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APPROACHES TO A MARKEDLY INCREASED SENSITIVITY OF THE RADIOIMMUNOASSAY FOR THYROTROPIN-RELEASING HORMONE BY DERIVATIZATION

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Summary

Antisera to thyrotropin-releasing hormone (pGlu-His-Pro-NH_2, TRH) have previously been produced in rabbits by immunization with a conjugate having TRH linked to a carrier protein by means of a dinitrophenylene (Dnp) moiety. Studies on the specificity of the antisera obtained suggested that the sensitivity of the radioimmunoassay for TRH may be increased substantially by prior conversion of the hormone into dinitrophenylene derivatives.

To test this possibility, several TRH-Dnp derivatives were prepared by reaction of TRH with equimolar amounts of 1,5-difluoro-2,4-dinitrobenzene yielding N^{im}-(5-fluoro-2,4-dinitrophenyl)TRH. This intermediate was reacted with ammonia, histamine, tyramine or N^{α}-acetyl-lysine methyl ester (N^{α}Ac-Lys-OMe) to yield the respective unsubstituted and N-substituted N^{im}-(5-amino-2,4-dinitrophenyl)TRH derivatives: TRH-Dnp-NH_2, TRH-Dnp-histamine, TRH-Dnp-tyramine and TRH-Dnp-N^{α}Ac-Lys-OMe. N^{im}-(2,4-Dinitrophenyl)TRH was prepared similarly by reaction of TRH with 1-fluoro-2,4-dinitrobenzene. The products were isolated by means of high-performance liquid chromatography (HPLC) and were found to be pure by HPLC and thin-layer chromatography using several solvent systems. TRH-Dnp-histamine and TRH-Dnp-tyramine were labelled with ^{125}I using the chloramine-T method. The labelled products were purified to homogeneity by ion-exchange chromatography on SP-Sephadex and adsorption chromatography on Sephadex LH-20, respectively, and were found by HPLC to be pure.

Abbreviations: TRH, thyrotropin-releasing hormone; Dnp, dinitrophenyl(ene); DFDNB, difluorodinitrobenzene; FDNB, fluorodinitrobenzene; FDNA, fluorodinitroaniline; F-Dnp, fluorodinitrophenyl; Bzl, benzyl.

It was observed that by employing these modified labels instead of radioiodinated TRH (having the same specific activity), antiserum could be used at up to 400-fold higher dilution to achieve the same level of binding. The sensitivity of the modified radioimmunoassay to the TRH-Dnp derivatives was increased approx. 100-fold compared with that of the conventional assay to TRH.

It is concluded that the sensitivity of the radioimmunoassay for TRH is improved considerably by using a radioiodinated TRH-Dnp derivative and by prior derivatization of the ligand. The lower limit of detection may then amount to less than 0.2 pg of TRH-equivalents per tube.

Introduction

It has been proven to be very difficult to measure endogenous thyrotropin-releasing hormone (pGlu-His-Pro-NH_2, TRH) levels by radioimmunoassay. The main problem is the low concentration of the hormone in body fluids. This is probably not only because of the low amounts of TRH secreted but also due to the extensive degradation occurring at several sites in the body such as plasma and various tissues [1]. Also, the specificity of existing antibodies used in the radioimmunoassay of TRH has been questioned (see, for example, Ref. 2).

The first radioimmunoassay for TRH was described by Bassiri and Utiger [3]. Their method of producing antisera has been adopted by most investigators. It involves the use of a conjugate in which TRH is coupled to a protein carrier with the aid of bis-diazotized benzidine. In another procedure, *p*-aminophenylacetic acid is diazotized and reacted with TRH. The derivative formed is subsequently coupled to protein with the use of a carbodiimide [4]. In an entirely different approach a TRH-protein conjugate is obtained which does not involve the imidazole of the tripeptide [5]. Here, an amide bond is formed between pGlu-His-Pro-OH and NH_2 groups of a protein carrier.

We have previously described the adoption of Tager's method [6] in the preparation of a TRH-protein conjugate using 1,5-difluoro-2,4-dinitrobenzene [7,8]. In this conjugate, TRH is linked to the carrier via a dinitrophenylene moiety (Fig. 1, R = protein). Antisera to TRH were readily produced by the immunization of rabbits with such a conjugate. Using these antisera and radioiodinated TRH ([^{125}I]TRH) a radioimmunoassay was developed which was sensitive to approx. 20 pg TRH/tube. It was noted, however, that the affinity of some analogues, i.e. pGlu-Phe-Pro-NH_2 and pGlu-N^{im}Bzl-His-Pro-NH_2, for the antibodies was greater than that of TRH itself. This was interpreted to indicate that TRH was only part of the antigenic determinant, since it lacked the complementary Dnp group. It was predicted [7,8] that the sensitivity should improve if TRH were converted into some Dnp derivative prior to assay.

We now report on the preparation of several TRH-Dnp derivatives (Fig. 1). Some of these contain phenol and imidazole groups to enable the synthesis of radioiodinated ligands. Compared with [^{125}I]TRH, the use of ^{125}I-labelled TRH-Dnp derivatives allowed up to 400-fold higher dilution of antiserum in the radioimmunoassay. The sensitivity of this new radioimmunoassay to some of these derivatives was increased approx. 100-fold compared with the sensitivity

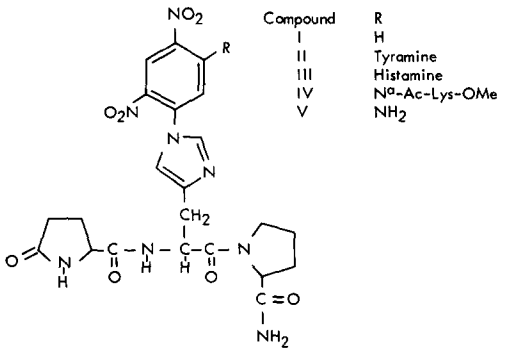

Fig. 1. Structures of TRH-Dnp derivatives.

of the conventional assay to TRH. The lower limit of detection of the modified method, therefore, amounts to approx. 0.2 pg TRH-equivalent per tube.

