate solubilized T_4 5′-deiodinating activity [13] However, detergent removal was required for optimal recovery of enzyme activity and this procedure rendered the enzyme insoluble This led us to explore, in detail, the solubilization of renal tetraiodothyronine 5′-deiodinase and to examine the ability of such preparations to catalyze the 5′-deiodination of rT_3

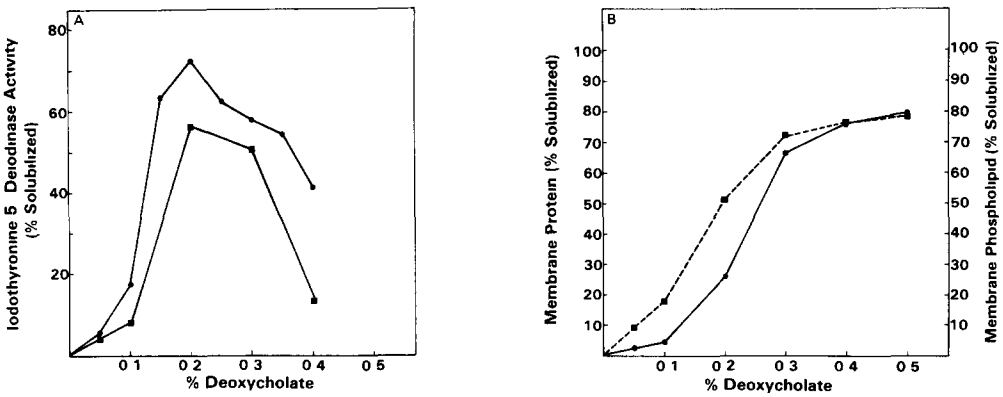

Fig 1 Effect of increasing concentrations of deoxycholate on the solubilization of iodothyronine 5′-deiodinase (A) Renal microsomes were suspended at a final concentration of 5 mg protein per ml in 250 mM sucrose, 20 mM Tris-HCl buffer, pH 7 5, at 4° C containing 1 mM EDTA, 1 mM dithiothreitol and increasing amounts of deoxycholate (0—5 mg/ml) The solubilization mixtures were kept at 4°C for 20 min and then centrifuged at 250 000 × g_{max} for 30 min The clear supernatants were collected and deoxycholate removed by Dowex 1 adsorption The corresponding pellets were suspended in 250 mM sucrose 20 mM Tris-HCl buffer, pH 7 5, at 4° C, containing 1 mM EDTA and 1 mM dithiothreitol Iodothyronine 5′-deiodinase activities (tetraiodothyronine and 3,3′,5′-triiodothyronine) were assayed at 0 5 μM T_4 and 0 5 μM rT_3, respectively T_3 formation was determined by radiometric methods ●——● 3,3′,5′-triiodothyronine 5′-deiodinase activity, ■——■ tetraiodothyronine 5′-deiodinase activity (B) Protein (■) and phospholipid (●) (expressed as total phosphate) content of the deoxycholate solubilized fractions as prepared above

The data in Fig. 1A show the effects of increasing concentrations of deoxycholate on the solubilization of renal tetraiodothyronine and 3,3′,5′-triiodothyronine 5′-deiodinase. Both enzyme activities showed solubilization optima between 0 15 and 0.25% deoxycholate, with 55—75% recovery of membrane-

TABLE I

EFFECT OF DEOXYCHOLATE ON IODOTHYRONINE 5′-DEIODINASE ACTIVITY

Renal microsomes and deoxycholate extracts were prepared and [^{3}H]deoxycholic acid was added to the detergent extracts to achieve a final spec radioact of 50 000 cpm/mg deoxycholate 25 mg of Dowex 1 was added to increasing volumes of the extract and the mixtures kept at 4°C for 20 min Resin was removed by filtration and 10 μl aliquots of the filtrate assayed for thyroxine and 3,3′,5′-triiodothyronine 5′-deiodinase activities at 10 μM T_4 and 1 μM rT_3, respectively T_3 formation was quantitated by radioimmunoassay Data are reported as the means of triplicate determinations Residual deoxycholate was determined on 50 μl aliquots of the filtrates counted in 10 ml of scintillation cocktail (Scintiverse, Fisher Chem Co)

