HOMOLOGOUS RADIOIMMUNOASSAY OF THYROTROPHIN IN RAT PLASMA

By
J. David Kieffer, Bruce D. Weintraub*, Walter Baigelman, Susan Leeman and Farahe Maloof

ABSTRACT
An homologous radioimmunoassay (RIA) using the highly purified rat thyrotrophin (TSH) and anti-rat TSH recently made available by NIAMDD is described in detail.
Evidence that the assay measures TSH and only TSH includes the following:
(a) Treatment of rats with TSH-releasing hormone (TRH) caused a significant increase (averaging 12-fold) and treatment with T_4_, a significant decrease (averaging 4.5-fold), in plasma TSH.
(b) Points for TSH standards and those for dilutions of plasma from TRH-treated rats fell on the same line, and regression lines calculated separately for standards and dilutions of plasma did not depart significantly from parallelism.
(c) At 14 days after gonadectomy of male rats, a time when plasma LH and FSH levels are known to be high, the assay showed no increase in plasma TSH. Moreover, reduction of plasma TSH levels by T_4_ was as great in gonadectomized rats as in controls.
(d) Assay of rat LH, rat FSH and rat prolactin, in 7 concentrations each, showed that cross-reaction averaged less than 1% in all cases. Other workers have calculated values greater than 1% for TSH contamination of rat LH and FSH. The slopes of regression lines for the 3 hormones tested for cross-reaction did not differ significantly from the slope for TSH standards. This result strengthens the hypothesis that the apparent slight cross-reactions are due to TSH contamination.

* Present address: Clinical Endocrinology Branch, National Institute of Arthritis, Metabolism and Digestive Diseases, Bethesda, Md. 20014, USA.
Findings for T₄-treated rats and saline-treated controls show that the homologous RIA is more sensitive than previous, heterologous assays: In previous studies, plasma TSH levels of most or all of rats treated with T₄ were not clearly greater than zero. By contrast, in the homologous RIA reported here, values for such rats did not overlap the range of the zero point on the one hand, nor the range for saline-treated controls on the other. Thus, distinct ranges were defined for both normal and low TSH levels. In view of the 1:20 final dilution of plasma during assay, it does not seem likely that non-specific effects of plasma were primarily responsible for the low TSH values of T₄-treated rats.

Additionally, the useful range of the homologous assay (about 200-fold) is greater than that of earlier assays (about 70-fold, or less). Finally, the use of highly purified rat TSH for standards has avoided inconsistencies previously encountered with bovine TSH standards.

Since 1966, several reports (Reichlin et al. 1966, 1970; Panda & Turner 1967; Peake et al. 1967; Wilber & Utiger 1967) have appeared of immunoassay of TSH in rat plasma. These studies have all involved the use of an heterologous immune reaction. Specifically, they have depended on the cross-reaction of rat TSH in the plasma sample with antibody raised against bovine TSH. Moreover, the iodinated antigens used have not been of rat origin; either bovine TSH (Reichlin et al. 1966, 1970) or mouse thyrotrophic tumour TSH (Peake et al. 1967; Wilber & Utiger 1967) has been employed. Additionally, bovine TSH has constituted the standards in the reports cited. Upon discovery of non-parallellism of bovine standards and crude rat pituitary extract, Reichlin et al. (1970) adopted the latter as a working standard. The present paper describes a completely homologous radioimmunoassay (RIA) for rat TSH: Highly purified rat TSH was used for iodinated antigen and for standards. The antibody employed was raised against rat TSH. Advantages of the homologous RIA over previous heterologous methods include greater sensitivity and wider useful range. Furthermore, standardization in terms of purified rat TSH has been validated for the homologous assay; the clarity and simplicity thus attained are detailed below. There is no a priori reason why such standardization should not be validated for other rat TSH assays as well.

MATERIALS AND METHODS

Reagents

(a) Hormones. – NIAMDD-Rat TSH-I-1, which has a biological potency of about 35 IU/mg in the McKenzie assay, according to information supplied with the hormone, was used for standards and for iodination. NIAMDD Rat LH-RP-1, NIAMDD Rat FSH-RP-1, and NIAMDD rat prolactin-RP-1 were assayed to help determine the specificity of the assay.
Procedures

(b) Antibodies. – NIAMDD-Anti-Rat-TSH serum-1, which has been absorbed with rat LH before distribution to users, constituted the “first” antibody in the double-antibody RIA. Since the “first” antibody was raised in rabbits, normal rabbit serum served as “carrier”; and goat-anti-rabbit-gamma-globulin (Antibodies, Inc., Davis, California) served as “second” antibody. Prior to use, each lot of anti-rabbit-gamma-globulin was titered to equivalence by determining the amount above which no further precipitation of radioactivity occurred, under assay conditions.