Materials and Methods

Materials

TRH was purchased from Beckman Bioproducts, Geneva, Switzerland; 1,5-difluoro-2,4-dinitrobenzene, 1-fluoro-2,4-dinitrobenzene (FDNB), tyramine, histamine and N^{α}-acetyl-lysine methyl ester (N^{α}Ac-Lys-OMe) from Sigma, St. Louis, MO, U.S.A.; 5-fluoro-2,4-dinitroaniline (FDNA) from Pierce, Rockford, IL, U.S.A.; [^{3}H]TRH (60 μCi/μg) from New England Nuclear, Dreieichenhain, F.R.G.; and $^{125}I^-$ (14 mCi/μg) from the Radiochemical Centre, Amersham, U.K.; Sephadex G-10, Sephadex LH-20 and SP-Sephadex from Pharmacia, Uppsala, Sweden.

Preparation of TRH-Dnp derivatives

Both thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC) were used to follow the reactions. TLC was performed on silica-gel 60 F254 plates (Merck) using as solvents *n*-butanol/ethylacetate/acetic acid/water, 1 : 1 : 1 : 1; *i*-propanol/benzene/water, 4 : 3 : 1; chloroform/methanol/conc. ammonia, 60 : 45 : 10; and chloroform/methanol/38% acetic acid, 3 : 2 : 1. Reversed-phase HPLC was done on a 30 × 0.4 cm μBondapak (C_{18}) column using a Model 6000A solvent delivery system and monitoring absorbance at 254 nm with a Model 440 fixed wavelength detector (Waters, Melford, MA, U.S.A.). As solvents, mixtures of water, 0.01 M ammonium acetate (pH 4) or 0.01 M sodium borate (pH 9) with methanol or acetonitrile of various compositions were used. Flow rate was 1.5 ml/min.

For the synthesis of TRH-Dnp (Fig. 1, compound I) to 5 μmol FDNB in 400 μl dimethylformamide was added a mixture of 1.25 μmol TRH (containing 1.25 μCi [^{3}H]TRH) and 1.25 μmol triethylamine in 100 μl dimethylformamide at room temperature. This was repeated three times at intervals of 15 min. After mixing for an additional period of 2 h at room temperature, the mixture was kept at 4°C under nitrogen in the dark until further processing.

This involved evaporation of the solvent at 50°C under reduced pressure, dissolution of the residue in a mixture of water and methanol (2 : 1, v/v) and purification by HPLC using this mixture as the solvent.

The synthesis of the other derivatives (Fig. 1, compounds II—V) was carried out as follows. N^{im}-(5-Fluoro-2,4-dinitrophenyl)TRH (TRH-F-Dnp) was prepared with the same procedure as for TRH-Dnp substituting DFDNB for FDNB. Without further purification the intermediate was reacted with 5 μmol tyramine or histamine and 5 μmol triethylamine, with 5 μmol N^{α} Ac-Lys-OMe (HCl salt) and 10 μmol triethylamine, or with 20 μl conc. ammonia in 400 μl dimethylformamide. After agitation for 2 h at room temperature, the mixtures were processed as described for TRH-Dnp. Purification on HPLC was done using as solvent water/methanol (1 : 1, v/v) for the separation of TRH-Dnp-tyramine (II), TRH-Dnp-N^{α}Ac-Lys-OMe (IV) and TRH-Dnp-NH_2 (V), and 0.01 M ammonium acetate (pH 4)/acetonitrile (5 : 1, v/v) for the separation of TRH-Dnp-histamine (III).

The concentration of the various derivatives in the final preparation was calculated from the recovery of added radioactivity and is expressed (on a weight basis) in terms of TRH-equivalents.

Preparation of [^{125}I]TRH, TRH-Dnp-[^{125}I]tyramine and TRH-Dnp-[^{125}I]histamine

Radioiodination of TRH, compound II or III was carried out by a modification of the chloramine-T method [9]. Approx. 2 μg TRH-equivalents were reacted with 1 mCi $^{125}I^-$ and 10 μg chloramine T in 50 μl 0.1 M phosphate, pH 7.5. After 1 min at room temperature the reaction was halted by the addition of 100 μg sodium metabisulfite.

In case of TRH, the reaction mixture was fractionated on a 7 × 0.5 cm Sephadex G-10 column with 0.01 M phosphate/0.15 M NaCl/0.25% bovine serum albumin (pH 7.5). Part of the radioactivity appearing in the first of the two peaks from this column was applied to a 15 × 1.5 cm SP-Sephadex column, which was eluted with a linear salt gradient obtained by mixing 100 ml 0.01 M ammonium acetate (pH 5.5) with 100 ml of this buffer containing 0.4 M NaCl (see also Ref. 10). Fractions of 2.5 ml were collected.

The iodination mixture with TRH-Dnp-histamine was directly applied to the SP-Sephadex column and conditions for elution were as described above for [^{125}I]TRH.

Radioiodination of TRH-Dnp-tyramine was followed by elution over a 7 × 0.5 cm Sephadex LH-20 column with 0.05 M sodium borate (pH 8.5)/ethanol (7 : 3, v/v). Fractions of 1.2 ml were collected.

