A Radio-Immunoassay for the Determination of Thyroid Stimulating Hormone

Thyroid stimulating hormone (TSH) is a mucoprotein of molecular weight between 20,000 and 30,000. A pure sample of this hormone is not at present available; the preparations of BATES and CONDLIFFE[1–3], although homogeneous in the ultracentrifuge, are found to contain several components after electrophoresis in starch gel. This heterogenicity is caused by the presence of several active fractions. The lack of a pure sample of TSH makes the design of an immunoassay for TSH especially difficult.

Despite the heterogenicity of the available preparations, several authors have established the antigenicity of TSH. This antigenicity has been demonstrated *in vitro*, using hemagglutination and agar diffusion techniques[4–8]. Different authors[6–10] have shown that antibodies against TSH neutralize the *in vitro* and *in vivo* biological effect of TSH. Antiserum against bovine TSH neutralized, in both systems, the activity of bovine and of human TSH. Ten to twenty times more antibovine serum is required to neutralize human TSH than is required to neutralize a similar quantity of bovine TSH. Similarly, twenty-five times more antibovine serum than antihuman serum is required to neutralize the thyrotrophic effect of myxoedemic serum[6].

UTIGER[11] has recently shown that the antibodies elicited by highly purified human and bovine TSH are species specific and no cross reaction occurs between antihuman serum and bovine TSH. Where a cross reaction has been shown to occur, it is probable that the samples of TSH used were not adequately purified and antibodies were formed against the several fractions of the TSH, producing an antiserum capable of neutralizing homologous and heterologous TSH.

Once TSH had been shown to be antigenic, several groups attempted to establish an immunoassay. An assay using the inhibition of hemagglutination of erythrocytes sensitized to TSH is very sensitive, provided that the TSH is determined in buffer. This assay can detect as little as 0.02 or 0.04 mU of TSH in buffer, but does not give any results when applied to biological liquids (serum or urine[12]). There seem to be non-specific inhibitors in the biological fluids; agglutination can be demonstrated but not inhibition of agglutination by the antiserum. In the early stages of the radio-immunoassay reported, similar difficulties were encountered. TSH could be determined in buffer but not in serum or plasma[11]. Experiments were then carried out to determine the optimal conditions for the assay in serum and it is now possible to assay TSH in biological fluids.

Material and methods. Theory: This assay is based on the HALES and RANDLE[13] immunoassay for insulin and on the assay for ACTH described by FELBER[14]. TSH bound to antibodies is precipitated by the addition of anti-γ-globulins; the precipitated complex is then isolated from the medium by filtration through Oxoid membrane filters. Details of the timing of the assay and the reactions taking place at each stage are given in Table I.

Labelling of TSH with I^{131}: Three preparations of TSH have been used in the development of the assay. The first is Armour bovine TSH, the second bovine TSH (initially 12 UI/mg, then 6 UI/mg) which was a gift from Dr. P. G. CONDLIFFE, and the third bovine TSH purified in this laboratory according to CONDLIFFE[3] from the fraction E of REISFELD[15].

The preparation of the I^{131} TSH is carried out using the chloramine T method of GREENWOOD and HUNTER[16], 10 μg TSH being labelled on each occasion. The reaction mixture, after quenching with sodium metabisulphite, is transferred to a 1.0×10.0 cm column of Sephadex G-50. The equilibration of the column is carried out with phosphate buffer pH 7.4 0.04 *M* containing beef serum albumin, and the elution is carried out with phosphate buffer pH 7.5 0.01 *M* containing 0.15 *M* NaCl. The eluate is collected in 1 ml portions in tubes containing 0.1 ml of 2% beef serum albumin. The first three fractions contain little or no radioactivity; tubes 4 to 6 contain the majority of the radioactivity and the purest fractions of TSH (Figure 1). Later fractions contain diminishing amounts of radioactivity which are composed of a mixture of labelled proteins – in particular labelled albumins. Paper electrophoresis is carried out on each fraction (10 μl of samples, phosphate buffer pH 7.4 0.04 *M*) for 1 h at 400 V with the tanks at + 4°C. The electrophoretograms are scanned in a Nuclear Chicago Actigraph (Figure 2A, B).