(c) Buffers. – A protein-free, 0.05 M phosphate buffer of pH 7.4 was used to dissolve TSH for iodination. A 0.1 % solution of human serum albumin in this buffer was the diluent for all other procedures in the assay, except that phosphate-buffered saline (0.01 M phosphate, 0.15 M sodium chloride, pH 7.4) with 0.02 % sodium azide was used for elution of columns, as noted below.

Procedures

(a) Iodination. – Preliminary experiments with bovine TSH demonstrated that the sensitivity of assays using tracer iodinated more than 2–3 weeks previously was substantially less than the sensitivity of assays using freshly iodinated tracer. To minimize the effect of this variable during development of the homologous assay, rat TSH was iodinated not more than 5 days before use in the assay. The effects of delays greater than 5 days have not yet been determined for rat TSH.

One to two days before the start of an assay, 5 µg of rat TSH was iodinated by the Chloramine-T method (Greenwood et al. 1963) in the presence of 700–900 µCi of 125I-NaI (Cambridge Nuclear Corporation). The reaction products were promptly applied to a column (90 x 1.5 cm) of Sephadex G-100 in a 4°C coldroom and eluted with the buffer noted above. A typical pattern of elution is shown in Fig. 1. Titration against anti-rat TSH ranging from 1:1000 to 1:5 000 000 in final dilution showed that the second peak (maximum at 78 ml in Fig. 1) was immunoreactive TSH. Preliminary experiments have indicated that the fraction of maximum radioactivity in the second peak and 1–2 fractions on either side have similar, high antibody-binding capacities. Strong immunoreactivity of the tracer was confirmed in each assay by inclusion of tubes with a final antibody dilution of 1:10 000. Binding in such tubes was 74.3 ± 1.4 % (mean ± se) of total counts, nearly twice the binding achieved by the smaller amount of antibody in the assay tubes per se. Volumes of elution indicated that the first peak (maximum at 69 ml in Fig. 1) was iodinated organic aggregates and the third peak (maximum at 146 ml in Fig. 1) was 125I-NaI. Incorporation of radioactivity into organic products varied from 43 % to 75 %, and the estimated specific activities of the iodinated TSH ranged from 70 to 130 µCi/µg.

(b) Assay. – The assay was carried out in 10 x 75 mm disposable tubes. These were kept at 4°C between additions. On day 1 of the assay, two additions were made to each tube: 50 µl of plasma sample (or of standard solution) and an 850 µl addition which contained 8.0 µl of normal rabbit serum, 1.1 µl of a 1:100 dilution of anti-rat TSH, and 840.9 µl of buffer. Control (“non-immune”) tubes which contained only normal rabbit serum and buffer were also prepared. All tubes were mixed on a Vortex mixer after the 850 µl addition and each subsequent addition. Tubes were made up in duplicate. Six to seven pairs were prepared for the zero point of the standard curve, to estimate the variability of the procedure.

Preliminary experiments indicated that assays in which the addition of labelled TSH tracer was delayed were more sensitive than those in which tracer was added
Elution of radioactive products of TSH iodination from Sephadex G-100. Each fraction was 1 ml. Iodinated TSH of fraction 78 was used in the assay. The peaks preceding and following the TSH peak represent, respectively, iodinated organic aggregates and $^{125}$I-NaI. See text. Multiplication of cpm on the ordinate by a factor of 769 yields values comparable to those in the assay tubes, because of differences in counting geometry.

at the same time as unlabelled TSH in standards or samples. Therefore, on day 3, about 48 h after the start of the assay, iodinated rat TSH was diluted (1:200-1:300) so that 100 µl contained 10 000–24 000 cpm; and this volume was added to each tube. Aliquots of 100 µl of labelled TSH were also added to 4 pairs of otherwise empty tubes, to measure total counts. In four assays, the tracer mass per tube, as calculated from specific activities, ranged from 100 to 175 pg.

Addition of iodinated TSH brought the final volume before precipitation to 1.000 ml, and the final dilution of “first” antibody to 1:90 000. In four typical assays, the mean ± se for binding at the zero point was 39.5 ± 1.1 % of total counts.