Columns were calibrated with $^{125}I^-$ and the tritiated forms of TRH and the derivatives. The purity of the ^{125}I-labelled derivatives was checked on HPLC using conditions similar to those described above for the non-radioiodinated compounds.

Radioimmunological procedures

Anti-TRH antisera 4094 and 4101, obtained as previously described using DFDNB [7,8], and two similarly but more recently produced antisera 4319 and 4619 were used in the present study.

Serial dilutions of antiserum were incubated for 3 days at 4°C with 15 000

cpm (approx. 1.5 pg TRH-equivalents) [^{125}I]TRH, TRH-Dnp-[^{125}I]tyramine or TRH-Dnp-[^{125}I]histamine in 1 ml 0.05 M sodium phosphate/0.05 M NaCl/ 0.1 M KCl/0.25% bovine serum albumin (pH 7.5). Antibody-bound radioactivity was precipitated using the second antibody technique, and counted with 85% efficiency in a Searle gamma spectrometer. In the radioimmunoassay, appropriately diluted antiserum was incubated with radioiodinated ligand in the absence or presence of increasing amounts of unlabelled TRH or compounds I—V. Conditions for incubations and separation of antibody-bound from free radioactivity were as described above.

Synthesis of the various TRH-Dnp derivatives, both unlabelled and radioiodinated, as well as the radioimmunological procedures (performed in duplicate) were repeated several times with closely agreeing results. Typical examples of these experiments are shown.

Results

Preparation on TRH-Dnp derivatives

Retention times of TRH and FDNB on HPLC using water/methanol (1 : 1, v/v) as the solvent amounted to approx. 1.5 and 6 min, respectively. Reaction of TRH (and [^{3}H]TRH) with FDNB yielded mainly one radioactive, ultraviolet-absorbing and Pauly-negative product as evidenced by HPLC (Fig. 2) and TLC. Retention time of this material in the above system was 3 min. After 1 h at room temperature the reaction had virtually proceeded until completion. The product was isolated by HPLC and was found by HPLC (Fig. 2) and TLC using several solvent systems to be pure.

Reaction of TRH with DFDNB yielded a product with similar characteristics as TRH-Dnp (Fig. 3). Retention time of the product also amounted to 3 min com-

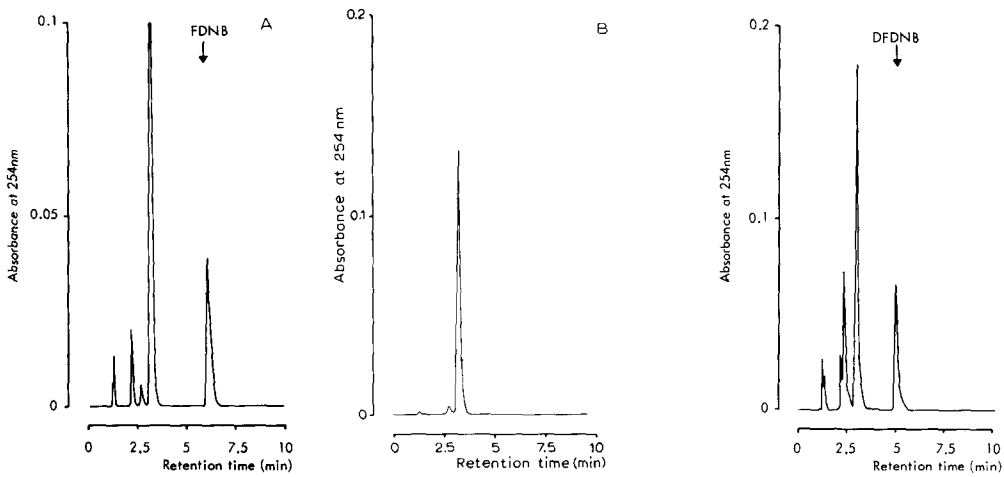

Fig. 2. A. HPLC of the reaction mixture of TRH with FDNB. B. HPLC of isolated TRH-Dnp (solvent, water/methanol, 1 : 1).

Fig. 3. HPLC of the reaction mixture of TRH with DFDNB (solvent, water/methanol, 1 : 1).

pared with 5 min for DFDNB. Reaction of the crude mixture containing TRH-F-Dnp with tyramine gave rise to a new product with a retention time of 7.5 min simultaneous with the disappearance of TRH-F-Dnp and the further disappearance of DFDNB (Fig. 4). Conversion into this product was almost complete within 2 h. It was shown to be radioactive and to form a brown colour on TLC plates with Pauly reagent. The derivative was isolated by HPLC and evidence for its purity was obtained by HPLC (Fig. 4) and TLC.