Table I. Time-table of TSH radio-immunological test at 4°C

Time	Reaction
0 h	0.1 ml TSH standard or plasma 0.1 ml diluted anti-TSH serum
	↓
30 min	(antibody-TSH)
	↓ ← 0.1 ml TSH labelled with I^{131}
	(antibody < TSH / TSH*) mixed 3 times with Vortex Mixer
	↓
5 h	0.2 ml anti-γ-globulin mixed 3 times with Vortex Mixer
	↓
	[(antibody < TSH / TSH*) -anti-γ-globulin]
	↓
22 h	filtration of the complex

[1] R. W. BATES, M. M. GARRISON, and T. B. HOWARD, Endocrinology *65*, 7 (1959).
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[14] J.-P. FELBER, Exper. *19*, 227 (1963).
[15] R. A. REISFELD, V. J. LEWIS, N. G. BRINK, and S. L. STEELMAN, Endocrinology *71*, 559 (1962).
[16] F. C. GREENWOOD and W. M. HUNTER, Biochemical J. *89*, 114 (1963).

The fractions which are the purest on electrophoresis are combined and the volume made up to 25 ml with phosphate buffer containing 0.2% albumin and 1/1000 merthiolate. Aliquots of the purified protein are stored at – 15°C. The labelled TSH is diluted to give a final dilution of 1/100 for each assay. The recovered labelled TSH contains about 20 to 50 mμg/ml with a specific activity of between 20 and 50 mc/mg.

Preparation of the TSH antiserum: A commercial preparation was used for the production of the antibodies. By the use of the relatively impure TSH, the importance of the specific difference between TSH samples is diminished.

Guinea-pigs were immunized with Armour TSH by weekly injections (0.4 ml of 1.00 IU/ml) of the protein in emulsion with Freund's adjuvant.

Titration of the TSH binding capacity of the antiserum: The titer of the antiserum was followed during the immunization. Dilutions of the antiserum were incubated with fixed quantities of labelled TSH and precipitating antiserum. The highest titer was found between the 4th and the 8th week of immunization.

Precipitating antiserum: In the early stages of the assay, the precipitating antiserum was produced by immunizing rabbits with guinea-pig γ-globulins. For the last 6 months the precipitating antiserum has been purchased from Burroughs Wellcome Ltd., England. The dilution at which the precipitating antiserum is used depends on the titer of the antibodies.

Standard curves: Armour TSH (bovine) is used as a standard. Standard dilutions are made in phosphate buffer 0.04 *M* pH 7.4 containing 1 mg/ml beef albumin. The addition of increasing amounts of unlabelled TSH to incubations containing fixed quantities of labelled TSH, antibodies, and precipitating antiserum produces a decrease in the radioactivity bound to the antibodies (measured as cpm retained on the filters). Plotting the cpm on the filter against the concentration of the unlabelled TSH gives an exponential curve (Figure 3), plotting the cpm against the logarithm of the TSH concentration produces a straight line (Figure 4). The straight line conversion is used routinely, as this curve enables the values of the unknown TSH to be read with precision.

Assay design: The incubations are carried out in small test-tubes of 1.5 ml capacity. For each point the following volumes of reagents are mixed (see Table I): (1) 0.1 ml of standard TSH (bovine TSH, concentration between 0 and 0.4 mU/0.1 ml) or of the unknown serum or plasma,

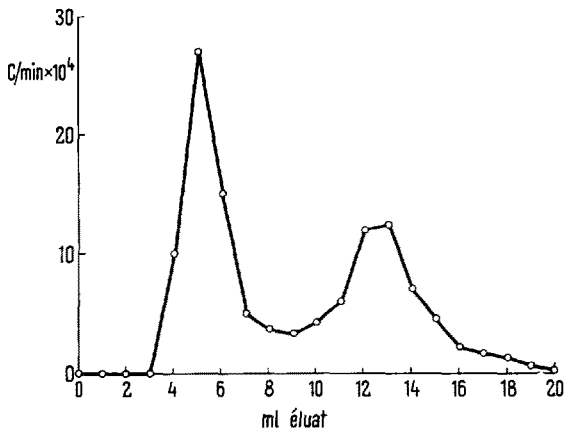

Fig. 1. Separation of the TSH labelled with I^{131} from the other reaction compounds on a Sephadex G-50 column (phosphate buffer 0.01 *M* pH 7.5 containing 0.15 *M* NaCl).

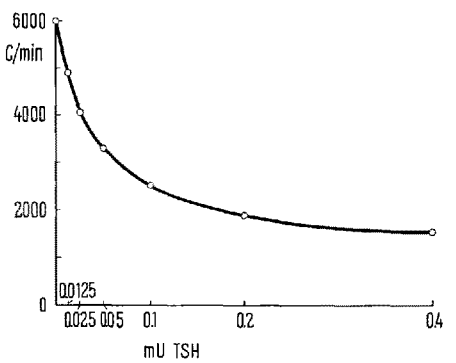

Fig. 3. Standard curve showing the decrease in radioactivity held on the filter as the non-labelled TSH (expressed in mU) increases.

