REVISTA ESPAÑOLA DE FISIOLOGIA, 32, 59-76. 1976

Validation of a Heterologous Radioimmunoassay for the Determination of Rat Thyrotrophic Hormone *

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(Received on August 19, 1975)

M. D. GARCIA, L. CACICEDO and G. MORREALE DE ESCOBAR. *Validation of a Heterologous Radioimmunoassay for the Determination of Rat Thyrotrophic Hormone.* Rev. esp. Fisiol., 32, 59-76. 1976.

A heterologous RIA system is described for the determination of TSH in rat plasma. The antibody was obtained by us in guinea-pigs and directed against bovine-TSH (B-TSH). It was chosen from several antisera as the one showing the greatest potency *in vivo* against *rat* TSH, using the McKenzie bioassay. Two different antigens were tried for radioiodination: B-TSH (B-TSH*) ** and a purified mouse tumour TSH (M-TSH*). The choice of labeled antigen proved to be critical. It was not possible to develop a reliable RIA with B-TSH*. Using M-TSH*, however, a RIA was developed which is sensitive enough to detect differences between normal and lower than normal plasma TSH levels. The reproducibility, sensitivity and specificity of the RIA are described, as well as several procedures which shorten the time spent on a given assay, and at the same time decrease inter and intra-assay variations. Some of the results obtained with experimental plasmas were compared to the *in vivo* potency of the samples in the McKenzie bioassay. The results obtained with the present RIA have also been validated physiologically by carrying out the determinations on plasmas obtained from rats submitted to situations known to decrease (hypophysectomy, treatment with thyroid hormones, ether anaesthesia) or increase (injection of TRH, thyroidectomy, treatment with goitrogens) circulating TSH levels. The circulating TSH levels of normal rats found with the present RIA compare well with values obtained with the homologous immunoreactants available from NIAMDD at NIH (U.S.A.).

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* Part of the present work has been reported previously at the IV Meeting of the European Thyroid Association, Berne, 1971. Request for reprints should be addressed to «Departamento de Endocrinologia Experimental, Facultad de Medicina, Universidad Autónoma, Avda. del Arzobispo Morcillo, s/n, Madrid - 34» (Spain).

** The asterisk denotes the I* labeled preparation. The same applies to M-TSH*.

When the present work was initiated several years ago, two laboratories (17, 18, 27) had reported that thyrotrophic hormone (TSH) could be measured by radioimmunoassay (RIA) in normal rat serum. In both laboratories the antisera used were directed against bovine TSH (B-TSH) preparations and obtained in the guinea-pig and rabbit, respectively. The antigens used for labeling were different: LEMARCHAND-BÉRAUD and VANOTTI (17) used purified B-TSH for labeling with radioiodine (I*). WILBER and UTIGER (27) used a mouse TSH (M-TSH) preparation purified from a TSH-secreting tumor, because they found that with their antiserum, bound B-TSH* could not be displaced by normal rat serum, though it was displaced by rat pituitary preparations. REICHLIN *et al.* (23), later confirmed the report by LEMARCHAND-BÉRAUD and VANOTTI (17) that using antisera against B-TSH obtained in the guinea-pig, B-TSH* could be displaced by rat sera; they developed a RIA system which they used for studies of rat physiology (19).

We have obtained an antiserum directed against B-TSH in the guinea-pig, and studied both systems, namely the one employing M-TSH* and the one employing B-TSH*. With our antiserum only the system employing M-TSH* was suitable for the detection of TSH in normal rat plasma. The system was validated physiologically by determining changes in TSH levels, known to occurr under different experimental conditions. The increase in plasma TSH observed by RIA in rats on a goitrogen for several days were compared to the *in vivo* responses elicited with the same plasmas in mice prepared for the MCKENZIE (20) bioassay.

Though recently the immunoreactants for an homologous RIA system are being generously distributed by Dr. A. Parlow on behalf of the Rat Pituitary Hormone Distribution Program of the National Institutes for Arthritis, Metabolic and Digestive Diseases (NIAMDD) of the National Institutes of Health (NIH, U.S.A.), we believe the present experience in the validation of a RIA for rat TSH may be of use to other investigators.

Materials and Methods

Hormones. The bovine TSH used was either Thytropar purchased from Armour Co. (Illinois), or the NIH-TSH-B_4 and B_5 generously supplied by the Pituitary Distribution Program of the Endocrinology Study Section of the NIAMDD at NIH (Bethesda, U.S.A.), with TSH potencies of about 2.0 USP (Bovine) U/mg. The mouse thyrotropic tumour preparation (M-TSH) was a gift of Dr. R. W. Bates, its potency being about 5 USP (Bovine) U/mg. The rat reference preparation (NIAMDD-Rat-TSH-RP 1) was kindly supplied by Dr. A. F. Parlow on behalf of the Rat Pituitary Hormone Distribution Program of the NIAMDD at NIH, and has a reported potency of 0.22 USP (Bovine) U/mg. The bovine LH preparation used (NIH-LH-B_7) was also supplied by the NIAMDD at NIH, with a reported LH potency of 1.16 U (NIH-LH-S 1)/mg, and a TSH potency of 0.010 U/mg. A highly purified LH-RH/FSH-RH preparation of porcine origin was generously made available to us by Dr. A. V. Schally.

Other reagents. Complete Freund's adjuvant was obtained from Difco Laboratories (Detroit, Michigan) and the goat anti-guinea pig gamma globulin antiserum from Antibodies Incorporated (Davis, California). Sephadex G-50 Fine and Sephadex G-100 were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden), carboxymethylcellulose (CM-C), bovine serum albumin (BSA) and ethylenediaminetetraacetic acid (EDTA) tetrasodium salt from Sigma Chemicals Co. (St. Louis, Missouri), and EDTA disodium salt, mono and disodium phosphates, and sodium metabisulphite from Merck Co. (Darmstadt, Germany). Chloramine T was obtained

from Fluka AG (Buchs SG, Germany). The ^{125}I (I*) for protein iodinations was obtained from the Radiochemical Center, at Amersham (England).

Buffers. PBS is a 0.01 M phosphate buffer, pH = 7.4-7.6, containing 0.15 M NaCl. BSA-PBS is PBS containing 2 % BSA. The RIA buffer is PBS containing 1 % of non immune guinea pig serum and 0.02 M EDTA.

Antisera. These were prepared by us against Thytropar in male guinea pigs, with complete Freund's adjuvant, following the general procedure described by LEMARCHAND-BÉRAUD and VANOTTI (17). The antisera were inactivated for complement at 56° for 30 minutes and tested against B-TSH and a rat pituitary extract by the double diffusion Ouchterlony technique. The most promising antisera were then assayed for their capacity to inactive the *in vivo* response of the mouse thyroid to stimulation by a rat pituitary extract, using the MCKENZIE (20) TSH bioassay as modified by us (9). The antisera were injected at different dilutions, the ones chosen being those which had the greatest effect against rat TSH at the highest dilution.