TRH-Dnp-N^{α}Ac-Lys-OMe (IV) and TRH-Dnp-NH_2 (V) were prepared simi-

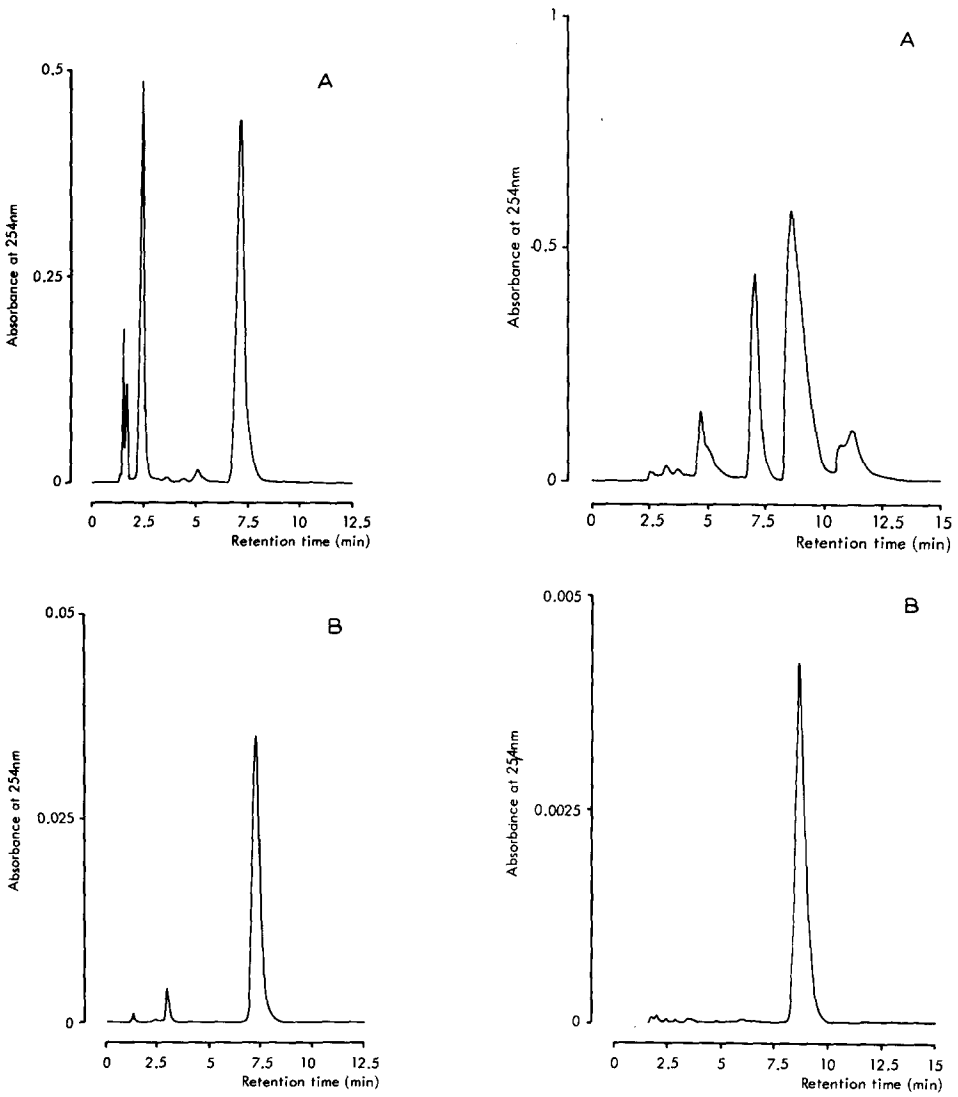

Fig. 4. (Left-hand figures.) A. HPLC of the reaction mixture of crude TRH-F-Dnp with tyramine. B. HPLC of isolated TRH-Dnp-tyramine (solvent, water/methanol, 1 : 1).

Fig. 5. (Right-hand figures.) A. HPLC of the reaction mixture of crude TRH-F-Dnp with histamine. B. HPLC of isolated TRH-Dnp-histamine (solvent, 0.01 M ammonium acetate (pH 4)/acetonitrile, 5 : 1).

larly by reaction of crude TRH-F-Dnp with N^{α}Ac-Lys-OMe or conc. ammonia, respectively. Retention times on HPLC with water/methanol (1 : 1, v/v) amounted to 6 and 4 min, respectively. The products isolated on HPLC were found to migrate as single spots on TLC, which did not colour with Pauly reagent. The properties of compound V were identical with the reaction product of TRH with FDNA in the presence of triethylamine in dimethylformamide at 50°C. The yield of the latter reaction, however, was low.

Purification of the reaction product of TRH-F-Dnp with histamine was not possible on HPLC with unbuffered water/methanol mixtures. Resolution improved by using acidic buffers and by substituting acetonitrile for methanol. TRH-Dnp-histamine was isolated using 0.01 M ammonium acetate (pH 4)/acetonitrile (5 : 1, v/v) as the solvent (Fig. 5). TLC in several solvents demonstrated a single substance forming an orange colour with Pauly reagent.

Preparation of radioiodinated TRH and derivatives

Ion-exchange chromatography on SP-Sephadex has been shown to yield the clear separation between iodide, unlabelled, mono- and diiodinated TRH [10]. This method was adopted to purify the iodination products of TRH (unpublished results) and TRH-Dnp-histamine (Fig. 6). Radioiodide eluted from the SP-Sephadex column in fractions 5—8, [^{3}H]TRH in fractions 28—34, [^{125}I]-TRH in 18—22, [^{3}H]TRH-Dnp-histamine in 40—46 and TRH-Dnp-[^{125}I]histamine in 27—33. Virtually all radioactivity in the latter peak eluted as a single component from the HPLC column with a retention time of 9 min in 0.01 M ammonium acetate (pH 4)/acetonitrile (3 : 1, v/v) (Fig. 6).

The radioiodination product of TRH-Dnp-tyramine — in contrast to the unlabelled derivative — was retarded on Sephadex columns. The ^{125}I-labelled compound was isolated on Sephadex LH-20 with a 0.05 M borate (pH 8.5)/30% ethanol mixture (Fig. 7). Over 80% of the product obtained eluted as a single peak from the reversed-phase column with a retention time of 5 min in 0.01 M borate (pH 9)/acetonitrile (2 : 1, v/v) (Fig. 7).

Under the conditions we tested, usually 40—80% of ^{125}I was incorporated in TRH and the derivatives. Based on the specific activity of ^{125}I^{-} that of the radioiodinated compounds was calculated to amount to approx. 4800 μCi/μg TRH-equivalent.