Enzyme source	Dowex 1/ deoxycholate (mg/mg)	Residual deoxycholate (mg/ml)	Tetraiodothyronine-5′-deiodinase (pmol T_3 formed/30 min)	3,3′,5′-triiodothyronine 5′-deiodinase (pmol I^- released/min)
Renal microsomes	0	0	9 4	8 6
Deoxycholate extract	0	2 0	4 1	3.8
	12	1 3	6 5	6 0
	15	1 1	7 4	6 8
	20	0.8	8 0	7 3
	30	0 6	8 0	7 3
	60	0 3	5.6	5 1

bound activity in the soluble fraction The composition of the solubilized fractions is shown in Fig 1B Optimal solubilization with respect to enzyme activity (i.e , 0 2% deoxycholate) was accompanied by partial delipidation, as judged by the relative recovery of phosphate and protein in the soluble fraction Treatment of microsomes with at least 0 3% deoxycholate restored the protein-phospholipid ratio of the detergent extract to that of the microsomal fraction but led to a considerable decrease in recovery of enzyme activity (Fig 1A)

The effects of increasing the Dowex 1 detergent ratio on the recovery of 5′-deiodinase activity are shown in Table I Enzyme assays done in the presence of 0 2% deoxycholate, the concentration used for solubilization, showed less than 10% of the enzyme activity found in the absence of detergent [13] A 10-fold dilution of the deoxycholate extract achieved during the enzyme assay restored 45% of the enzyme activity. Treatment of the deoxycholate extract with increasing amounts of Dowex 1 prior to enzyme assay resulted in increased 5′-deiodinating activity, optimal recovery of enzyme activity was achieved with a resin detergent ratio of 20 (Table I). Although the deoxycholate concentration was further reduced by treatment with larger amounts of Dowex 1 (resin detergent ratio of at least 50), the recovery of enzyme decreased, possibly due to adsorption of the enzyme on the anion-exchange resin In all subsequent experiments, enzyme solutions containing 0 2% deoxycholate were treated with Dowex 1 (40 mg/ml) for 20 min at 4°C, the resin removed by filtration on glass wool and the filtrate used for enzyme activity measurements

Isopycnic banding of solubilized iodothyronine 5′-deiodinase

Deoxycholate removal from detergent extracts of renal microsomes invariably rendered the 5′-deiodinating activity insoluble To study the nature of the enzymatically active aggregates obtained from the solubilized preparation, the density of the particulate enzyme(s) was determined on 10—60% linear sucrose gradients Detergent removal resulted in the formation of a heterogeneous population of sedimentible particles as judged by the protein distribution in the gradients (Fig 2). In contrast, both tetraiodothyronine and 3,3′,5′-triiodothyronine 5′-deiodinase showed single symmetrical peaks of activity with a density of approx 1 17 g/ml (Fig 2) The ratio of rT_3 5′-deiodination to T_4 5′-deiodination was 27.2 ± 2 (U/U) and remained constant across the peak, indicating that no fractionation of the enzymatic activities was achieved by sedimentation after detergent removal The density of the active aggregates was similar to that of microsomal membranes and suggested that the aggregates were composed of protein and lipid [25].

Effects of phospholipid on enzyme activity in renal microsomes

Since disruption of protein-lipid interaction was the expected mechanism of solubilization by deoxycholate, the effects of membrane components on enzyme activity were studied Soybean phospholipid was partially purified from Asolectin by the method of Kagawa and Racker [26] and unilaminar liposomes prepared by sonication. The data in Fig 3 show that exogenous phospholipid inhibited microsomal tetraiodothyronine 5′-deiodinase activity in a