Labeled antigens. i) *B-TSH:* Two procedures have been usually employed to purify the B-TSH preparations before or after their labeling with I*. In a few instances we used Thytropar purified on carboxymethylcellulose (CM-C) in the manner proposed by CONDLIFFE *et al.* (5) for B-TSH, as applied by LEMARCHAND-BÉRAUD and VANOTTI (17, 18). The purified protein appearing at about 0.3 NaCl during gradient elution with 0-1.1 M NaCl, visualized and quantitated by measuring optical density at 260 and 280 mμ in a Beckman DU-2 Spectrophotometer, were then used for labeling. These highly purified fractions are, however, extremely unstable. For this reason we prefer to label NIH-TSH-B_4 preparations, and then purify the most homogenous proteins fraction on the CM-C column (1 × 40 cm). The newly CM-C-purified B-TSH*, chosen as the labeled protein peak appearing during elution with an NaCl-gradient, could then be stabilized by collecting the 1.0 ml eluates on 0.1 ml of a protein containing buffer, such as the BSA-PBS, a procedure which cannot be applied prior to labeling. These B-TSH* fractions are pooled, diluted five-fold in BSA-PBS and stored frozen in 0.25 ml aliquots at —20° till the day of their use in the RIA.

In other instances, NIH-TSH-B_4 or B_5 was labeled, freed from inorganic I* on Sephadex G-50, diluted five-fold in BSA-PSB, divided into aliquots and stored frozen until purified on Sephadex G-100, as described further on.

ii) *M-TSH:* 1 mg of M-TSH was dissolved in 1 ml of pH = 7.5, 0.01 M phosphate buffer, divided into 10 μl aliquots, and stored frozen in liquid N_2 for labeling.

iii) *Labeling procedure:* The general Chloramine T procedure outlined by GREENWOOD *et al.* (12) was followed and carried out in the cold with 1.5-2 mCi of ^{125}I for 10 μg of the antigen, 88 μg of freshly dissolved Chloramine T in 25 μl PBS and a 90-120 seconds reaction time. It was stopped by the addition of 100 μg of freshly dissolved sodium metabisulphite in 100 μl PBS. Except when CM-C columns were used for B-TSH* the mixture was passed through a 1 × 20 cm Sephadex G-50 column which had been pre-treated with 1 ml BSA-PBS and washed with 20-30 ml PBS. PBS was used for elutions and 0.5 ml fractions were collected into tubes contaning 0.1 ml of BSA-PBS. Fractions corresponding to the upper part of the descending I* labeled protein peak were diluted five-fold with BSA-PBS and stored frozen. When the products of the labeling were purified on CM-C, the fractions eluting at 0.3 M NaCl

were diluted five-fold with BSA-PBS and stored at —20° C.

iv) *Purification of labeled antigens on Sephadex G-100:* The labeled antigens are passed thorugh 1.2 × 40 cm column containing Sephadex G-100 which had been pre-treated with 30 ml of BSA-PBS and washed with 50-60 ml PBS. The fractions (1.2-2 ml) are eluted with PBS and collected into tubes containing 0.1 ml of BSA-PSB. Those corresponding to the upper part of the descending limb of the «retarded» (second) protein peak are pooled for immediate use in a RIA. This purification is always applied, whether CM-C purified B-TSH*, or Sephadex G-50 purified B-TSH* or M-TSH* are involved.

Procedure for RIA proper. i) *Plasma used for dilutions:* Adult male or female rats are injected with 50 μg L-thyroxine (T_4), at about 4 p.m. and the morning after with 5 μg triiodo-L-thyronine (T_3). Two hr later they are injected heparin into the vena cava inferior and bled thoroughly under ether anaesthesia. The plasmas are separated, frozen and later tested in a RIA. The degree of binding of the labeled antigen to the antibody, in tubes containing 100 μl of these plasma pools and 500 μl of RIA-buffer is compared to that containing 100 μl PBS instead of the plasmas. The plasmas showing the least effect on the amount of labeled antigen bound to the antibody, as compared to that observed in the tubes containing PBS instead of plasma, are then considered the most adequate for dilutions, pooled and stored at —20° C. This pool is referred to as (T_4 + T_3)-Pl and is used for serial dilutions of standards and unknowns.

ii) *Preparation of the Standard Rat Pituitary Extract (R-TSH):* Before the NIAMDD Rat TSH 1 Reference preparation was distributed, no international rat TSH standard was available and therefore a rat pituitary extract was used by us as an internal standard for the RIA. The anterior pituitaries from male rats were homogenized in PBS (1 pituitary/ml), centrifuged in the cold at 3,500 r.p.m. for 30 minutes, and the supernatant was divided into 200 μl aliquots, which were stored frozen at —20°. This extract will be referred to here as R-TSH.

For a large part of the present work, this crude R-TSH was used as standard preparation. An arbitrary unit system was used, 1 U R-TSH corresponding to 1 ml (1 pituitary) of the extract. As will be described in detail elsewhere (10), the potency of this extract in the McKenzie bioassay was 0.23 USP (Bovine) U/ml (with 95 % fiducial limits of 0.17 and 0.31 USP U/ml).

iii) *Preparation of the Standard curve:* A 200 μl aliquot of the R-TSH extract is diluted to 10 ml in (T_4 + T_3)-Pl (2,000 R-TSH μU/100 μl). This diluent is used to prepare 10-14 further dilutions containing 1,000, 750, 500 μU/100 μl, etc., down to about 15 R-TSH μU/100 μl. Sufficient amount of each dilution is prepared to pipette thirty to forty 100 μl aliquots of each into disposable glass tubes (9 × 80 mm). Three times as many tubes were prepared containing 100 μl of (T_4 + T_3)-Pl and R-TSH. All tubes are then kept frozen at —20° C until used in a RIA. This is the procedure later used with the NIAMDD-Rat TSH RP 1 preparation. With the latter, the standards were diluted in (T_4 + T_3)-Pl from 300 ng/100 μl down to about 5 ng/100 μl.

iv) *Setting up the RIA:* 100 μl aliquots of each unknown serum or plasma are pipetted in triplicate. When high TSH values are expected, the samples are first diluted four to ten-fold with the (T_4 + T_3)-Pl, and 100 and 50 μl, or 50 and 25 μl aliquots are used in duplicate or triplicate. Whenever the aliquot is smaller than 100 μl, the volume is completed using the same (T_4 + T_3)-Pl pool. Once all unknowns have been pipetted, three tubes of each of the 10-14 standards are thawed,

plus nine of the tubes containing only (T_4 + T_3)-Pl. Six of them, are used for the «0» point (no R-TSH added) and three as «blanks» (no anti-TSH serum added).