Radioimmunoassays

Binding of [^{125}I]TRH, TRH-Dnp-[^{125}I]tyramine and TRH-Dnp-[^{125}I]histamine as a function of dilution of antiserum 4094 is shown in Fig. 8. By substituting normal rabbit serum for antiserum it was found that only approx. 1, 2 and 1%, respectively, of total radioactivity was bound nonspecifically. Fig. 8 demonstrates that with the radioiodinated TRH-Dnp derivatives the antiserum may be used at a much higher dilution in order to achieve the same degree of binding as with [^{125}I]TRH. This is not due to differences in the concentrations of the radioactive ligands, since the amount of radioactivity was the same in the several experiments, as was the specific radioactivity of these tracers. From the curves in Fig. 8 the dilution of antiserum was chosen which afforded the binding of approx. 40% of the total radioactivity added. These dilutions amounted to 1 : 10^{4}, 1 : 10^{6} and 1 : $4 \cdot 10^{6}$ for [^{125}I]TRH, TRH-Dnp-[^{125}I]tyramine and

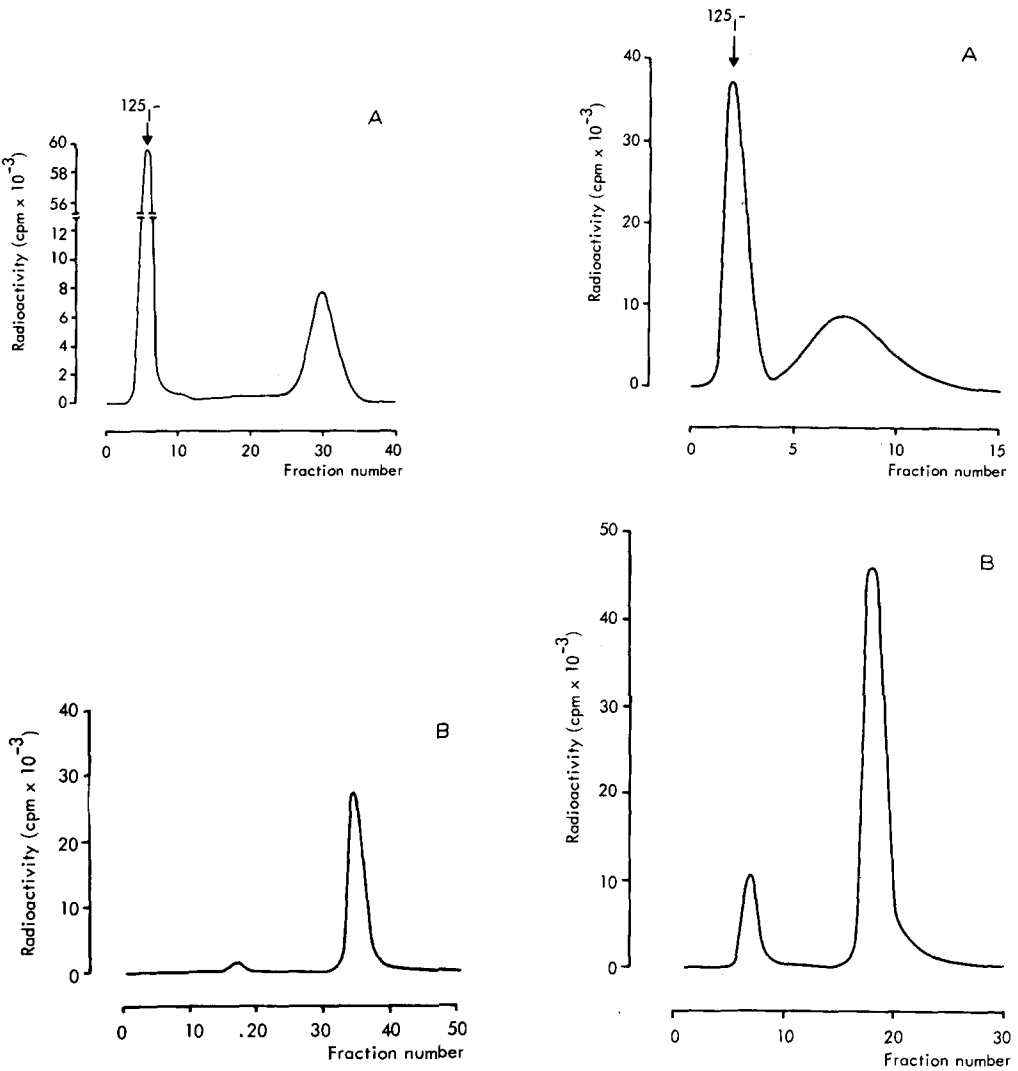

Fig. 6. (Left-hand figures.) A. SP-Sephadex chromatography of iodination mixture with TRH-Dnp-histamine (solvent, 0—0.4 M NaCl in 0.01 M ammonium acetate, pH 5.5). B. HPLC of isolated TRH-Dnp-[^{125}I]histamine. Fractions of 0.375 ml (15 s) were collected (solvent, 0.01 M ammonium acetate (pH 4)/acetonitrile, 3 : 1).

Fig. 7. (Right-hand figures.) A. Sephadex LH-20 chromatography of iodination mixture with TRH-Dnp-tyramine (solvent, 0.05 M sodium borate (pH 8.5)/ethanol, 7 : 3). B. HPLC of isolated TRH-Dnp-[^{125}I]-tyramine. Fractions of 0.375 ml (15 s) were collected (solvent, 0.01 M sodium borate (pH 9)/acetonitrile, 2 : 1).

TRH-Dnp-[^{125}I]histamine, respectively.