About 72 h after the addition of iodinated TSH (day 6), “second” antibody, diluted to provide the appropriate amount in 100 µl, was pipetted into all but the “total counts” tubes. After another 20–24 h incubation (day 7), all but the “total counts” tubes were centrifuged for 45 min at 3000 r. p. m. in a Sorvall RC-3 refrigerated centrifuge. The supernatants were decanted, and the tubes were inverted and drained for at least 15 min. The inside of each tube was then wiped with cotton-tipped applicators to maximize removal of supernatant. Radioactivity of the precipitates was determined in a well-type Packard Scintillation Spectrometer. Counts for duplicate tubes routinely agreed to within 5 %, virtually always to within 10 %. Non-specific counts, including any counts from residual supernatant, were consistently low. In four representative assays, such “non-immune” counts constituted 2.8 ± 0.2 % (mean ± se) of the total counts. Non-specific counts were subtracted from total counts as well as from the counts for each sample.

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Calculations. – Data were processed according to Method 1 of Rodbard et al. (1969). Briefly, this involves reduction of the standard curve to the linear form logit
\( Y = a + b \log_{10} X \), where \( Y \) is the relative per cent bound, \( X \) is the concentration of unlabelled TSH, and logit \( Y = \log_{10} (Y/100-Y) \). In general agreement with the report of Rodbard et al. (1968), the transformed standard curve was linear over at least the range of 12–88 relative per cent bound; only points within this range were used to determine the regression line. This determination, and the subsequent computations of values for unknowns, were performed on a Wang desk-top calculator.

In each assay, the value falling 2 so below the mean of 12–14 replicates of the zero point of the standard curve was taken as the limit of detection. Points lying between this limit and the most dilute standard were assigned values by numerical extrapolation of the logit-log regression line. The accuracy of this procedure was confirmed by close agreement between extrapolated readings and readings for the same samples made graphically from the simple arithmetic standard curve (Fig. 2 a), which is linear in this region.

Animal experiments

To demonstrate that the assay would detect predictable variations in TSH, the effects of TSH releasing hormone (TRH) and of \( T_4 \) on plasma TSH were measured. To show that high gonadotrophin levels would not result in spurious TSH values, plasma TSH was determined in both control and \( T_4 \)-treated gonadectomized rats.

Rats of the Charles River CD strain were used throughout; tap water and Purina Lab Chow were always available. The TRH studies were done on immature females. All other experiments involved adult males.

TRH (Abbott Laboratories) in isotonic saline was administered iv. Sodium L-thyroxine pentahydrate (Sigma Chemical Co.) was dissolved in 1 n NaOH and brought to the appropriate volume with saline, at a finally normality of 0.01 n NaOH. This preparation, or alkaline saline for controls, was injected ip in a volume of 0.5 ml. \( T_4 \) doses cited are in terms of the free acid.

Blood was collected into heparinized tubes by decapitation under light pentobarbital anaesthesia. After separation, the plasma was stored frozen until assay. In view of reports of variations in TSH levels due to anaesthesia (Ducommun et al. 1966; Wilber & Utiger 1967) and to diurnal variation (Bakke & Lawrence 1965), care was taken that experimental and control groups were anaesthetized by the same procedure and killed at approximately the same time of day. Preliminary studies using an heterologous assay similar to that of Reichlin et al. (1970) have tended to confirm the findings of somewhat lower TSH levels in anaesthetized rats and not to confirm the existence of diurnal variations of TSH. Use of the more sensitive homologous RIA to repeat and extend the preliminary studies should help to clarify these issues.

RESULTS

Standard curves

Fig. 2 a and 2 b show, respectively, the simple arithmetic and the logit-log forms of the standard curve for one performance of the assay. Table 1 shows the reproducibility of salient statistical features of the logit-log form, as calculated for 4 representative performances of the assay. The limit of detection
Arithmetic plot of a standard curve for rat TSH and of dilutions of plasma from TRH-treated rats. TSH values on the abscissa represent final concentrations in the assay tubes. The location of the μl scale with respect to the scale for standards was based on the mean of assayed values for all dilutions.

is cited in terms of plasma levels; that is, the figure given in Table 1 is 20 times the minimum detectable concentration in the assay tubes, to correct for the 20-fold dilution of sample during assay. The comparable figure for the maximum concentration that can be measured with accuracy is 20 × 2.5 ng/ml—that is, 50 ng/ml. The potentially useful range of the assay is thus about 200-fold.