The labeled antigen, which has just been purified on Sephadex G-100, is added to enough RIA buffer to ensure 0.5 ml/tube. Thus, for instance, if the RIA consists of 500 tubes, about 250-275 ml of RIA buffer are prepared, and the M-TSH* added. After dispensing 0.5 ml into the 3 «blank» tubes, 500-550 μl of Ab diluted 1/200 are added, and 0.5 ml of this complete RIA mixture are dispensed to the other remaining «0» points, Standards and unknowns, using an automatic dispensing device (Model R Pipettor from Oxford Laboratories, California). Using the M-TSH* system, the addition of the antibody, labeled antigen and buffer in a single aliquot decreases both the time spent on the procedure and the «residual» (error) variance considerably, without affecting sensitivity.

Tubes are incubated for 5 days at 4-10°, after which 100 μl of an adequate dilution of the second antibody (anti-guinea-pig gamma globulin) are added and the incubation prolonged for a further 24 hours. The tubes are then centrifuged for 30 minutes in the cold at 2,500 r.p.m., 288 at a time (MSE Mistral L centrifuge), after addition of 4 ml of ice-cold PBS containing 1 % BSA or 10 % beef plasma. The supernatant is rapidly sucked off as completely as possible and the tubes are counted in a Packard Autogramma Spectrometer.

TSH bioassay: We initially followed the method described by McKENZIE (20), and later modified it in the following manner:

Young male or female mice of a Swiss strain were obtained from a local breeder and two to three days after arrival placed on a low iodine diet (LID) and distilled water. In many instances the «low iodine regime» was used. This consisted in placing the mice on LID, and supplementing this diet with 1 mg PTU/mouse/day or with 1 % $KClO_4$ in the drinking water for one or two weeks, respectively, after which the drugs were again withdrawn and the animals continued on the LID till the end of the assay. About 4-7 days after withdrawal of the antithyroid drugs, the mice were injected i.p. with 10-15 μc of I*, usually ^{125}I. For the «suppression» of endogenous TSH release we followed more closely the schedule described by RERUP and MELANDER (24): thus, instead of a 10 μg L-T_4 injection followed by 0.066 % USP thyroid powder, we injected s.c. 0.2 ml of saline containing 20 μg of L-T_4, just after the I* dose, and 10 μg L-T_4 every other day, or, more recently 0.2 μg of L-T_3 every day. Starting 3-4 days after the I* dose, the assay was carried out by withdrawing 0.1 ml of blood by orbital sinus puncture, the same fine-pointed micropipette being used for all the animals. Groups of 6-7 mice each were injected ip. with saline containing 2 % BSA, or with TSH, rat plasmas or pituitaries, etc. The second 100 μl blood sample was withdrawn after three hours (6). The first and second blood aliquots are referred to as «basal» and «response» samples. The log of the % response

$$\left(\log \frac{\text{net counts in «response»}}{\text{net counts in «basal»}} \times 100\right)$$

have been used for the calculations. This log transformation avoids the considerable heteroscedasticity observed with the % response data.

Experimental situations. To validate the results obtained by the present RIA, rats were submitted to several experimental procedures known to change circulating TSH levels.

A) To decrease circulating levels below the normal value, rats were i) hypophysectomized via the parapharyngeal route and bled a month later, when growth stasis was clearly established; ii) anesthetized with ether and bled 1-2 minutes and

15 minutes after onset of contact with ether; iii) treated with T_4 and T_3 [see preparation of (T_4 + T_3)-Pl].

B) To increase circulating TSH levels markedly rats were i) injected 1-2 μg TRH iv and bled 15 minutes later; ii) thyroidectomized ($\overline{T}$) surgically and fed a low iodine diet, or fed the same diet supplemented with 1 % $KClO_4$ in the drinking water or with 5 mg/10 g diet of 6-propyl-2-thiouracil (PTU). These animals were bled a month, or longer, after $\overline{T}$ or onset of goitrogen treatment.

C) To increase circulating TSH levels more moderately i) groups of 5 rats each were injected 0.25 ml of saline, or of saline containing 20, 40, 60 or 80 μg synthetic TRH (Abbott Laboratories, Chicago, Ill.); ii) Groups of 6 rats each were bled 3, 6 or 10 days after onset of feeding the low iodine diet supplemented with 5 mg PTU/10 g; another group was bled after 11 days on PTU and 18 hours after a single 50 μg T_4 injection. Normal controls were also bled on days 3, 6, 10 and 11.

D) To induce fluctuations of the plasma TSH levels around the physiological range in the same experiment, male $\overline{T}$ rats were injected ip every morning with 1.8 μg T_4/100 g body weight (BW) or 0.4 μg T_3/100 g BW. Groups of 7 rats each were bled at different time intervals after the last daily T_4 or T_3 dose.

In each case plasmas were collected using heparinized syringes and glass-ware, spun off and stored at —20° C until used in the RIA or the bioassay.

CALCULATIONS

The mean value of the counts obtained for the «blanks» are subtracted from those of all other tubes and the net values are transformed into RPB. These are the «relative-percentage bound» and are calculated from the net counts corresponding to a given standard or unknown, taking the mean net counts corresponding to the «0» point tubes as 100 %. The RPB are also transformed into logit RPB ($\ln \frac{RPB}{100 - RPB}$). The mean ± SD of the RPB or logits are calculated for each standard point or unknown. Either the RPB are plotted against the logs of the TSH concentration and the unknowns read visually from the standard displacement curve, or the straight line of best fit is calculated by the least squares method using the logit-log plot. Linearity of the curve thus obtained is then tested. In most cases the portion between RPB values of 10-90 % is the one which is both linearized using the logit-RPB-log TSH plot and shows homogeneous variances. Outside of these limits, either there is no longer good linearity, or the variances increase markedly (fig. 1). TSH concentrations (and 95 % fiducial limits) of unknown samples with RPB values comprised within such limits are then calculated automatically.

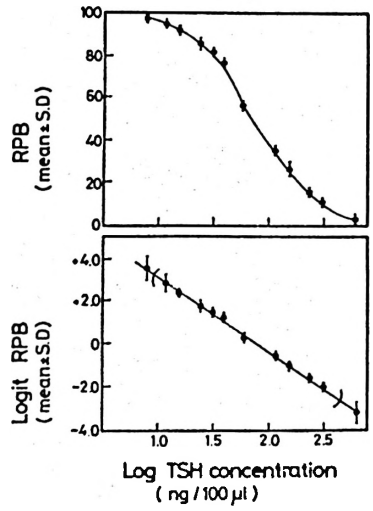

Fig. 1. *The upper panel shows a standard M-TSH* displacement curve obtained by using the NIAMDD-Rat TSH-RP 1 preparation, and given in RPB versus log TSH concentration.* The lower panel shows the logit RPB-log TSH plot of the same data. The portion included between brackets is the linear portion, with homogenous variances, used for further calculations of TSH values for unknown samples.