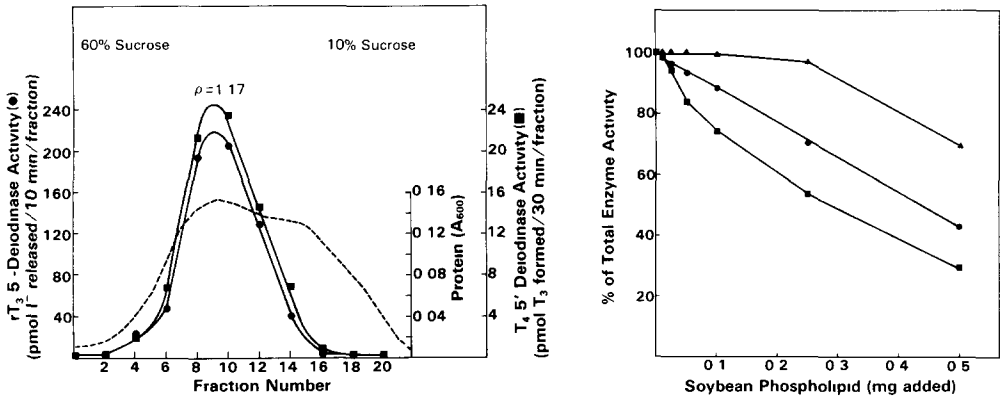

Fig 2 Isopycnic banding of solubilized iodothyronine 5′-deiodinase A standard deoxycholate extract from renal microsomes was prepared and the deoxycholate removed by Dowex 1 adsorption (40 mg resin per ml deoxycholate solution) After filtration, 200 μl of the opalescent enzyme solution was layered on 4 8 ml 10—60% linear sucrose gradients containing 20 mM Tris-HCl buffer, pH 7 5, 1 mM EDTA and 1 mM dithiothreitol Protein was determined by the method of Bradford [23] Data are reported as the means of three separate gradients (■) tetraiodothyronine 5′-deiodinase activity assayed at 0 5 μM T_4 with T_3 formation determined by a radiometric method (see Methods) (●) 3,3′,5′-triiodothyronine 5′-deiodinase activity assayed at 0 5 μM rT_3

Fig 3 Effects of lipids on iodothyronine 5′-deiodinase activity of renal microsomes Partially purified soybean phospholipids were suspended at 5 mg per ml in 50 mM Tris-HCl buffer, pH 7 5, containing 1 mM EDTA and 1 mM dithiothreitol and sonicated to clarity Renal microsomes (1 mg protein) and increasing concentrations of lipid were combined in a final volume of one ml and the resultant mixtures assayed for iodothyronine 5′-deiodinase activity at 0 5 μM T_4 (■), 5 μM T_4 (●) or 0 5 μM rT_3 (▲) T_3 formation was determined by radiometric assay in triplicate Data are reported at the percentage of control, enzyme assays were done in the absence of added lipid Data obtained at 0 5 μM T_4 and 5 μM T_4 were tested by two-way analysis of variance and found to be significant ($p < 0\ 002$) Combined S D of these data ranged from 4—12% of control

concentration dependent manner and that this inhibition was partially overcome by a 10-fold increase in the T_4 concentration. 3,3′,5′-Triiodothyronine 5′-deiodinase activity was also inhibited at lipid concentrations greater than 250 μg/ml. These results suggested that there was competition between lipid and the 5′-deiodinase for the iodothyronine substrates In all subsequent experiments, the T_4 and rT_3 concentrations were 10 μM and 1 μM, respectively, to diminish effects of exogenous phospholipid on enzyme activity.

Gel filtration on Sepharose CL-6B

In agreement with the results of the buoyant density measurements, gel filtration of deoxycholate extracts on Sepharose CL-6B in the absence of 0.2% deoxycholate resulted in quantitative recovery of both T_4 and rT_3 5′-deiodinating activities at the column void volume (data not shown) By contrast, gel filtration in the presence of 0.2% deoxycholate resulted in substantial losses of enzyme activity (over 90%), however, a small peak of activity with an elution volume similar to that of bovine serum albumin was routinely found (Fig. 4A, B). Since gel filtration in the presence of detergent might lead to separation of membrane components (i.e., protein and lipid) [27—29], the possibility that the proteins associated with 5′-deiodinating activity might require phospholipid for optimal activity was considered