To determine the applicability of the purified TSH standards to the measurement of plasma TSH, the pooled plasma of 4 TRH-treated rats was assayed in 5 dilutions ranging from 1:1 to 1:6. Fig. 2a suggests strongly that standards and plasma dilutions lie on the same line, for TSH concentrations from zero to at least 17 ng/ml (0.85 ng/ml in the assay tube). Logit-log regression lines were calculated separately for standards and for dilutions of plasma (Fig. 2b). A t-test for slopes revealed no significant difference, $P \geq 0.05$. Thus, there was no significant departure from parallelism in the range tested. Extension of the
Fig. 2 b. Logit-log plot of the data of Fig. 2 a. Observed data appear as points. The solid lines are calculated regression lines. The line for dilutions of plasma has been shifted to the right to facilitate graphical comparison of the slopes. The values on the left-hand ordinate are based on setting the mean binding at the zero point of the standard curve equal to 100%. Because of the logarithmic abscissa, zero points are not shown.

Zero points and their relation to other points can be examined in Fig. 2 a.

demonstrated range of close similarity of standards and plasma to or toward the 50 ng/ml upper limit of the assay will be attempted if plasmas with sufficiently high endogenous TSH can be obtained. Assay of an additional dilution of plasma, to reduce the range of interpolation between the zero point and the 1:6 dilution is planned as a confirmatory study.

Table 1.
Statistical features of standard curves for 4 TSH assays in logit-log form.

<table>
<thead>
<tr>
<th></th>
<th>Mean ± se</th>
</tr>
</thead>
<tbody>
<tr>
<td>Correlation coefficient (r)</td>
<td>-0.993 ± 0.002</td>
</tr>
<tr>
<td>Slope (b)</td>
<td>-2.260 ± 0.040</td>
</tr>
<tr>
<td>y-intercept (a)</td>
<td>-1.229 ± 0.047</td>
</tr>
<tr>
<td>Standard error of estimate (Sy, x)</td>
<td>0.136 ± 0.022</td>
</tr>
<tr>
<td>Lowest plasma TSH level detectable, ng/ml</td>
<td>0.23 ± 0.03</td>
</tr>
</tbody>
</table>
Table 2.
Effect of T₄ and gonadectomy on plasma TSH concentrations.

<table>
<thead>
<tr>
<th>Surgical status of rats</th>
<th>Plasma TSH, ng/ml</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± se</td>
<td>No. of rats in brackets</td>
<td></td>
</tr>
<tr>
<td>Saline treated</td>
<td>T₄-treated (a)</td>
<td>(8)</td>
<td>(9)</td>
</tr>
<tr>
<td>Intact</td>
<td>2.06 ± 0.47</td>
<td>0.43 ± 0.04 (c)</td>
<td></td>
</tr>
<tr>
<td>Sham-gonadectomized (b)</td>
<td>1.37 ± 0.23</td>
<td>0.40 ± 0.04 (c)</td>
<td></td>
</tr>
<tr>
<td>Gonadectomized (b)</td>
<td>2.00 ± 0.23</td>
<td>0.37 ± 0.05 (c)</td>
<td></td>
</tr>
</tbody>
</table>

(a) T₄, 50 µg/rat/day for last 3 days before autopsy.
(b) Surgery 14 days before autopsy.
(c) Significantly less than saline controls, P < 0.001.

To assess the reproducibility of values assigned to unknowns, the plasma TSH levels of 8 normal rats (intact controls of Table 2) were determined in 2 separate assays. The mean values ± se were 2.1 ± 0.5 and 2.2 ± 0.5 ng/ml. Moreover, the mean of the differences of the 2 values for each plasma was only 0.1 ng/ml. A t-test for paired samples showed that these differences were not significant, P > 0.05.

Cross-reaction with other rat pituitary hormones

Rat LH, rat FSH, and rat prolactin were assayed in 7 concentrations each, ranging from 10 to 1000 ng/ml in the assay tubes.

The extent of cross-reactivity was calculated on a per cent basis; that is,

\[
\text{Cross-reaction} = \frac{\text{TSH concentration as assayed}}{\text{known hormone concentration of LH, FSH, or prolactin}} \times 100
\]

was determined for each sample. The mean ± se percentages of cross-reaction were 0.61 ± 0.04 %, 0.79 ± 0.07 %, and 0.11 ± 0.01 %, for LH, FSH, and prolactin, respectively. The values for LH and FSH are well within the per cents of TSH contamination calculated by Reichlin et al. (1970) from bioassay data; no estimate was given for prolactin. Moreover, we found that the slopes of the regression lines for the 3 hormones tested did not differ significantly from the slope for TSH standards, P > 0.05 in all cases. This constitutes further evidence that the apparent cross-reactions actually represent contamination of the LH, FSH, and prolactin preparations by TSH.
Effects of $T_4$ and gonadectomy on plasma TSH concentrations

The data of Table 2 show that gonadectomy of male rats did not result in increased plasma TSH levels 14 days later: The value for intact controls was slightly higher than that for gonadectomized rats; sham-operated animals had a slightly, but not significantly ($P > 0.05$) lower value than did gonadectomized rats. In view of the reports (Gay & Midgley 1969; Root & Russ 1972) of highly elevated serum LH and FSH levels 14 days after gonadectomy of male rats, the lack of elevation of TSH in such rats indicates that the gonadotrophins have little if any influence on our TSH assay.