Logit RPB-log TSH plots are also the ones used for the calculation of the index of precision ($\lambda = \frac{\sqrt{s^2}}{b}$) of the standard curve, for tests of parallelism between the standard curve and those obtained by serial dilutions of pituitary and plasma preparations, as well as for the potency estimates and their 95 % fiducial limits. These calculations are carried out automatically using programs we have developed for the Olivetti P-602 microcomputer. They are based on the principles outlined for bioassays by BLISS (3) and differ somewhat from those adapted to RIA systems by VIVIAN and LABELLA (26). The main differences with regard to the procedures described by these authors (26) is that they use an arcsine RPB transformation, instead of the logit RPB one; moreover they use only the residual («error») variance and the slope obtained from the standard curve, and disregard the residual variance and, when tested at more than one dilution, the slope of the unknowns, for calculation of the potency and 95 % fiducial limits of the samples. We have used a program which, as is the case for bioassays (3) takes into consideration the residual variance and slope of the unknown sample as well. This has the advantage that the same microcomputer programs may be used for calculations of the results obtained in bioassays (using the log % response-log dose plot for linearization).

For experiments in animals, differences between mean values for different groups were calculated using Student's test as outlined by SNEDECOR (25). Degree of significance is given by * for $p < 0.05$; ** for $p < 0.01$ and *** for $p < 0.001$. For $p > 0.05$ the difference was considered as not significant (n.s.).

Results and Comments

Antisera. Of the 15 antisera raised against B-TSH, 2 gave a clearcut immunoprecipitation reaction on Ouchterlony plates, both against B-TSH and a crude rat pituitary (R-TSH) extract. All antisera obtained were tested in the McKenzie mouse assay against both B-TSH and R-TSH. Figure 2 shows results obtained at a 1:125 dilution with the antisera which had the most intense inhibitory effects on the thyroid-stimulating activity of B-TSH and R-TSH. These coincided with the two antisera showing a clearcut reaction on Ouchterlony plates. Antiserum n.° 2 is the one used throughout the present work.

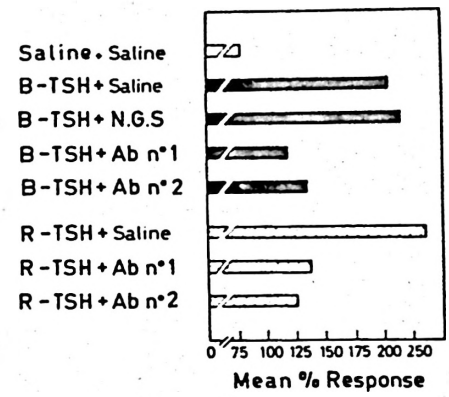

Fig. 2. *Mice (7-8/group) prepared for the McKenzie TSH bioassay were injected ip just after withdrawal of a basal blood sample with 0.125 ml/mouse of saline, or of the same volume of saline containing 0.5 USP (bovine) mU or 0.002 of a rat pituitary.* At a separate site they were injected i.p. with 0.125 ml/mouse of saline, non-immune guinea pig serum (N.G.S.), or of an antiserum (Ab) raised in the guinea pig against B-TSH. Blood samples were taken again 3 hours later and their radioactivity expressed a «% response» over the basal counts.

Purifications of labeled antigens. The Sephadex G-50 elution pattern of B-TSH* obtained just after labeling shows one radioactive protein peak (and the iodide one). With M-TSH*, two labeled protein peaks appear (fig. 3). Both with B-TSH* and M-TSH* the radioactive materials corresponding to the upper and descend-

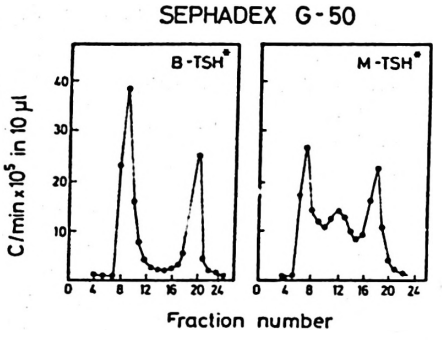

Fig. 3. *I* profile obtained after Sephadex G-50 separation of labeled B-TSH* and M-TSH* from I* iodide, immediately after labeling.*

ing slope of the first protein peak are those showing the highest degree of immunoreactivity towards the anti-B-TSH serum. Both with B-TSH* and M-TSH* the radioactivity of these fractions can be displaced by preparations containing rat TSH.

When they are purified on Sephadex G-100, the patterns of elution are the usual ones obtained with other labeled protein hormone preparations: a first non-retarded and a second «retarded» peak (plus a radioiodide one) (see, for instance, the upper part of figure 5). Both with B-TSH* (9) and M-TSH* the fractions of the upper and descending limb of the «retarded» labeled protein peak show the maximum degree of binding to the anti-B-TSH serum, and the maximum displacement by R-TSH preparations. Using CM-C purified B-TSH*, these properties are found for the radioactive peak eluting at about 0.3 M NaCl (9). As results obtained with B-TSH* purified on Sephadex G-100 and on CM-C were comparable, the Sephadex G-100 procedure was later used routinely both for B-TSH* and M-TSH*.

Antibody concentration. The upper half of figure 4 shows the degree of binding of B-TSH* and M-TSH* to the anti-B-TSH serum. As may be seen, the max-

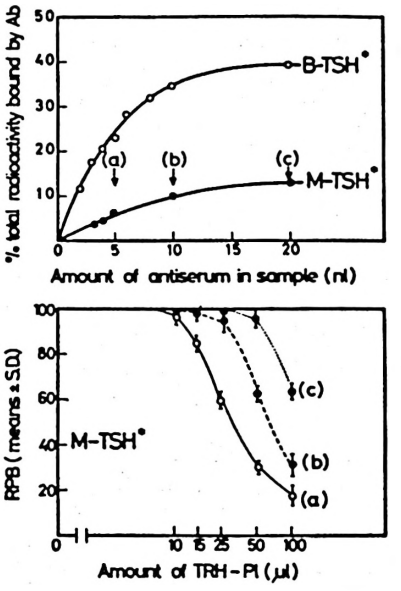

Fig. 4. *Sephadex G-100 purified B-TSH* and M-TSH* were used to asses the degree of binding to different amounts of Ab.*
Upper half: the «net» % bound is shown, the «blank» radioactivity having been substracted. No cold R-TSH was added. The arrows show the Ab concentrations (a), (b) and (c), used to test M-TSH* displacement by different volumes of a plasma pool obtained from rats injected 15 minutes before 1 µg TRH iv. The serial dilution curves found using the Ab at concentrations (a), (b) and (c) are shown in the lower half.

imum binding did not exceed 40 % of the total radioactivity present even when B-TSH* was used. It was lower where M-TSH* was used and it did not exceed about 13 %. Therefore, it was not possible to work at an Ab concentration precipitating about 50 % of the total radioactivity, as done in most other RIA systems. But, as shown in the lower half of figure 4 for the system using M-TSH* as an example, the Ab concentration precipitating 50 % of the «bindable» radioactivity (a) did permit detection of the TSH contained in 10-15 µl of a plasma from TRH injected rats (curve a). At higher Ab concentrations (curves b and c) the

sensitivity of the system was too low to be of any use for the detection of normal rat TSH levels. Thus, concentration (a), giving a final Ab dilution of 1:120,000, was used routinely though less than 10 % of the total radioactivity is bound. With such a low degree of maximum binding, procedures which measure the «free» labeled fraction (charcoal, for instance) are not suitable. The second antibody method has, however, been used successfully, if care is taken to dilute adequately the large proportion of «free» radioactivity just before final centrifugation. In this manner the contamination of the precipitate by «free» labeled material is avoided as far as possible.