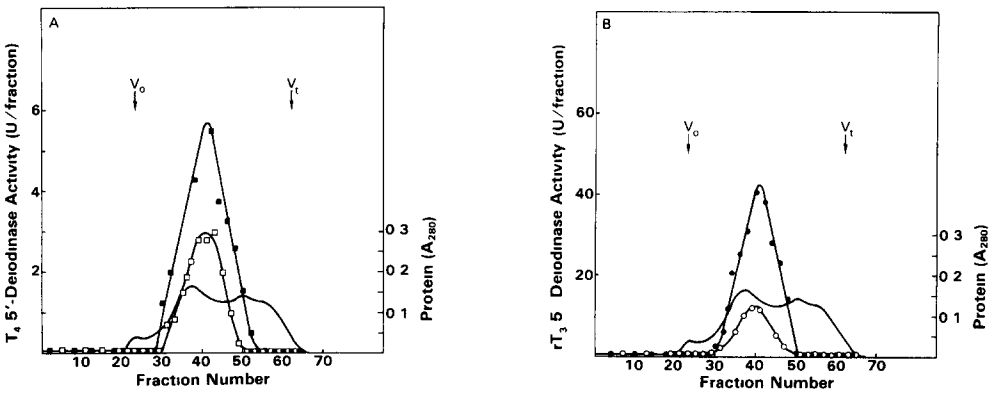

Fig 4 Effect of soybean phospholipids on iodothyronine 5′-deiodinase activities after gel filtration on Sepharose CL-6B in the presence of 0 2% deoxycholate The standard deoxycholate extract from renal microsomes was concentrated 10-fold by ultrafiltration (PM-10 membrane, Amicon) and two ml of the concentrate applied to a 1 5 X 85 cm column of Sepharose CL-6B equilibrated in 20 mM Tris-HCl buffer, pH 7 5, at 4°C, 1 mM EDTA, 1 mM dithiothreitol and 0 2% deoxycholate The column was developed with equilibration buffer at a flow rate of 15 ml/h and 2 ml fractions collected A clear solution of soybean phospholipid was prepared in equilibration buffer at 4 mg phospholipid per ml 50 μl of the phospholipid solution was added to 950 μl of selected column fractions and the mixture kept at 4°C for 1 h, after which the deoxycholate was removed by Dowex 1 adsorption and the fractions assayed for 5′-deiodinase activity V_0 and V_t represent the elution volumes of Blue Dextran and ^3H_2O, respectively (A) Tetraiodothyronine 5′-deiodinase activity before (□) and after (■) the addition of 200 μg phospholipid Enzyme assays were done with 10 μM T_4 and T_3 formation determined by radioimmunoassay (B) 3,3′,5′-Triiodothyronine 5′-deiodinase activity before (○) and after (●) the addition of 200 μg phospholipid.

The data in Fig 4A and B show that addition of 200 μg soybean phospholipid to column fractions prior to Dowex 1 treatment resulted in a 2- to 4-fold stimulation of enzyme activity Both tetraiodothyronine and 3,3′,5′-triiodothyronine 5′-deiodinase activities showed identical elution profiles, and the addition of phospholipid did not alter the enzyme distributions (Fig. 4A, B).

Phospholipid reconstitution of enzyme activity

The phospholipid reactivation of enzyme activity was further studied using partially purified soybean phospholipid and lipid-depleted enzyme obtained by gel filtration in the presence of 0.2% deoxycholate Such enzyme preparations obtained after gel filtration on Sephadex G-200 contained approx 40% of the phospholipid present in the renal microsomal fraction.

Increasing amounts of phospholipid were added to aliquots (100 μg protein) of the G-200 eluate and the reactivation mixtures were kept at 4°C for 1 h prior to detergent removal Shorter periods of standing prior to Dowex 1 treatment led to variable and inconsistent reactivation. The data in Fig. 5 show the results of a representative reconstitution experiment. Addition of increasing amounts of lipid resulted in a concentration dependent restoration of iodothyronine 5′-deiodinase activity, with maximal reactivation achieved between 75 and 100 μg phospholipid added per ml. Addition of 100 μg of soybean phospholipid resulted in a phospholipid.protein ratio in the reactivation mixture of 0.81 μmol total phosphate/mg protein, comparable to that of the renal membranes Since the molecular weight of the proteins in the pooled G-200

eluate ranged from 30 000—65 000, 0.81 μmol total phosphate/mg G-200 protein corresponds to 25—53 molecules phospholipid per protein molecule.

