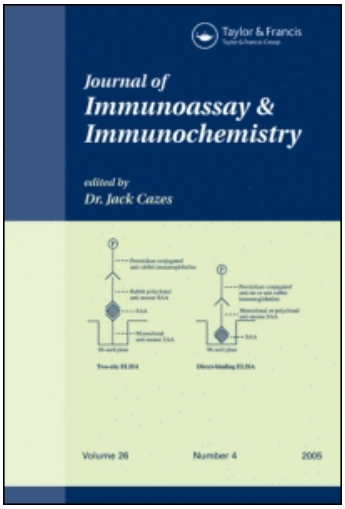

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DEVELOPMENT OF A RADIOIMMUNOASSAY FOR TRIIODOTHYRONINE SULFATE

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ABSTRACT

This paper is the first description of a radioimmunoassay (RIA) for triiodothyronine sulfate (T_3S). Rabbits were immunized against T_3S coupled to bovine serum albumin using carbodiimide. All animals produced antibodies to T_3S but also even higher titers of T_3 antibodies. Ka values for binding of T_3 and T_3S to these antisera varied between $2x10^{10}$ and $8x10^{10}$ M^{-1}. One of the antisera (#8193) was selected for use in the T_3S RIA because of a high titer of T_3S antibodies (final dilution 1 : 50,000), a high sensitivity to T_3S (<2.5 fmol/tube), and a low crossreactivity by T_3 (0.4%). This RIA provides a tool for the study of the importance of sulfation as a metabolic pathway for T_3. (KEY WORDS: thyroid hormone, triiodothyronine sulfate, radioimmunoassay)

INTRODUCTION

Triiodothyronine (T_3) is the bioactive form of thyroid hormone. Roughly 20% of circulating T_3 is derived from thyroidal secretion while the majority is produced by phenolic ring deiodination of thyroxine (T_4) in peripheral tissues, especially the liver (1,2). In rats, T_3 is metabolized by deiodination of the tyrosyl ring and by conjugation of the phenolic hydroxyl group

with sulfate or glucuronic acid (3,4). Although direct deiodination of T_3 occurs in extrahepatic tissues (2,3), sulfation enhances T_3 deiodination in the liver (5,6). In contrast, T_3 glucuronide (T_3G) is a stable conjugate. Thus, after incubation of T_3 with isolated rat hepatocytes, iodide and T_3G are the major products (5). Accumulation of T_3 sulfate (T_3S) in these cultures occurs only after inhibition of deiodinase activity, for instance with propylthiouracil (PTU) (5). To further investigate the role of sulfation in T_3 metabolism we decided to develop a radioimmunoassay (RIA) for T_3S. Here we report the characteristics of this RIA.

METHODS

Production of Antiserum. The T_3S-protein conjugate used for immunization was prepared as follows. To 100 mg anhydrous T_3 (Sigma) and 1 μCi [^{125}I]T_3 (Amersham) was added 0.5 ml of a mixture of chlorosulfonic acid and di-methylformamide (1/4, v/v) at 0 C (7). After reaction for 40 h at room temperature, T_3S was precipitated by addition of this mixture to 5 ml H_2O at 0 C. The pellet was dissolved in 1 ml 2 M NH_4OH and reprecipitated with 5 ml 1 M HCl. The pellet was further washed by repeated suspension in 3 ml 0.1 M HCl. Analysis of this material by HPLC (7) indicated that it consisted of T_3S and only 1% T_3. The T_3S was dissolved in 5 ml dimethylformamide and added to a solution of 100 mg bovine serum albumin (BSA) in 20 ml H_2O. After adjusting the mixture to pH 5 with 0.1 M NaOH, 50 mg 1-ethyl-3-(3-dimethylaminopropyl)-

carbodiimide (Sigma) was added in 5 ml H_2O (8). This was repeated after 3 h, and the mixture was further stirred for 16 h at room temperature. The product was dialysed at 4 C against successively 2x2 1 H_2O, 2 1 0.1 M NaOH and 3x2 1 H_2O, changed twice daily. Analysis of remaining radioactivity indicated a low degree of T_3S incorporation (<5%), corresponding to <5 mol T_3S/mol BSA. The immunogen was stored at -20 C in a concentration of 2 mg protein/ml.