Standard displacement curves. Figure 5 shows typical displacement curves simultaneously obtained with serial dilutions of the standard rat pituitary extract, the same Ab concentration, and B-TSH* or M-TSH* as labeled antigen. As may be seen, the slope of the linear portion of the RPB-log TSH plot is steeper using M-TSH*. The labeled antigen is almost completely displaced with high TSH concentrations. This does not occurr with B-TSH*. Moreover, the 0.1 mU R-TSH standard results in a more marked displacement of M-TSH* than of B-TSH*. However, the use of B-TSH* seemed to have the advantage of covering a wider range of TSH concentrations. But when 100 μl of plasmas from rats submitted to different experimental situations were assayed simultaneously with B-TSH* or M-TSH* against the same R-TSH standards, it became apparent that there was at least a ten-fold difference in the TSH values obtained, according to whether B-TSH* of M-TSH* were used as labeled antigen. Though, as expected, the RPB of rats treated with $T_4 + T_3$ was higher than that of rats with a presumably high circulating TSH ($\overline{T}$ or on goitrogens) both when B-TSH* and M-TSH* had been used, it was necessary to resolve the ob-

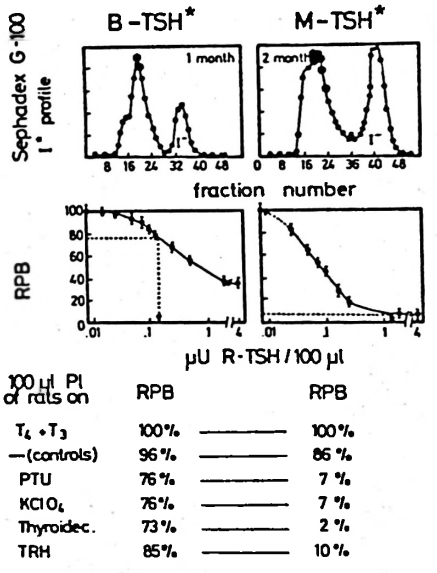

Fig. 5. *Purifications of B-TSH* and M-TSH* fractions 1 and 2 months, respectively, after labeling, and displacement curves obtained with the same antibody dilution and the R-TSH standard, using pools of the purified B-TSH* o M-TSH* fractions shown by* ⊙. The lower part of the figure shows the mean RPB values obtained with 100 μl of different plasma pools in the RIA system using B-TSH* (left hand column) or M-TSH* (right hand column). The RPB corresponding to the plasma of the PTU or $KClO_4$-treated rats are read off the corresponding standard curves, as shown by the dotted lines (---→). As may be seen, the TSH concentration read off the curve obtained with B-TSH* is ten times smaller than the one read of the curve obtained with M-TSH*.

servation that there was a marked discrepancy in the values obtained with the two different labeled antigens.

Figure 6 shows results obtained when two RIA were carried out simultaneously with serial dilutions of the same plasma and pituitary preparations and using the same conditions, except for the labeled antigen. When M-TSH* was used, parallelism of curves prepared with different samples obtained from rats was excellent, whether pituitary homogenates, the more purified NIAMDD Reference preparation,

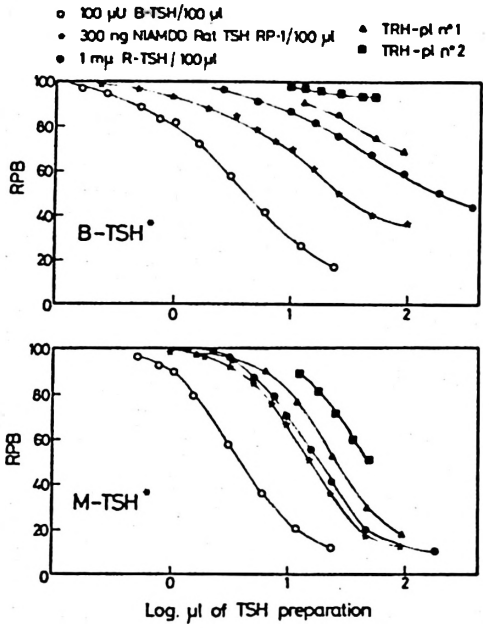

Fig. 6. *Displacement curves obtained simultaneously with B-TSH* and M-TSH* and serial dilutions of different preparations containing bovine or rat TSH.*
The final Ab dilution was 1:120,000 and conditions of the RIA were the standard ones. TRP-Pl n." 1 and n." 2 correspond to two different pools of plasma obtained from rats injected iv with 0.5 µg TRH 10-15 minutes before bleeding.

or plasmas from TRH injected rats were used. On the basis of displacement curves obtained in the same RIA using the R-TSH and the NIAMDD-Rat-TSH-RP 1 preparations, it appears that 1 of our arbitrary R-TSH units is equivalent to 0.29 mg of the NIAMDD-Rat-TSH-RP 1 (with 95 % fiducial limits of 0.28 and 0.31). The displacement curves obtained with the NIH-TSH-B_3 preparation were parallel to those obtained with R-TSH. One of our arbitrary R-TSH units is equivalent in the RIA system to 0.026 USP (bovine) TSH units (with 0.022 and 0.030 as 95 % fiducial limits).

The addition of B-LH to the RIA system only affected binding of the M-TSH* to the Ab appreciably, when it exceeded 0.5 µg B-LH/tube. The degree of displacement then observed agreed with the amount of TSH known to contaminate the LH preparation.

In accordance with this finding ten different pools of rat plasmas, obtained a month or more after thyroidectomy or goitrogen treatment, were diluted ten -fold with (T_4 + T_3)-Pl and then assayed using 100 µl and 50 µl duplicates (results are given in table I). The mean (±SE) of the ratios of the TSH concentrations found using the two different volumes of each of the 10 pools was 0.97±0.028. This value is not significantly different from 1.