Physical parameters of the deoxycholate soluble tetraiodothyronine and 3,3',5'-triiodothyronine 5'-deiodinase

Although solubilized preparations of the enzymes were inhibited by deoxycholate and detergent removal resulted in the formation of macromolecular complexes, the ability to restore enzyme activity after fractionation by addition of lipids allowed the physical parameters of the deoxycholate soluble enzyme protein(s) to be estimated.

Estimation of $s_{20,w}$

Determination of $s_{20,w}$ for T_4 and rT_3 5'-deiodinase were done by the method of Martin and Ames [15] on 5—20% linear sucrose gradients, 0 2% deoxycholate was present to prevent reaggregation of the enzyme(s) during centrifugation The fractional radial migration (r/r_0) of six reference proteins was a linear function of their $s_{20,w}$ values (Fig 6) indicating that deoxycholate did not alter the relative sedimentation of these proteins. Iodothyronine 5'-deiodinase, assayed after supplementing gradient fractions with soybean phospholipid and detergent removal, migrated as single symmetrical coincident peaks with an estimated $s_{20,w}$, relative to the standards of 3 5 S (Fig 6). The effects of deoxycholate on the sedimentation behavior of iodothyronine 5'-deiodinase was further studied using gradients formed in 90% ^2H_2O.

Sedimentation of reference proteins in ^2H_2O sucrose gradients was, again, a linear function of the $s_{20,w}$ The data in Table II show the radial migration (r/r_0) in H_2O and ^2H_2O sucrose gradients for three reference proteins and for iodothyronine 5'-deiodinase Partial specific volumes ($\bar{v}$) for catalase, aldolase, and ovalbumin were calculated by the method of Clarke [16] and showed good agreement with published values. The $\bar{v}$ of both tetraiodothyronine and 3,3',5'-triiodothyronine 5'-deiodinase was 0.74 cm^3/g (Table II) This is within the range for globular proteins and suggested that only a small amount of deoxycholate was bound to the enzyme(s) under these conditions [17,18]

The co-sedimentation of enzyme activities during gradient centrifugation and the co-elution during gel filtration suggested that the protein(s) catalyzing the respective 5'-deiodination reactions shared common physical properties and are consistent with the proposal that a single enzyme catalyzes both reactions [3,11,14].

Estimation of Stokes' radius

The effects of deoxycholate on the sieving properties of the gel or on the effective Stokes' radius of the proteins were studied by calibration of the Sephadex G-200 column in the absence and presence of 0 2% deoxycholate [30] Reference proteins were chromatographed on a 0.9 × 58 cm column of Sephadex G-200 and the effective pore radius of the gel, r, was calculated as described by Ackers [31]. The mean r was 18.4 ± 2 3 mm (n = 5) in the absence and 18 1 ± 1.0 nm (n = 5) in the presence of 0 2% deoxycholate, in close agreement with the value obtained by Ackers [31] and indicating that 0.2% deoxycholate did not alter the sieving properties of the gel.