Using these antiserum dilutions, the effect of unlabelled TRH and compounds I—V on the binding of the three radioactive ligands to the antibodies was studied. In Fig. 9 are depicted the several dose-response curves using [^{125}I]-TRH as the tracer. Although all curves fall closely together it is noteworthy

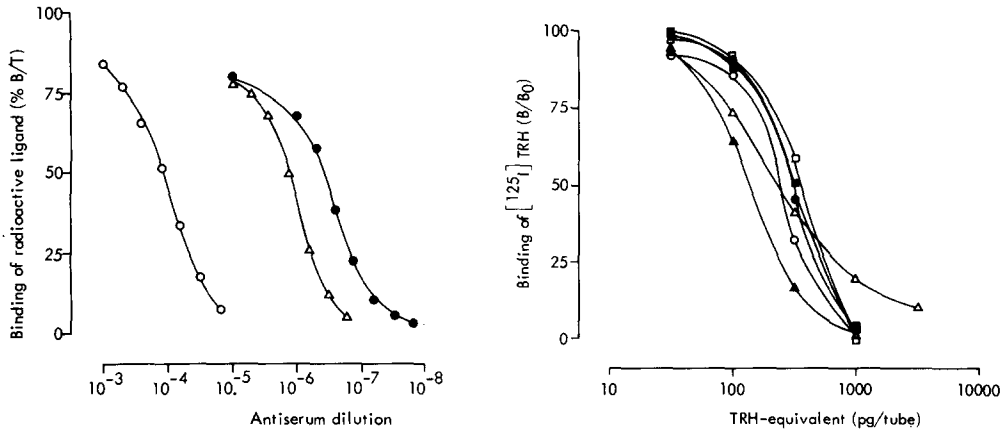

Fig. 8. Binding (*B*) of radioiodinated TRH and derivatives as a function of antiserum dilution and expressed as a percentage of the total amount of radioactivity added (*T*). Antiserum 4094. ○, [^{125}I]-TRH; △, TRH-Dnp-[^{125}I]tyramine; ●, TRH-Dnp-[^{125}I]histamine.

Fig. 9. Inhibition of the binding of [^{125}I]TRH to antiserum 4094, diluted 1 : 10^4, by increasing concentrations of TRH or TRH-Dnp derivatives. Binding (*B*) is expressed as a percentage of that in the absence of unlabelled compounds (B_0). △, TRH; ▲, compound I; ●, II; ○, III; □, IV; ■, V.

that those of the TRH-Dnp derivatives are much steeper than that of TRH. Under the conditions tested, TRH-Dnp (I) appeared to be the most potent in competing with [^{125}I]TRH for the binding sites in the antiserum. The concentrations of the various compounds needed to displace 50% of [^{125}I]TRH from

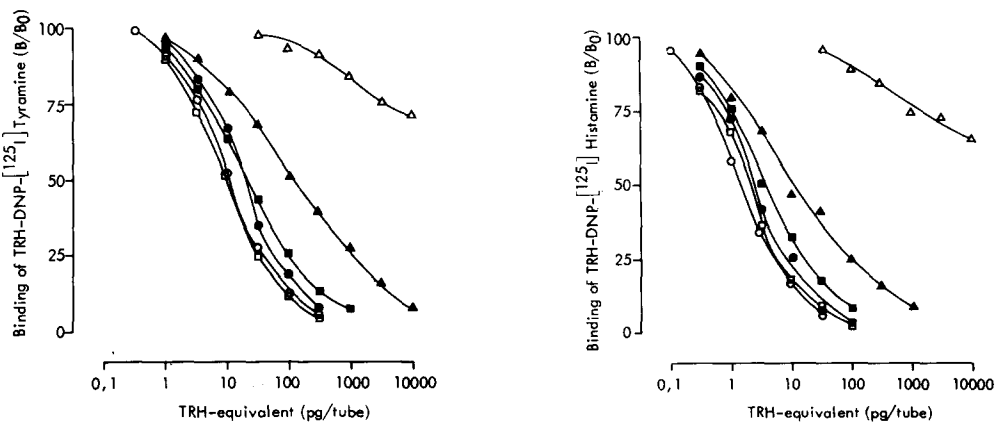

Fig. 10. Inhibition of the binding of TRH-Dnp-[^{125}I]tyramine to antiserum 4094, diluted 1 : 10^6, by increasing concentrations of TRH or TRH-Dnp derivatives. Binding (*B*) is expressed as a percentage of that in the absence of unlabelled compounds (B_0). △, TRH; ▲, I; ●, II; ○, III; □, IV; ■, V.

Fig. 11. Inhibition of the binding of TRH-Dnp-[^{125}I]histamine to antiserum 4094, diluted 1 : $4 \cdot 10^6$, by increasing concentrations of TRH or TRH-Dnp derivatives. Binding (*B*0 is expressed as a percentage of that in the absence of unlabelled compounds (B_0). △, TRH; ▲, I; ●, II; ○, III; □, IV; ■, V.

the antibodies vary between 100—400 pg TRH-equivalents/tube (Table I).

In Fig. 10 the results with TRH-Dnp-[^{125}I]tyramine and more diluted antiserum are shown. Compared with [^{125}I]TRH (Fig. 9) the situation has changed drastically. Here, TRH has become virtually inactive, inhibiting the binding of TRH-Dnp-[^{125}I]tyramine by only 30% at a concentration of as high as 10 ng/tube. Among the derivatives, TRH-Dnp (I) is now the least effective. Nevertheless, under the conditions tested similar amounts of the compound are needed to inhibit the binding of TRH-Dnp-[^{125}I]tyramine and that of [^{125}I]-TRH (Table I). However, the activity of compounds II—V is increased considerably. Only 10—20 pg of TRH-equivalents are needed to inhibit the binding of radioiodinated compound II by 50%, compared with 200—400 pg in the displacement of [^{125}I]TRH (Table I). Among these compounds, TRH-Dnp-histamine (III) and TRH-Dnp-N^{α}Ac-Lys-OMe (IV) are the most active, both with an ID_{50} of only 10 pg TRH-equivalents per tube.