As may also be seen from figure 6 that parallelism was very poor when B-TSH* was used as labeled antigen. This was most striking when plasma samples were used. Lack of parallelism was visually evident, but was also confirmed by calculation. Addition of B-LH displaced B-

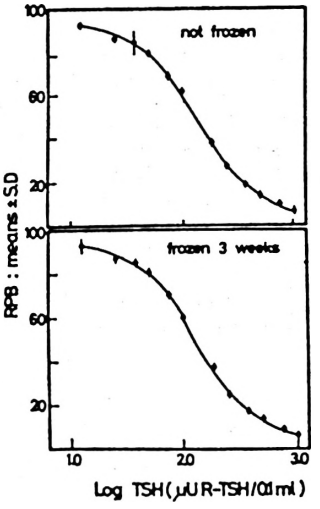

Fig. 7. *Comparison of RIA curves obtained with the same purified M-TSH* and Ab dilution, using the R-TSH standards which had been freshly diluted to different concentrations in (T_4 + T_3)-Pl, or another series of standards which had been prepared with the same diluent 3 weeks before, pipetted in 100 µl aliquots and stored frozen.*

Table I. *TSH concentrations In pooled rat plasmas.*

Treatment	Dilution prior to assay	Volume for RIA (μl)	RPB (mean±SD)	TSH concentration μg/ml NIAMDD Rat TSH-RP 1
Hypophysectomy	None	100	104.3±1.5	«0»
(T_4+T_3)-PI	»	»	100.0±3.3	«0»
None	»	»	86.2±1.0	0.10
»	»	»	86.6±1.2	0.10
»	»	»	79.5±1.3	0.13
»	»	»	72.1±2.4	0.22
»	»	»	80.0±0.6	0.16
Thyroidectomy	×10	100	30.5±2.3	10.5
		50	45.5±1.0	10.1
PTU	×10	100	53.6±4.8	4.6
		50	70.7±3.0	5.3
»	×10	100	56.9±2.5	4.2
		50	77.1±0.4	4.2
»	×10	100	48.7±0.6	5.7
		50	68.0±5.8	5.8
»	×10	100	52.3±4.3	5.0
		50	71.5±2.5	5.3
»	×10	100	66.3±6.1	3.2
		50	83.5±3.8	2.8
$KClO_4$	×10	100	54.6±4.1	4.5
		50	75.9±2.3	4.6
»	×10	100	55.3±6.8	4.4
		50	75.5±5.1	4.5
»	×10	100	50.9±1.5	4.2
		50	70.1±0.3	5.2
»	×10	100	53.9±1.6	4.7
		50	72.9±4.7	5.0

TSH* to a considerable degree; prior absorption of the Ab with B-LH did not completely correct the undesirable features of the system. These appeared whenever B-TSH* was used, whether purified on Sephadex G-100 or on CM-C, or both. Therefore, it was considered that displacements of B-TSH* bound to Ab did not reflect TSH concentrations in a reliable manner and the use of this labeled antigen was abandoned, despite initial preliminary results reported by us (9). M-TSH* was chosen for the remaining work described herein.

*Stability of the standard dilutions and the M-TSH**. Figure 7 compares a curve obtained with R-TSH standards freshly diluted in (T_4 + T_3)-PI, with the one obtained with pre-pipetted frozen standards. As may be seen, the standards may be prepared and kept frozen. This reduces considerably the time required for setting up a given assay. Figure 8 shows that comparable RIA curves may be obtained with M-TSH* which has been kept frozen for up to a month and a half, provided the M-TSH* is purified on Sephadex G-100 just before use. Despite this, when plasmas with a very low TSH content are going to be assayed, it is advisable to use the M-TSH* within 2-3 weeks after labeling.

The mean (±SE) index of precision λ corresponding to 15 different assays chosen at random was 0.062±0.004. These

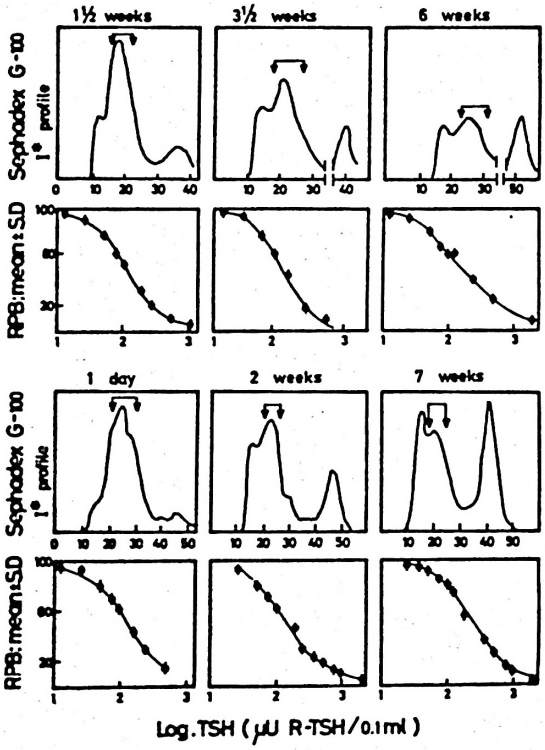

Fig. 8. *Different standard curves are shown, which were obtained on separate occasions using M-TSH* which had been stored frozen for the lenghts of time indicated in the illustration, and then purified on Sephadex G-100 just before use.*

The fractions indicated between the arrows were those pooled to obtain the standard curves shown underneath each Sephadex G-100 I* profile. The upper and lower half of the illustration correspond to M-TSH* from two different labelings.

included curves obtained with freshly prepared series of standards and M-TSH* and other obtained with series of standards which had been kept frozen, and with M-TSH* which had been labeled up to 6 weeks previously.

Sensitivity and reproducibility of the system. In a few instances, the degree of binding of the M-TSH* to the antiserum was tested using as «unknown» 100 μl of the RIA-buffer, or of plasma from hypophysectomized ($\overline{H}$) rats, or of the (T_4 + T_3)-Pl. The bound radioactivity was somewhat lower for the tubes containing the plasma pools, as compared to that of the RIA buffer. Thus, when the «unknowns» to be assayed are rat plasma samples, a plasma containing «0» TSH has to be added to the standard displacement curve. Considering that no statistically significant difference was found between data obtained with $\overline{H}$ plasma and those found with (T_4 + T_3)-Pl, and that the latter is more easily accessible to most laboratories, we have used (T_4 + T_3)-Pl for all dilutions. In most RIAs the mean RPB of plasmas from male and female rats, sacrificed by decapitation or under slight ether anaesthesia were significantly different from the RPB corresponding to H plasma or the (T_4 + T_3)-Pl (for instance, see table I). In several sensitive assays it was found that serial dilutions of plasma from normal animals gave RPB which were parallel with the standard curve. Figure 9 shows results obtained after serial dilutions of a pool of plasma from normal females, and of another from males gonadectomized 2 months earlier. As may be seen, results obtained with different volumes