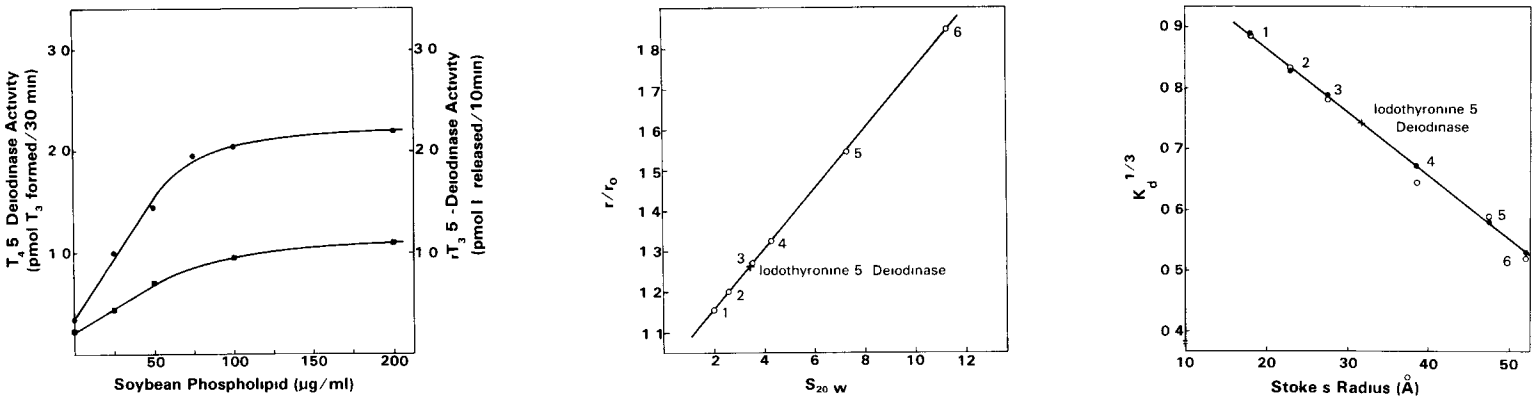

Fig 5 Effect of soybean phospholipid on restoration of iodothyronine 5′-deiodinase activity to lipid depleted enzyem Lipid-depleted enzyme and soybean phospholipid in 20 mM Tris-HCl buffer, pH 7 5, 1 mM EDTA, 1 mM dithiothreitol and 0 2% deoxycholate were prepared Reconstitution mixtures done in duplicate contained in a total volume of 300 µl 100 µg enzyme protein, increasing amounts of soybean phospholipid and buffer After 1 h at 4°C, 15 mg of Dowex 1 resin was added to the mixture, and 20 min later the resin was removed by filtration on glass wool 25 µl aliquots of the filtrates were assayed in parallel for tetraiodothyronine (T_4, 3,5,3′,5′-tetraiodothyronine ■) and 3,3′,5′-triiodothyronine (rT_3, 3,3′,5′-triiodothyronine ●) 5′-deiodinase activity at 10 µM T_4 and 1 µM rT_3 Enzyme assays were done in triplicate and the data are reported as the means of values obtained from duplicate reconstitution mixtures Attention is directed to the difference in the ordinate scales for tetraiodothyronine and 3,3′,5′-triiodothyronine 5′-deiodinase activity

Fig 6 Determination of the sedimentation coefficient for iodothyronine 5′-deiodinase in the presence of 0 2% deoxycholate Protein standards were (1) ribonuclease A, (2) chymotrypsinogen, (3) ovalbumin, (4) bovine serum albumin, (5) aldolase, (6) catalase Tetraiodothyronine and 3,3′,5′-triiodothyronine 5′-deiodinase activities were assayed at 10 µM T_4 and 1 µM rT_3, respectively

Fig 7 Determination of Stokes' radius for iodothyronine 5′-deiodinase on Sephadex G-200 in the presence of 0 2% deoxycholate The data are presented as proposed by Porath [21] Reference proteins, chromatographed in the absence (●) or presence (○) of 0 2% deoxycholate were (1) ribonuclease A, (2) chymotrypsinogen, (3) ovalbumin, (4) bovine serum albumin, (5) aldolase, (6) catalase

TABLE II

DETERMINATION OF PARTIAL SPECIFIC VOLUME ($\bar{v}$) AND SEDIMENTATION COEFFICIENT ($s_{20,w}$) FOR IODOTHYRONINE 5'-DEIODINASE

Sedimentation was done on linear sucrose gradients Iodothyronine 5'-deiodinase activities (tetraiodothyronine and 3,3',5'-triiodothyronine) were assayed at 10 μM T_4 and 1 μM rT_3, respectively Data are reported as the means of values obtained from three separate gradients for each sample