Four New Zealand white rabbits were immunized by s.c. injections with 1 mg conjugate in 1 ml of a 1 : 1 mixture of water and complete Freund's adjuvant at multiple sites in the back. This was repeated after 2 and 4 months and subsequently at irregular intervals of 1-6 months. High-titer antibodies to T_3 and T_3S were detected in serum of all animals from the second immunization onwards. Two rabbits (#8190 and #8193) produced high-affinity T_3S antibodies, and the characteristics of antisera obtained 2 weeks after the 10^{th} injection (15 months after start of immunization) are described here.

Radioimmunoassay. Radioactive T_3S was prepared by reaction of [^{125}I]T_3 with chlorosulfonic acid and purified as previously described (7). In general, approximately 25,000 cpm (~8 fmol) ^{125}I-labeled T_3 or T_3S were incubated for at least 48 hours at 4 C with appropriate dilutions of antiserum #8190 or #8193 in a final volume of 1 ml RIA buffer (0.06 M barbital, 0.15 M NaCl, 0.1 % BSA, pH 8.6). Preliminary results had shown that this incubation period was necessary to obtain equilibrium, and that tracer binding was constant over a broad pH range. Antibody-bound

radioactivity was precipitated by addition of goat anti-rabbit IgG antiserum and polyethylene glycol 6000 (2.5 % final concentration). After incubation for 1 h at 4 C, the tubes were centrifuged, the supernatants were aspirated and the pellets were counted for radioactivity. Reference iodothyronines were obtained from Henning, Berlin, FRG, and sulfate conjugates were synthesized in our laboratory (7). Solutions of standards and analogs were prepared in 0.01 M NaOH and assayed in duplicate in 50-100 µl aliquots.

RESULTS

Figure 1 shows the binding of T_3 and T_3S tracers to progressive dilutions of antisera #8190 and #8193. Both antisera contained high titers of antibodies to T_3 and T_3S. However, higher dilutions of antiserum could be used to obtain the same degree of T_3 binding compared with T_3S. Binding of biosynthetic T_3G tracer to even low dilutions of antiserum was negligible (not shown). As a suitable condition for the RIA roughly 35% binding of T_3 tracer to either antiserum occured at a final dilution of 1 : 800,000 and in case of T_3S tracer at 1 : 50,000-60,000.

Using these antiserum dilutions the characteristics of T_3 and T_3S binding were further investigated. Significant displacement of T_3S tracer was observed with <2.5 (#8193) or 5 (#8190) fmol unlabeled T_3S per tube (Fig. 2). In case of the T_3 RIA lower limits of detection amounted to 3-5 fmol T_3 per tube. Logit-log

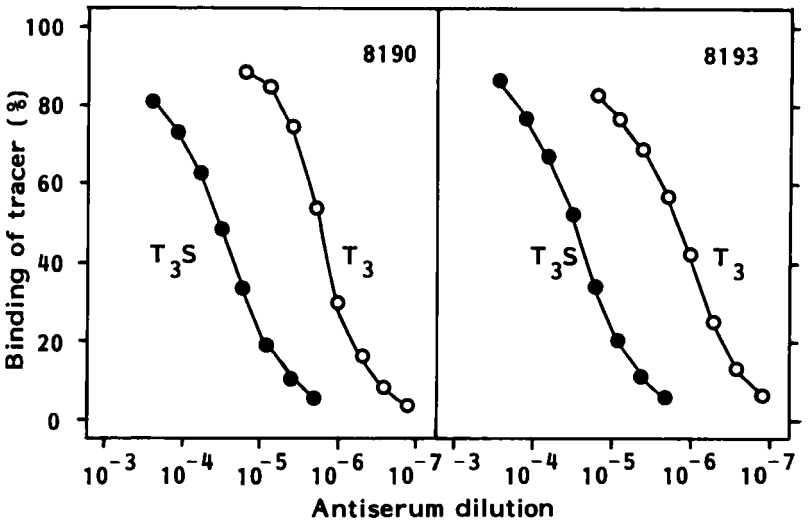

FIGURE 1. Binding of [^{125}I]T_3S (●) or [^{125}I]T_3 (○) to progressive dilutions of antiserum #8190 (left) or #8193 (right).

plots of the standard curves showed excellent fits ($r>0.99$). Linear transforms were also constructed using the method of Scatchard (9) with r values >0.95, which permitted estimation of average binding parameters of the antisera. Binding of T_3S to antisera #8190 and #8193 was characterised by Ka values of $2x10^{10}$ and $8x10^{10}$ M^{-1} and by values for the maximal binding capacity (MBC) in undiluted antiserum of 1.7 and 0.4 μM, respectively. In case of T_3, Ka values were $8x10^{10}$ and $3x10^{10}$ M^{-1} and MBC values 8 and 15 μM, respectively.