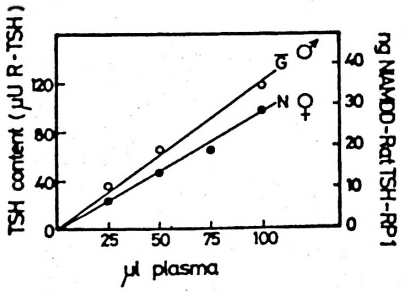

Fig. 9. *TSH content of different volumes of a plasma pool from normal female rats (N) and from male rats gonadectomized ($\overline{G}$) 2 months before bleeding.*

Volumes were completed to 100 μl with (T_4 + T_3)-Pl. Data were obtained against R-TSH as standard and are shown on the left ordinate; on the right hand ordinate are the same data recalculated in terms of the NIAM-DD-Rat-TSH-RP 1 preparation.

of plasma agreed very satisfactorily. Normal adult female and male rats bled quickly in the morning under slight ether anaesthesia usually had plasma TSH levels equivalent in the RIA to 0.13-0.29 μg/ml of the NIAMDD Rat-TSH-RP 1 preparation. On some occasions less sensitive assays are obtained and such values then fall very near to, or below, the limit of detection. Seven different pools of plasmas from normal animals were assayed on two different occasions, the mean (±SE) of the values being 0.137±0.012 and 0.140±0.014 μg/ml of the NIAMDD-Rat TSH-RP 1 preparation; the difference between the mean values obtained on the two different occasions was not significant. With plasmas having a high TSH content, the coefficient of interassay variation is larger. Thus, a pool of plasma from hypothyroid rats was diluted four-fold and assayed on 4 different occasions, giving a mean (±SE) value of 1.91±0.15 μg/ml of the NIAMDD-Rat TSH-RP 1, which represents a 7.9 % coefficient. After further dilution, the value from 4 different assays was 0.48±0.016 μg/ml, which represents a 3.0 % coefficient.

Changes of the plasma TSH level ot rats submitted to different experimental situations:

As indicated by data shown in table I, it was possible to differentiate between the circulating plasma TSH levels of normal rats and of those with presumably low TSH, namely, the hypophysectomized ones, and those treated with $T_4 + T_3$ [for $(T_4 + T_3)$-Pl]. Table II shows that

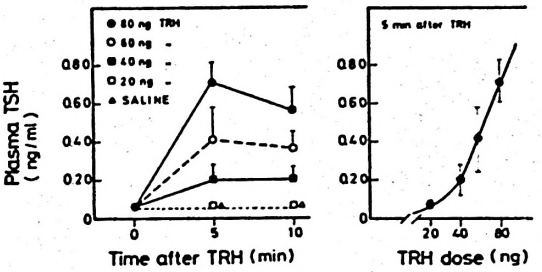

Fig. 10. *Changes in circulating THS levels of normal male rats (5/group) injected i.v. with the doses of TRH indicated.* Values are means ± SD.

with the present RIA it was also possible to detect a decrease in circulating TSH levels due to ether anaesthesia, reported by DUCUMMUN *et al.* (7) on the basis of bioassay results. Data included in table I also show the marked increase in plasma TSH induced by $\overline{T}$ or treatment with goitrogens. Figure 10 shows the increase induced with moderate TRH doses. Figure 11 shows the gradual increase in circulating TSH levels of rats treated with PTU. The changes, as measured by RIA, parallel the increase in thyroid weight, and also the increase in thyroid stimulating potency of the plasmas, as assessed from their effects in mice prepared for the McKenzie TSH bioassay. Figure 12 shows that with the present RIA it is possible to follow fluctuations of circulating plasma TSH in $\overline{T}$ rats on a daily T_4 or T_3 «maintainance» dose. TSH levels are high at the moment of minimal thyroid hormone availability, namely, 24 hours after a dai yldose, and then decrease after the T_4 or T_3 injections. Changes are more

Table II. *Plasma TSH levels In normal female rats bled after decapitation, or during ether anaesthesia.*

Groups	mU R-TSH/ml	μg/ml NIAMDD-Rat TSH-RP 1	Statistics
I: Decapitated	0.69±0.13	0.27±0.05	I-II: n.s.
II: Ether for 1-2 minutes	0.63±0.11	0.24±0.04	I-III **
III: Ether for 15 minutes	0.33±0.17	0.13±0.06	II-III **

** $P < 0.01$

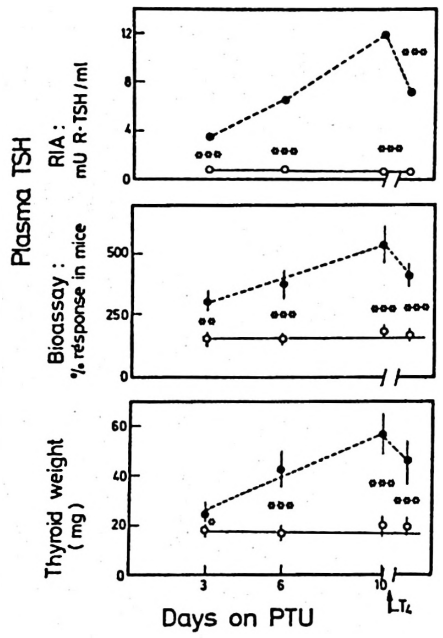

Fig. 11. *Changes in thyroid weight and in plasma TSH of rats during the first days of treatment with PTU (●) as compared to those of age-paired controls (○).*
One group of rats was treated with PTU of 11 days and injected 18 hours before bleeding with 50 μg T_4. Data shown for thyroid weights are means ± SD. The RIA data correspond to the value obtained with a plasma pool/group. The same pools were injected into mice prepared for the McKenzie bioassay (300 μl/mouse into 5-6 mice/pool) and the data shown are the mean responses ± SE.

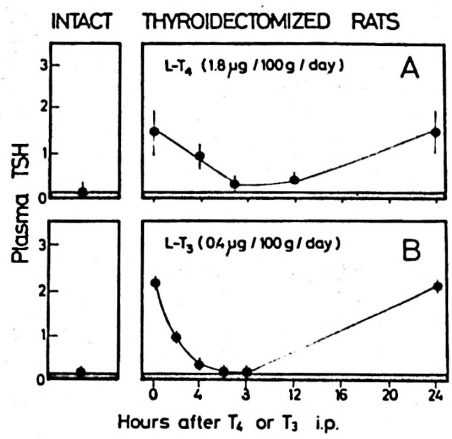

Fig. 12. *Panels A and B show, respectively, the changes occurring at different intervals after the injection of the last daily hormonal dose in the circulating levels of TSH of T rats injected once daily with 1.8 μg T_4/100 g BW or with 0.4 μg T_3/100 g BW.*
Values shown are expressed in μg/ml of the NIAMDD-Rat TSH-RP 1 preparation and are means ± SE.

marked and more rapid after the injection of T_3 than of T_4. A smaller dose of T_3 than of T_4 induces comparable effects. All this agrees with the changes which could be predicted to occurr in this experimental set-up on the basis of knowledge available prior to the use of a RIA (22).