Sample	r/r (H_2O)	r/r_0 (^2H_2O)	$\bar{v}$ * (cm^3/g)	$\bar{v}$** (cm^3/g)	$s_{20,w}$ (S)
Catalase ***	1 85	1 53	0 73	0 730	11 3
Aldolase	1 54	1 35	0 74	0 742	7 35
Ovalbumin	1 27	1 16	0 74	0 746	3 55
Iodothyronine 5'-deiodinase	1 26	1 16	0 74		3 46

* Values calculated by the method of Clarke [16]

** Values for reference proteins taken from the literature [19,20]

*** Sedimentation was performed on gradients which did not contain 1 mM dithiothreitol

Gel filtration of the deoxycholate extract of renal microsomes was done in the presence of 0.2% deoxycholate and column fractions were supplemented with soybean phospholipid (200 μg/ml) prior to Dowex 1 treatment and enzyme assay. Both enzyme activities showed single symmetrical peaks, with a Stokes' radius of 32 Å (Fig 7)

Molecular weight and frictional ratio

A molecular weight for deoxycholate soluble iodothyronine 5'-deiodinase of 49 900 was computed from the $s_{20,w}$ (3.5 S), $\bar{v}$ (0 74 ± 0.1 cm^3/g) and R_s (32.3 ± 0 5 Å) [20]. Similarly, a frictional ratio (f/f_0) of 1.32 was calculated [20] indicating a modest asymmetry in the enzyme protein which is common to intrinsic membrane proteins [27].

Discussion

Previous studies on iodothyronine 5'-deiodination have depended on broken cell or crude membrane preparations as the source of enzyme. In light of the limitations imposed by these impure preparations and the ambiguity concerning the number of 5'-deiodinating enzymes, attempts were made to obtain a soluble iodothyronine 5'-deiodinase The data in this paper characterize the deoxycholate solubilized enzyme and its phospholipid dependency and extend the observations to the 5'-deiodination of rT_3.

Solubilization of iodothyronine deiodinases from rat liver microsomes has been recently reported by Fekkes et al. [32]. These investigators found that cholate and a mixture of nonionic Brij detergents were the most effective solubilizing agents, although inactivation of the deiodinases (over 40%) was noted. Removal of the detergent from the solubilized fraction by dilution or ion-exchange chromatography resulted in a doubling of catalytic activity and reaggregation of the enzyme These findings are in agreement with those reported previously by us for the kidney enzyme [13] and presented in this report.

Deoxycholate solubilization of enzyme activity was optimal at a detergent to protein ratio (w/w) of 0 4, with 55—75% recovery of the activity in the soluble fraction and little or no activity present in the deoxycholate insoluble fraction Solubilization also resulted in partial delipidation (approx 35%) and it is therefore possible that delipidation may have been a factor in the incomplete recovery of enzyme activity

Successful reconstitutions of the mitochondrial proton pump [26] and several membrane bound enzymes [27—29,33] from crude bile salt extracts or from mixtures of lipid-detergent micelles and purified membrane proteins have been reported. In most cases, removal of the detergent, an obligate step in reconstitution, resulted in the formation of enzymatically active macromolecular complexes Iodothyronine 5′-deiodinase showed similar properties, detergent removal from inactive deoxycholate extracts resulted in the formation of enzymatically active particles with a density of 1 17 g/ml consistent with the formation of a lipid-protein aggregate [25]

Substantial inactivation of the enzyme occurred when deoxycholate extracts were chromatographed on Sepharose CL-6B in the presence of detergent Initial attempts to improve the yield of enzymes by addition of lipid to the column fractions resulted in further decreases in residual catalysis rather than the expected enhancement of enzyme activity Since iodothyronines are lipophilic [34] these results suggested that phospholipid might paradoxically activate the enzyme via lipid reconstitution and inhibit the enzyme via sequestration of the substrate Added phospholipid was found to decrease renal microsomal iodothyronine 5′-deiodinase activity and this inhibition was partially overcome by increasing the substrate concentration Column fractions assayed using concentrations of iodothyronine sufficiently high to minimize the inhibitory effects of phospholipid on the membrane bound activity showed the stimulatory effect of lipid replacement