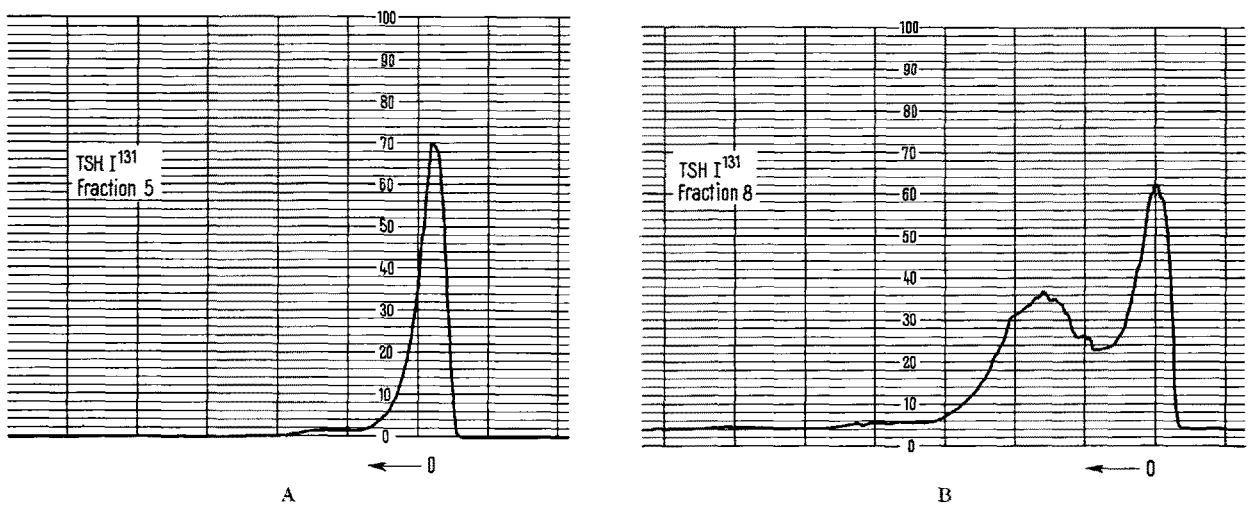

Fig. 2. TSH labelled with I^{131}. Electrophoresis of a TSH fraction separated on a column of Sephadex G-50, registered on a gas flow actigraph (nuclear). (Phosphate buffer pH 7.4, 1 h, 400 V). A, pure fraction of labelled TSH. B, impure fraction.

Table II. TSH values according to the conservation of blood (mU/ml)

	Not frozen			Frozen and thawed			
				one time		2 times	3 times
Number of days after the taking off:	0 day	2 days	4 days	0 day	2 months	2 days	4 days
Plasma	0.38	0.32	0.19	0.40	0.37	0.40	0.17
Serum	0.31	0.16	0.19	0.37		0.15	0.19

(2) 0.1 ml of diluted antibodies (the dilution varies between 1/600 and 1/1600 depending on the antiserum used). Leave 30 min. There is no difference between 30 min and 24 h of incubation before adding labelled TSH.

After mixing with Vortex mixer, the tubes are left for 4 h at 4°C and the antibodies precipitated by the addition of 0.2 ml pf precipitating antiserum (dilution between 1/4 and 1/8).

Filtration is carried out 16 h after the addition of the precipitating antiserum. The filters are washed with phosphate buffer containing 20 mg/ml of bovine albumin. The filters are glued to cardboard discs and dried at 60°C. The radioactivity on the filters is determined in a Nuclear Chicago Gas-Flow counter.

Results. The standard curves, obtained with standard concentrations of unlabelled TSH in buffer, show that the assay functions for the determination of TSH in buffer. As reported in the discussion, determinations of plasma and serum TSH are possible if the relative concentrations of the two antibodies are optimal. Table II contains the results of determinations carried out in fresh serum and plasma, and in the same fluids after freezing and thawing. Fresh plasma and serum from the same subject give the same value for TSH; the TSH in serum is however unstable, as the concentration diminishes rapidly with freezing and thawing. The TSH in plasma is less sensitive to freezing and thawing. All determinations are carried out on plasma; if the determination cannot be carried out on fresh plasma, the plasma is frozen immediately after preparation. Standard bovine TSH added to the plasma is recovered as seen in this following example. Plasma alone, 0.020 mU/0.1 ml; TSH added, 0.062 mU/ml; theoretical, 0.082 mU/0.1 ml; plasma + TSH found, 0.089 mU/0.1 ml = 109%. As seen in Table IV the injected TSH is also recovered.