Not only the affinity but also the specificity of the binding of T_3S was different between the antisera (Fig. 2 and Table 1). Binding of T_3S to antiserum #8190 showed a high degree of

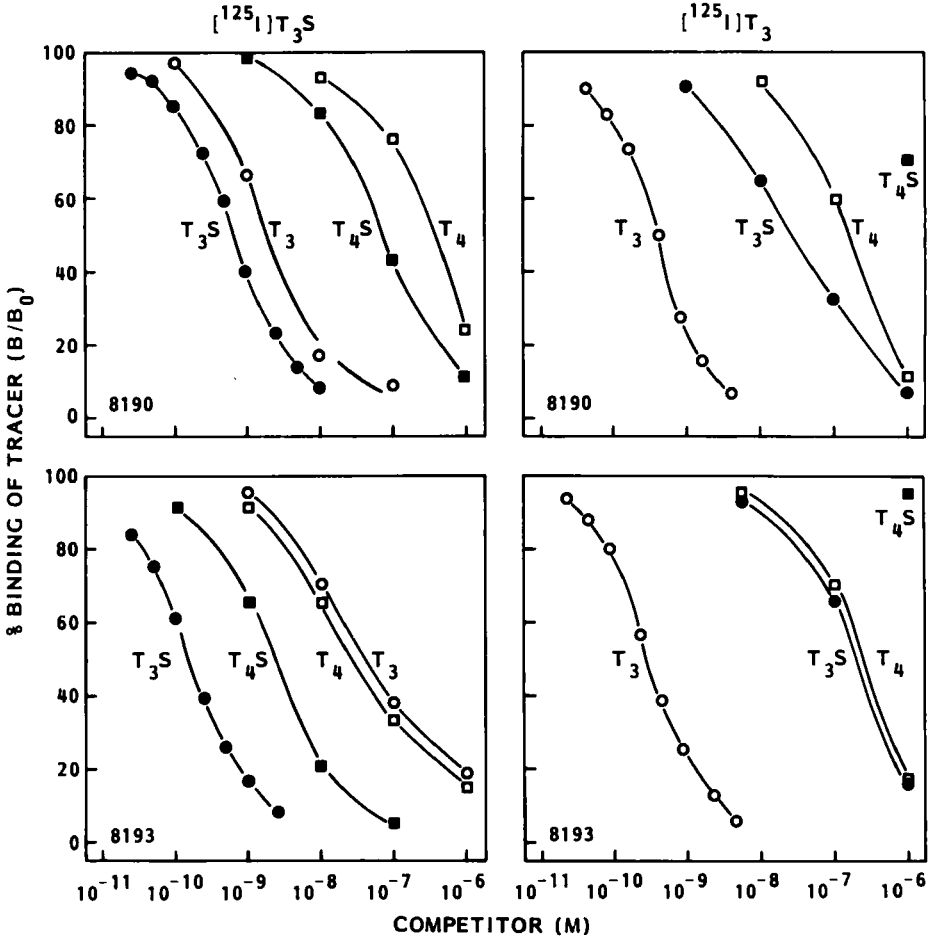

FIGURE 2. Inhibition of the binding of $[^{125}I]T_3S$ (left) to antiserum #8190 (1 : 60,000) or #8193 (1 : 50,000) or that of $[^{125}I]T_3$ (right) to antiserum #8190 or #8193 (both 1 : 800,000) by increasing concentrations of unlabeled T_3S (●), T_3 (○), T_4 (□) or T_4S (■). Results with antiserum #8190 are given in the upper panels and those with antiserum #8193 in the lower panels.