The additional finding (Table 2) that the plasma TSH levels of both gonadectomized and control rats were strongly suppressed by $T_4$ constitutes further evidence for the specificity of the assay.

Of the 24 $T_4$-treated rats represented in Table 2, 23 exhibited TSH values strictly above the limit of detectability, which was 0.22 ng/ml in that particular assay. The other TSH value coincided exactly with the limit. Similarly, of the 26 saline-treated rats represented in Table 2, 25 showed TSH values strictly greater than the highest value for $T_4$-treated rats. The remaining value fell near the top of the range for $T_4$ treatment. These results indicate that the assay can discriminate clearly between normal and low TSH levels.

Effect of TRH on plasma TSH concentrations

Four immature female rats bled 15 min after the administration of TRH (25 ng/100 g body weight), had mean ± se plasma TSH levels of 9.6 ± 0.8 ng/ml. Four saline-treated control rats had 0.8 ± 0.1 ng/ml. This difference is significant, $P < 0.02$. Detection of this predictable rise in plasma TSH constitutes further evidence that the assay in fact measures TSH.

DISCUSSION

One major problem encountered in previous efforts to measure TSH in rat plasma by means of heterologous assays has been the selection of appropriate standards. Reichlin et al. (1970) demonstrated that bovine TSH and a crude rat pituitary extract produced standard curves that were not parallel, while curves for the extract and those for dilutions of rat plasma with high TSH levels were parallel. For this reason, Reichlin et al. (1970) calculated their results in terms of the extract as a working laboratory standard and converted to USP units on the basis of bioassay of the extract. However, their findings also showed that conversion to USP units does not eliminate inconsistencies among standards: They assayed 3 groups of normal rat plasmas against both rat and bovine standards. Even after conversion to USP units, the values based

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on the rat standards averaged 8-fold higher than the values based on the bovine standards. Moreover, the group with the highest values in the case of rat standards had the lowest values according to the bovine standards. We suggest that NIAMDD-Rat-TSH-I-1 constitutes an excellent assay standard. This material produced a standard curve parallel to that of dilutions of plasma of TRH-treated rats. Because of its high purity, NIAMDD-Rat-TSH-I-1 yields numerical values that probably approach true absolute values. Moreover, this material can be expected to be available to all qualified investigators from a single, reliable source. Therefore results calculated in terms of NIAMDD-TSH-I-1, unlike those calculated in terms of a local laboratory standard, will not require conversion to USP units. Additionally, our results show that extremely small amounts of NIAMDD-Rat-TSH-I-1 are adequate for the preparation of standards: Standards sufficient for hundreds of assays have been made from 5 μg, the amount consumed in a single iodination.

A second major problem encountered in the heterologous assay of TSH has been lack of sensitivity to low plasma TSH levels. Because of the difficulties of standardization described above, the numerical values cited as limits of detectability have varied considerably and are difficult to interpret. However, a consistent finding has been that TSH was undetectable in the plasmas of rats treated with T₄ (Wilber & Utiger 1967; Reichlin et al. 1970) or subjected to hypophysectomy (Panda & Turner 1967). The homologous RIA has detected TSH in rats treated with the same T₄ regimen reported by Reichlin et al. (1970). It is possible that a non-specific effect of plasma may account for some part of the low TSH values found for such animals, despite the 1:20 final dilution of plasma during assay. Further experiments with other T₄ regimens are underway in our laboratory to investigate this matter more fully. Nevertheless, with reference to a sure (buffer) zero point, the homologous RIA has given TSH values for normal rats that show virtually no overlap with those for T₄-treated rats; in turn, the values of the T₄-treated rats do not overlap the range of the zero point.

An additional advantage of the homologous assay is its wide useful range — about 200-fold, as noted above. Previous heterologous assays have had a useful range of about 70-fold or less, as estimated from data presented graphically.

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* Plasma TSH was undetectable in 7 of 7 rats subjected to both hypophysectomy and and prolonged treatment with T₄ (50 μg/rat/day for 10 days).
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