Iodothyronine 5′-deiodinase showed an absolute requirement for lipid. Addition of increasing concentrations of phospholipid to the delipidated enzyme, followed by detergent removal, resulted in a lipid-dependent restoration of the enzyme activity. Interestingly, reconstitution reached a maximum when sufficient lipid was added to restore the phospholipid.protein ratio to that of the renal microsomal membrane

Sedimentation analysis of deoxycholate extracts of renal microsomes was done on linear sucrose gradients containing 0.2% deoxycholate in H_2O and ^2H_2O solvents The sedimentation coefficient for iodothyronine 5′deiodinase was 3 5 S This is in reasonably good agreement with the value (4 3 S) obtained for the liver enzyme in the presence of 0 6% cholate [32]. Fekkes et al [32] found that omission of detergent or alteration of the detergent type greatly influenced the sedimentation rate of the enzyme, suggesting that changes in binding of detergent to enzyme or perhaps variable delipidation altered the 'molecular size' of the liver deiodinase Our studies comparing the sedimentation in detergent-containing sucrose gradients in H_2O and ^2H_2O using the method of Clarke [16] showed that the deoxycholate-soluble renal iodothyronine 5′-deiodinase had a partial specific volume of 0 74 cm^3/g, a value similar to that of detergent-free globular proteins and thus suggesting that little detergent was bound to the enzyme Although a $\bar{v}$ of 0 74 cm^3/g might seem

low for a membrane protein it is by no means unique, the studies of Neer [17] and Asbury [18] showed that Triton X-100 soluble adenylate cyclase had a $\bar{v}$ of 0 74 cm^3/g and this led to the conclusion that little detergent was bound to the solubilized membrane protein

Unlike the observations of Fekkes et al [32], we found lipid reconstitution in gradient fractions to be essential for enzyme activity The Stokes' radius of 32 Å for the renal enzyme in the presence of 0 2% deoxycholate is in good agreement with the 35—37 Å reported for the cholate soluble liver enzyme [32]

Both tetraiodothyronine and 3,3',5'-triiodothyronine 5'-deiodinase activities were associated with protein(s) which shared common properties. Molecular parameters of the enzyme(s) were indistinguishable, neither sucrose gradient centrifugation nor gel filtration separated these enzymic species Both activities were rendered soluble by similar concentrations of deoxycholate and showed similar lipid reconstitution patterns Although there is no direct evidence that the soluble enzyme catalyzing the respective 5'-deiodination reactions is a single species, these data are consistent with and complement earlier work on T_4 and rT_3 5'-deiodination It has been shown that both T_4 and rT_3 5'-deiodination follow ping-pong type reaction kinetics [13,14,35] and both reactions are inhibited by thiouracil in an uncompetitive fashion [11,36]. In addition, inhibition of tetraiodothyronine 5'-deiodinase by chemical modification of essential sulfhydryls [13,14] has been extended to the 5'-deiodination of rT_3, 6-*n*-propylthiouracil, tetraiodothyronine and 3,3',5'-triiodothyronine individually protected both deiodinase reactions from inhibition by iodoacetate pretreatment [14] Taken together, these data support the proposal that both T_4 and rT_3 are 5'-deiodinated by a single enzyme.

The renal enzyme has been shown to be an intrinsic membrane protein with a requirement for a structural relationship between lipids and the enzyme molecule for optimal activity Although a phospholipid requirement for the hepatic 5'-deiodinase has yet to be demonstrated, the recently reported aggregation upon dilution, enhancement of activity by detergent removal and the sedimentation and gel filtration behavior as influenced by detergents [32] all suggest that lipids play a role in the detergent soluble liver enzyme Purification of a catalytically active enzyme from crude membrane preparations will most likely require lipid reconstitution The results presented in this report provide a basis for further studies on more purified enzyme preparations necessary to resolve the question as to the identity of the 5'-deiodinases of tetraiodothyronine and 3,3',5'-triiodothyronine

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