Table III contains the values for the TSH in the plasma of 18 normal subjects. The mean value is 0.38 mU/ml plasma and the values range between 0.13 and 0.81 mU/ml. These values are of the same order as those obtained with the more sensitive bioassays[17].

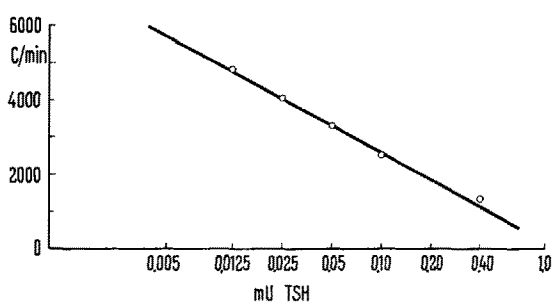

Fig. 4. Standard curve as shown in Figure 3. The non-labelled TSH is plotted as the log of the concentration (mU).

Table IV shows our first results of TSH values obtained in patients with thyroid disorders.

They are only preliminary results but, as can be seen, hyperthyroids, whether treated or not, have normal values of TSH. This is not surprising, as blood of hyper-

Table III. TSH values in human plasma

Methods	Authors	Euthyroidism
Histological (colloid)	DE ROBERTIS[18]	0.25–0.5
Tadpole growth	DI GEORGE et al.[19]	0–1.0
I^{131} discharge:		
in guinea-pig	ADAMS et al.[20]	0–0.01
in mouse	MACKENZIE[6,21]	0–0.16
in chicken	GILLILAND and STRUDWICK[22]	0.29–0.54
	BATES et al.[23]	0.50 (0.1–1.0)
in vitro:		
I^{131} discharge by thyroid slices	BOTTARI[24]	0.37 (women) 0.22 (men)
Radio-immunological test	UTIGER et al.[11], our own results	0 0.38 (0.13–0.81)

Table IV. Preliminary results of TSH value in the plasma (mU/ml)

	Number of cases	Mean	Values range
1. Euthyroidism	18	0.38	(0.13–0.81)
2. Hyperthyroidism	8	0.26	(0.04–0.68
3. Hyperthyroidism treated with I^{131}	6	0.43	(0.10–0.98)
4. Hypothyroidism	4	2.2; 3.8; 1.0; 8.8	
5. 24 h after a 3-day treatment with TSH	2	2.6; 1.7	

[17] J. ROBBINS, J. RALL, and P. G. CONDLIFFE, in *Hormones in Blood* (Ed.: C. H. GRAY and M. L. BACHARACH; Academic Press, 1961), p. 49.
[18] E. DE ROBERTIS, J. clin. Endocr. Metab. *8*, 956 (1948).
[19] A. M. DI GEORGE, S. A. D'ANGELO, and K. E. PASCHKIS, J. clin. Endocr. Metab. *17*, 842 (1957).
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[22] I. C. GILLILAND and J. I. STRUDWICK, Brit. Med. J. *1956 i*, 378.
[23] R. W. BATES, M. M. GARRISON, and T. B. HOWARD, Endocrinology *65*, 7 (1959).
[24] P. M. BOTTARI, J. Endocrinol. *17*, 19 (1958).

thyroids contains another TSH stimulating factor: the long acting thyroid stimulator (LATS)[9,25] which is not measured in this radio-immunologic assay for TSH.

Discussion and conclusions. The radio-immunoassay for TSH described here is, in principle, simple, but in practice it displays several difficulties. The first problem is that of the specificity of the TSH and of the antibodies. Although UTIGER[11], working with highly purified human and bovine TSH, has demonstrated absolute species specificity, we have taken advantage of the lesser purity of commercial TSH to produce antibodies which bind both human and bovine TSH. These antibodies enable the assay to be used for the determination of human TSH while using bovine TSH both as a standard and as the labelled protein.