Discussion

Present data show, in agreement with previous reports (17, 23, 27, 28), that heterologous RIA systems may be developed successfully to measure TSH in normal rat plasma. To set up the present RIA, we chose the antiserum which showed the most intense inhibitory effects against stimulation by a *rat* TSH preparation when injected *in vivo* into mice prepared for the McKenzie TSH assay. With this antiserum, however, the choice of labeled antigen proved to be critical. Thus, contrary to findings with the antiserum used by LEMARCHAND-BÉRAUD and VANOTTI (17) and by REICHLIN *et al.* (23) we were unable to use B-TSH* to assess rat TSH reliably. The undesirable features of the assay system using B-TSH* were not overcome by absorption of the antiserum with B-LH, or purification of the B-TSH preparation on CM-C, Sephadex G-100, or both.

Using a purified murine TSH preparation as labeled antigen, the system may be used reliably. As shown here it is possible to differentiate between the TSH content of plasma from normal rats, and plasma from hypophysectomized, or thyroid hormone-treated rat. The values for

normal rats, bled under slight ether anaesthesia, ranged usually between 0.13-0.29 μg/ml of the NIAMDD-Rat TSH-RP 1 reference preparation, and appear to be comparable to those obtained with the homologous NIAMDD rat TSH immunoreactants (2, 4). They are considerably lower than those reported with the heterologous systems using B-TSH* (1, 13, 23). It detects a decrease below normal values, induced by ether anaesthesia or treatment with thyroid hormones. Using plasma from the thyroid hormone-treated rats as diluent for the unknowns and the rat standard preparation, good parallelism of serial dilution curves is obtained; in very sensitive assays, this may be confirmed even with plasma from normal rats. If the plasma TSH concentrations are read off a standard curve which has been prepared without plasma from the thyroid hormone-treated rats, they are likely to be erroneously high, as appears to be the case for the human TSH RIA (14).

It would appear that the present RIA system, using M-TSH*, is not appreciably influenced by very high circulating gonadotropin levels, as show by the data with the pool from gonadectomized male rats (fig. 9). This was confirmed in experiments carried out later (10), where TSH was measured in the plasma from ovariectomized rats, with or without treatment with estrogen + progesterone, LH-RH, and T_4. It was observed that values detected with the present TSH RIA were within or slightly above the normal range and could be depressed by prior administration of a T_4 dose, whereas data obtained in the homologous LH RIA were extremely high and were not affected by T_4 treatment.

With the present heterologous system, when M-TSH* was used as antigen, all changes in circulating levels of TSH found under different experimental conditions agreed closely with the predicted ones. In an experiment where the increase in circulating TSH was not only measured by the present RIA, but also by the changes in thyroid weight of the rat, and by changes in I* secretion from the thyroid of test mice prepared by the McKenzie bioassay, a very good agreement was found among these different parameters. Again, the present RIA detected quite clearly the rapid fluctuations in circulating TSH levels ocurring in thyroidectomized, T_4 or T_3 maintained rats, after the injection of the daily hormonal dose.

We should like to draw attention to the fact that results of displacement by an unknown rat plasma or pituitary preparation should only be read off RIA standard curves obtained with a *rat* preparation. Now that the NIAMDD-Rat TSH-RP 1 is widely available as a reference preparation, data should be expressed in terms of *weight equivalents* of this standard. Though with the M-TSH* system the dilution curves obtained with a rat TSH preparation and B-TSH appeared parallel, data obtained using *rat* materials should *not* be read off a curve obtained using B-TSH, and then given in USP (bovine) units, as done by WILBER and UTIGER (27). We have found by bioassay that this might under-estimate TSH values by a factor of ten, or more, as reported elsewhere (10). Even if the biological potency of the internal rat standard preparation is known in terms of the USP bovine standard, data obtained in the RIA against it should not be converted to potency units: we have found that at least with the present RIA system, TSH values obtained by direct bioassay of rat plasmas tend to be about 1.5 times higher than those obtained by RIA, and then converted to USP units on the basis of the known potency of the RIA standard (10). It is not known if bioassay data and RIA data coincide when the homologous NIAMDD immunoreactants for rat TSH are used.

The present report also shows that it is possible to use prepipetted frozen standards, and the same labeled antigen over

a period of months, provided special precautions are taken; these simple procedures as well as the addition of the antibody, labeled antigen and buffer in a single aliquot, shorten considerably the time spent on a given assay. The present RIA system is being used succesfully for studies of thyroid physiology (8, 11, 15, 16, 21).

Acknowledgements

We are extremely grateful to Dr. R. W. Bates for his gift of highly purified mouse tumour TSH, used for radioiodination. We wish to thank Drs. Wilber, Utiger and Lemarchand-Béraud for useful advice; Dr. A. Portolés, Instituto J. Ferrán, C.S.I.C. for immunoprecipitation studies on Ouchterlony plates. We are grateful to Dr. A. Parlow for supplying us with NIAMDD-Rat TSH-RP 1 preparation before it became more widely available, and to NIAMDD for the supply of B-TSH and B-LH standard preparations. We aknowledge gratefully the assistance of Ms. B. Sánchez, M. Ruiz and M. de la Fuente for technical assistance and skilful preparation of figures and manuscript.

Resumen

Se describe la puesta a punto de un RIA heterólogo que permita la valoración de TSH en plasma de rata. El anticuerpo fue obtenido por nosotros en cobayos contra TSH bovino (B-TSH). Se escogió el antisuero que mostró mayor actividad *in vivo* en contra de TSH de *rata*, usando para ello en bioensayo de McKenzie. Se usaron dos antígenos diferentes para radioiodación: B-TSH (B-TSH*) y TSH de un tumor murino (M-TSH*) altamente purificados. Se encontró que el antígeno empleado juega un papel crítico, pues fue imposible desarrollar un RIA para TSH de rata empleando B-TSH*. Sin embargo, esto se consiguió con M-TSH*, pudiéndose diferenciar plasmas con TSH normal de aquellos en los que los niveles de TSH circulante son inferiores a lo normal. Se describen la sensibilidad, reproductibilidad y especificidad del RIA, y algunos procedimientos que acortan su ejecución y al mismo tiempo disminuyen la variabilidad intra e inter-ensayo. Algunos de los resultados obtenidos se compararon con la actividad *in vivo* de las mismas muestras, en el biensayo de McKenzie. Se validó el método fisiológicamente, llevando a cabo determinaciones en plasmas de ratas sometidas a situaciones experimentales que se sabe disminuyen (hipofisectomía, tratamiento con hormonas tiroideas, anestesia con éter) o aumentan (inyección de TRH, tiroidectomía, tratamiento con bociógenos) los niveles de TSH circulante. Los valores encontrados en ratas normales son comparables a los obtenidos con los inmunorreactivos homólogos distribuidos por los NIAMDD del NIH (U.S.A.).

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