TABLE 1

Specificity of Binding of T_3S and T_3 to Antisera #8190 and #8193

Competitor	Crossreactivity (%)			
	$[^{125}I]T_3S$		$[^{125}I]T_3$	
	#8190 (1:60,000)	#8193 (1:50,000)	#8190 (1:800,000)	#8193 (1:800,000)
T_4	0.2	0.6	0.2	0.2
T_4S	0.9	9	<0.1	<0.01
T_3	31	0.4	100	100
T_3S	100	100	0.9	0.2
rT_3	<0.01	<0.01	<0.01	<0.01
rT_3S	<0.1	0.2	<0.01	<0.01
$3,5\text{-}T_2$	2	<0.01	0.6	0.4
$3,3'\text{-}T_2$	0.4	<0.01	0.3	0.5
$3,3'\text{-}T_2S$	2	0.5	<0.01	<0.01
$3\text{-}T_1$	<0.01	<0.01	<0.1	<0.01
$3'\text{-}T_1$	<0.01	<0.01	<0.01	<0.1
T_0	<0.01	<0.01	<0.01	<0.01

Percentage crossreactivity is defined as the ratio (x100) of the concentration of ligand divided by the concentration of competing substance that each produce 50 % displacement of tracer. Results are calculated after logit-log transformation of the dose-response curves and represent the means of at least three different experiments. rT_3, 3,3′,5′-triiodothyronine; T_2, diiodothyronine; T_1, iodothyronine; T_0, thyronine; S, sulfate.

crossreactivity by T_3 but much less interference by T_4 and T_4 sulfate (T_4S). On the other hand, T_4S was a potent competitor for binding of T_3S to antiserum #8193 while T_3 was much less reactive. Low to absent crossreactivity was observed with all other iodothyronine analogs whether sulfated or not. Less differences were seen between the specificities of T_3 binding to the two antisera (Fig. 2 and Table 1). T_3S was more effective in competing

with T_3 for antiserum #8190 compared with #8193. However, crossreactivity remained below 1% as was the case with all analogs tested, including T_4.

DISCUSSION

This study represents the first report of a RIA for the measurement of an iodothyronine conjugate. Previous studies had already demonstrated that it is feasible to measure sulfate conjugates of steroids by RIA. An established method for the determination of plasma dehydroepiandrosterone sulfate makes use of the high crossreactivity of the conjugate with antiserum produced against the nonconjugated steroid (10). However, recent reports have also described the production of specific antibodies to sulfate conjugates, e.g. estradiol-3-sulfate, that do not crossreact with the free steroid (11).

Although we immunized rabbits against a conjugate prepared by coupling pure T_3S to albumin, the antisera produced contain distinct types of antibody, the most abundant of which specifically binds T_3. The reason for this is unknown but it is possible that part of the sulfate conjugate is hydrolysed during immunogen preparation or in vivo. Extensive hydrolysis has been observed during coupling of phenolic steroid sulfates to protein using the mixed anhydride method but not with carbodiimide (12), the coupling reagent also used by us. The crossreactivity of T_3S with the binding of T_3 tracer to the antisera was of similar magnitude as that observed with antibodies raised against free T_3 as the antigen (7). It is not excluded that part of this

crossreactivity by T_3S is due to slight contamination with free T_3.

Of greater interest to our studies is the other class of antibodies that show preference for T_3S as the ligand. Especially T_3S binding to antiserum #8193 is little affected by T_3, although this favorable property may in part result from the sequestration of T_3 by the high-capacity T_3-specific antibodies. Notwithstanding the low crossreactivity of T_4 in the T_3S RIA, it may still create a significant problem for the measurement of T_3S in samples with high T_4 levels such as plasma. The similar effects of T_4 and T_3 in the T_3S RIA with antiserum #8193 suggest that in this case the steric hindrance of the extra iodine in T_4 is counter-balanced by the favorable influence of the dissociated hydroxyl group. The latter introduces a negative charge in the same region of the molecule as the sulfate group.

In conclusion, the present study demonstrates the feasibility of the development of a RIA for T_3S. Preliminary studies have shown accumulation of immunoreactive T_3S in incubations of T_3 with isolated rat hepatocytes if subsequent deiodination of the conjugate is inhibited (13). T_3S immunoreactivity has also been detected in plasma of PTU-treated rats (14). Studies are now in progress in our laboratory to adapt this RIA for the measurement of T_3S in human plasma.

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