Even greater sensitivity is obtained using TRH-Dnp-[^{125}I]histamine and a still higher dilution of antiserum (Fig. 11). Only 10 pg TRH-equivalents of compound I are required for 50% inhibition (Table I), with a lower limit of sensitivity of less than 1 pg/tube. Of the other derivatives, TRH-Dnp-histamine (III) appeared to be the most active, followed closely by the other compounds (Table I). Only approx. 0.2 and 1 pg TRH-equivalents of compound III are needed to inhibit binding of radioactive ligand by 10 and 50%, respectively. Also in this case TRH at a concentration of 10 ng/tube displaces only 30% of the tracer.

Very similar observations were made with antisera 4101, 4319 and 4619 (Table I). Noteworthy is the finding that binding of ^{125}I-labelled compound III to antiserum 4619 is as effectively inhibited by TRH-Dnp as by the other deri-

TABLE I

CONCENTRATIONS OF TRH AND SEVERAL DERIVATIVES WHICH INHIBIT THE BINDING OF [^{125}I]TRH, TRH-Dnp-[^{125}I]TYRAMINE OF TRH-Dnp-[^{125}I]HISTAMINE TO ANTISERA BY 50% (ID_{50})

Concentrations of the various compounds are given in pg TRH-equivalents per tube (1 ml). Compound numbers refer to structures shown in Fig. 1.

Antiserum	Dilution	[^{125}I]Ligand *	ID_{50}					
			TRH	I	II	III	IV	V
4094	$1:1\cdot10^4$	TRH	200	100	300	200	400	300
	$1:1\cdot10^6$	II	$>10^4$	100	20	10	9	20
	$1:4\cdot10^6$	III	$>10^4$	10	2	1	2	4
4101	$1;5\cdot10^3$	TRH	400	300	600	400	600	600
	$1:5\cdot10^5$	II	$>10^4$	200	20	9	9	9
	$1:2\cdot10^6$	III	$>10^4$	20	3	1	2	3
4319	$1:1\cdot10^4$	TRH	50	30	40	30	40	40
	$1:1\cdot10^5$	II	$>10^4$	90	30	10	20	40
	$1:3\cdot10^5$	III	$>10^4$	10	3	2	2	3
4619	$1:6\cdot10^3$	TRH	60	70	80	80	100	100
	$1:2.5\cdot10^5$	II	$>10^4$	20	8	5	6	8
	$1:1\cdot10^6$	III	$>10^4$	1	1	1	1	1

* 150 000 cpm (approx. 1.5 pg TRH-equivalent) of each tracer was used.

vatives. With all compounds a significant response is observed with as low as 0.2 pg TRH-equivalents and 50% inhibition with 1 pg.

Discussion

It has been known for a long time, especially in the field of steroid radioimmunoassay [11], that the type of 'bridge' used in the conjugation of the hapten to the carrier protein and the site of its attachment to the hapten determine the specificity of the antiserum to be obtained. More specifically, it has been shown that radioiodinated derivatives of steroids have greatly increased affinities for antisera raised against conjugates in which similar derivatives of the hapten were coupled to protein [11,12]. Similar observations were made in the development of radioimmunoassays for prostaglandins [13]. Noteworthy in this respect are the findings that antibodies raised against Dnp-protein complexes bind N^{ϵ}Dnp-lysine 10—100 times more avidly than they bind dinitroaniline [14]. It appears, therefore, that the moiety linking the hapten to the carrier backbone is recognized by the antibody even if this side-chain was originally part of the protein.

Also in case of TRH, the specificity of the antiserum is dependent on the type of conjugate used in its production. Thus, antibodies raised against pGlu-His-Pro-(NH-protein) do not bind [^{125}I]TRH, where the iodine is introduced in the imidazole ring at C^2 or C^4. In addition, pGlu-N^{im}Bzl-His-Pro-NH_2 does not compete with TRH for the binding sites in the antiserum. The difference between the amide in TRH and the methyl ester in pGlu-His-Pro-OMe is, however, not recognized [5]. On the other hand, antibodies obtained by immunization of rabbits with conjugates, where the site of attachment is at the imidazole, have quite different properties. Here it is possible to develop a radioimmunoassay using [^{125}I]TRH. This holds irrespective of whether conjugation involved C^2 or C^4 as with the diazotized reagents [3,4] or one of the nitrogens in position 1 and 3 of the imidazole ring as with DFDNB [7,8]. Not only pGlu-N^{im}Bzl-His-Pro-NH_2 but also pGlu-Phe-Pro-NH_2 show even greater affinity for these antibodies than TRH itself [7,8]. Replacing the NH_2-terminal amide by the methyl ester, however, greatly reduces the affinity for the binding sites in these antisera.