The labelled TSH as produced by the technique detailed here is free from non-TSH contaminants; it is not yet known which fractions of the TSH are labelled. The labelled protein can be stored for up to three weeks at – 15°C. The greatest difficulty in establishing this method has been that of determining the optimal dilutions of the antibodies and of the precipitating antiserum. In Figure 5 the results of a dilution curve of the antibodies are shown. The concentration of the precipitating antiserum was constant throughout and at each concentration of antibody buffer, standard TSH and plasma were incubated. As can be seen, the serum and standard curves cross and re-cross as the antibodies are diluted. At high concentrations of the antibodies (1/200) the cpm held on the filter in the presence of the serum are higher than the cpm in the presence of buffer alone. At such concentration of the antibodies, there may be a non-specific precipitation of the labelled TSH in the presence of serum; this non-specific effect renders high concentrations of antibodies unsuitable for serum determinations. At higher dilutions of the antibodies (1/800), the standard TSH concentrations displace the labelled TSH proportionally to their concentrations and the serum value falls in the zone between the 0 and the highest standard. At the highest dilutions (1/3200), the antibodies do not bind enough labelled TSH, and displacement is complete by the lowest standard. At low antibody concentrations, non-specific reactions have been observed similar to those reported by PASCASIO[8] in the hemagglutination test and by UTIGER[11] in radio-immunological tests. The dilution of the antibodies to 1/800 gives a system which appears free from artefacts, and solely isotopic dilution causes a change in the TSH bound to the antibodies; under suitable conditions, the diminution in the labelled TSH bound is proportional to the unlabelled TSH present.

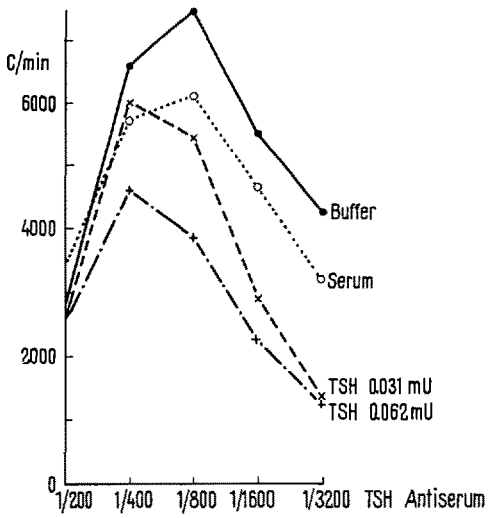

Fig. 5. Antibody titration curve. Varying dilutions of antibodies are added to constant amounts of labelled TSH and of anti-γ-globulins. At each dilution of the antibody buffer alone, a standard of TSH and a plasma are incubated. The optimal dilution for the antibodies tested lies between 1/600 and 1/800.

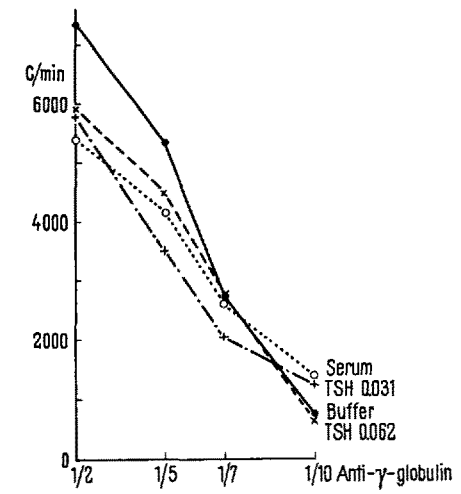

Fig. 6. Precipitating antiserum curve. Varying dilutions of precipitating serum are added to constant amounts of labelled TSH and of antibodies. At each dilution of the precipitating antiserum buffer alone, a standard of TSH and a plasma are incubated. The optimal dilution for the precipitating antiserum tested lies between 1/4 and 1/5.

The amount of precipitating antiserum added to each tube must be adequate to produce complete precipitation of the antibodies. At very high and low concentrations of the precipitating antiserum, non-specific reactions occur which interfere with the precipitation (see Figure 6). The Burroughs Wellcome precipitating antiserum is used at a dilution of between 1/4 and 1/5.

The importance of the relative concentrations of the antibodies and of the precipitating antiserum is such that the concentrations must be re-calibrated each time either of the two antisera is changed.

Despite the difficulties of the method, this radio-immunoassay for TSH is the only assay which is sufficiently sensitive to determine not only the hyper and normal values but also the sub-normal values[25,26].

Résumé. Les auteurs présentent un dosage radio-immunologique quantitatif de la thyréostimuline. Les conditions optimales pour son application en vue de mesurer la TSH endogène sont étudiées. Cette méthode est très sensible et permet de mesurer jusqu'à 5 μU de TSH non marquée. Les premiers résultats obtenus chez l'homme sont présentés.

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[25] D. D. ADAMS, J. clin. Endocr. Metab. *18*, 699 (1958).

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