The high activity of pGlu-N^{im}Bzl-His-Pro-NH_2 and pGlu-Phe-Pro-NH_2 in the radioimmunoassay for TRH suggested that besides TRH other parts of the immunogen have played a role in determining the specificity of the antibodies it elicited. This would include principally the Dnp moiety and perhaps also the residues in the protein it was attached to. These considerations are substantiated by the results described here. Testing identical amounts of the radioactive ligands, it was noted that up to 400-fold lower concentrations of antiserum sufficed to achieve the same percentage binding of the labelled TRH-Dnp derivatives as with [^{125}I]TRH (Fig. 8). This may indicate that the affinity of the derivatives for the antibodies is much higher than that of TRH. An alternative explanation would be the possible heterogeneity of the antiserum containing antibodies which bind the derivatives but not TRH, as well as antibodies binding to which does not require the presence of the Dnp group. The existence of the latter population of antibodies is concluded from the finding

that the TRH-Dnp derivatives completely inhibit the binding of radioiodinated TRH (Fig. 9). On the other hand, TRH hardly affects the binding of the labelled TRH-Dnp derivatives (Figs. 10 and 11). Other studies have shown that, indeed, maximum inhibition by TRH of the binding of the derivatives amounts to only 30%, indicating that TRH binds primarily to a subpopulation of antibodies.

This was confirmed by the Scatchard analysis [15] of the data presented in Figs. 9—11 (not shown). For this it was assumed that the unlabelled and radioactive forms of the ligands bind with the same affinity to the antiserum. In case of the [^{125}I]TRH/TRH and TRH-Dnp-[^{125}I]tyramine/TRH-Dnp-tyramine systems, non-linear Scatchard plots were obtained, each of which could be resolved in two lines. These results illustrate the heterogeneity of the antiserum containing two main populations of antibodies. The maximal binding capacity of the high-affinity antibodies was found to be 0.9 and 2.5 μg/ml TRH-equivalent per ml antiserum, with K_a values of $4 \cdot 10^9$ and $60 \cdot 10^9$ M^{-1} for TRH and the tyramine derivative, respectively. In case of the TRH-Dnp-[^{125}I]histamine/TRH-Dnp-histamine system, one set of binding sites appeared mainly to be involved. Here the K_a could not be calculated from the Scatchard plot, as these sites were already completely saturated in the presence of tracer only. The maximal binding capacity was 2.4 μg TRH-equivalents per ml antiserum, being in close agreement with the value obtained with the tyramine derivative.

These findings suggest that immunization with a TRH-Dnp-protein conjugate gives rise to the production of distinct types of antibody. The main difference between these populations appears to reside in the antigen-binding site depth as operationally defined in [14]. The deeper the cleft in the combining region of the antibody the better the interaction with the hapten, resulting in increased values of K_a. Deletion of the hapten tail, i.e., the N-substituted dinitroaniline moiety, would then result in a decreased affinity of the antigen for the deeper binding sites. The less deep combining sites would, however, not suffer a great deal from the deletion of this hapten tail. Our studies suggest that the antiserum contains a subpopulation of antibodies with shallow combining sites binding TRH and the Dnp derivatives with similar but low affinity. Antibodies in the second population have deeper antigen-binding clefts, which recognize the difference between TRH and the hapten. These antibodies only bind the TRH-Dnp derivatives with high affinity.

It seems, therefore, inevitable that sensitivity is lost if a radioimmunoassay is developed which is aimed to detect only part of the antigen. Our observations indicate that sensitivity is regained if the antigenic structure is restored prior to assay. No attempts have been made to optimize the radioimmunoassay with respect to the lowest concentrations to be detected. The results demonstrate, however, that by derivatization the limits of detection may be lowered by at least two orders of magnitude. Further studies are therefore planned to bring these limits even further down and to develop a method for the isolation and derivatization of TRH from body fluids. Measurements of TRH-Dnp derivatives instead of the hormone itself may eventually proove to be more reliable than existing assays for TRH. In this respect the results obtained with antiserum 4619 are promising since they demonstrate that maximum sensitivity is obtained with TRH-Dnp, which is easily prepared compared with the two-stage synthesis of the other derivatives.

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References

1 Griffiths, E.C., Kelly, J.A., Forbes, R., Jeffcoate, S.L., White, N., Molner, R.D.G. and Visser, T.J. (1979) in Brain Peptides: A New Endocrinology (Gotto, Jr., A.M., Peck, Jr., E.J. and Boyd, III, A.E., eds.), pp. 197—209, Elsevier/North-Holland Biomedical Press, Amsterdam
2 Youngblood, W.W., Lipton, M.A. and Kizer, J.S. (1978) Brain Res. 151, 99—116
3 Bassiri, R.M. and Utiger, R.D. (1972) Endocrinology 90, 722—727
4 Visser, T.J., Klootwijk, W., Docter, R. and Hennemann, G. (1976) Neuroendocrinology 21, 204—213
5 Visser, T.J., Docter, R. and Hennemann, G. (1974) Acta Endocrinol. 77, 417—421
6 Tager, H.S. (1976) Anal. Biochem. 71, 367—375
7 Visser, T.J., Klootwijk, W., Docter, R. and Hennemann, G. (1977) FEBS Lett. 83, 37—40
8 Visser, T.J., Klootwijk, W., Docter, R. and Hennemann, G. (1978) in Radioimmunoassay and Related Procedures in Medicine, Vol. II, International Atomic Energy Agency, Vienna, pp. 469—477
9 Greenwood, F.S., Hunter, W.M. and Glover, J.S. (1963) Biochem. J. 89, 114—123
10 Ling, N., Leppäluoto, J. and Vale, W. (1976) Anal. Biochem. 76, 125—133
11 Pratt, J.J. (1978) Clin. Chem. 24, 1869—1890
12 Gomez-Sanchez, C., Milewich, L. and Holland, O.B. (1977) J. Lab. Clin. Med. 89, 902—909
13 Maclouf, J., Sors, H., Pradelles, P. and Dray, F. (1978) Anal. Biochem. 87, 169—176
14 Halsey, J.F., Cebra, J.J. and Biltonen, R.L. (1975) Biochemistry 14, 5221—5224
15 Scatchard, G. (1949) Ann. N.Y. Acad. Sci. 51, 660—672