REVISTA ESPAÑOLA DE FISIOLOGIA, 33, 119-128. 1977

Validation of a Radioimmunoassay for Rat Thyrotrophic Hormone. II. Comparison with Results Obtained by Bioassay

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(Received on September 17, 1976)

M. D. GARCIA, L. CACICEDO and G. MORREALE de ESCOBAR. *Validation of a Radioimmunoassay for Rat Thyrotrophic Hormone. II. Comparison with Results Obtained by Bioassay.* Rev. esp. Fisiol., 33, 119-128. 1977.

As previously reported a heterologous radioimmuoassay (RIA) system was developed for the determination of rat thyrotrophic hormone (TSH), using guinea-pig antiserum against bovine TSH and a purified murine TSH for labeling. With this RIA system it is possible to detect TSH in plasma from normal rats, to follow its decrease during ether anaesthesia, and to differentiate between the TSH levels of normal rats and those of hyphophysectomized or thyroid hormone-treated rats; in appears to be free from interferences due to high circulating gonadotrophin levels.

Several preparations containing high concentrations of rat TSH were assayed simultaneously by this RIA and the McKenzie TSH bioassay. Bioassay values, especially for plasmas, tended to be higher than those obtained by RIA. The mean ratio of bioassay to RIA values for the limited number of samples tested so far is about 1.5 $\pm$ 0.2. Therefore, it does not appear correct to express rat TSH values obtained by RIA in biological potency units; they should only be given in terms of weight equivalents of an international rat reference preparation.

A heterologous radioimmunoassay (RIA) system which was developed for the determination of thyrotrophic hormone (TSH) in the plasma and pituitary of rats was previously described (11). This RIA system uses an antiserum against bovine TSH, obtained in the guinea-pig, and a purified mouse tumour TSH (M-TSH) as antigen for labeling, and was validated on the basis of numerous and different criteria. Though some data suggested that high gonadotrophin levels did not interfere with the determination of TSH, the specificity of the RIA system

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for TSH appeared to require further validation. Moreover, to our knowledge, TSH values, as determined by RIA in samples from rats, have not been compared to those obtained by bioassay. Despite this, many investigators express rat TH results obtained by RIA in terms of biological potency units.

The present paper describes the results obtained with the rat TSH RIA previously described (11), in plasmas from rats with very high circulating levels of pituitary gonadotrophins. It also describes the preliminary result obtained when several rat samples were submitted simultaneously to the RIA and to a bioassay.

Materials and Methods

Hormones. The mouse thyrotropic tumour preparation (M-TSH) had a potency of about 5 USP (Bovine) U/mg. The rat reference preparation (NIAMDD-Rat TSH-RP 1) had a reported potency of 0.22 USP (Bovine) U/m. The bovine LH preparation used was NIH-LH-B_7 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 used for some experiments.

Antisera. These were prepared against Thytropar (B-TSH, Armour Co., Ill.) in male guinea pigs (9-11) and assayed for their capacity to inactivate the *in vivo* response of the mouse thyroid to stimulation by a *rat* pituitary extract, using the TSH McKenzie bioassay (19) as previously modified (9).

Labeled antigen. One mg of M-TSH was dissolved in 1 ml of pH = 7.5, 0.01 M phosphate buffer, divided into 10 μl aliquots, stored frozen in liquid N_2, and thawed immediately before labeling.

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.

Standard and reference preparations. The anterior pituitaries of adult male rats were homogenized in PBS (1 pituitary/ml), centrifuged in the cold at 3,500 r.p.m. for 30 min, and the supernatant divided into 200 μl aliquots, which were stored at —20° and thawed only once. The arbitrary unit (1 U R-TSH) was defined as the TSH content of 1 ml of this crude extract. As will be shown here, 1 U R-TSH had a potency of 0.23 USP (Bovine) U/ml, with 95 % fiducial limits of 0.17 and 0.31, in two different McKenzie bioassays. This crude extract was used as internal standard until NIAMDD-Rat TSH-RP 1 was distributed. Unless stated otherwise, serial dilutions of this extract and of the NIAMDD standard, were carried out with plasma from rats treated with thyroxine (T_4) and triiodothyronine (T_3) for the last 24 h [referred to as (T_4 + T_3)-Pl]. It was also used for the «0» points (no cold TSH added) and «blanks» (no first antibody added).

Rat pituitary and plasma samples. To obtain pituitaries or plasmas from rats with high circulating TSH or gonadotropin levels, the rats were either. i) injected into the femoral vein with 0.5-2.0 μg synthetic TRH (Abbot Laboratories, Ill.) 10-20 min before bleeding; ii) fed a low iodine diet containg 0.05 % 6-propyl-2-thiouracil (PTU) for 0-10 days before bleeding; iii) thyroidectomized surgically as described by ZARROW *et al.* (25); iv) ovariectomized bilaterally (25) and injected, one and a half months later, with 25 mg progesterone (P) and 50 μg estradiol (E) sc once, three days before bleeding, as described by RAMÍREZ and MCCANN (20).

Procedure for the RIA. The prepa-

ration and storing of standard curves as well as the labeling procedure, the purification on Sephadex G-50 after radioiodination, and on Sephadex G-100 just prior to use of the M-TSH* [1] in the RIA, as well as incubation conditions and second antibody procedure, are described in detail elsewhere (11).

Bioassay. The McKENZIE assay (19) was used, with modifications described elsewhere (9). In brief, after a «low iodine regime», the mice are injected with I*, and 20 μg T_4 sc, followed by 0.2 μg T_3/day sc. About 3-4 days after the I* injection, a basal blood sample is withdrawn by orbital sinus puncture, and a response sample is again withdrawn 3 h after the ip injection of the TSH standard, or the «unknown» preparation. For each dilution of the different samples, 5-7 mice were used. The intensity of response was calculated as the log of the % response (taking the basal value as 100 %). The log transformation avoids the considerable heteroscedasticity observed with the % response data.

Calculations. The RIA data were automatically transformed into RPB (relative percentage bound) using the mean net radioactivity of the «0» points (no cold TSH added) as 100 %. The logit RPB-log TSH plots were used for tests of linearity and parallelism, calculation of the index of precision λ ($\lambda = \frac{\sqrt{s^2}}{b}$), and of the TSH concentration of «unknowns» with 95 % confidence intervals. Only the linear portion of the logit-log plots was used, and this usually comprised the RPB values between 10 and 90 %. The automated calculations (Olivetti P 602 desk microcomputer) were based on the principles outlined for bioassays by BLISS (5). The same programs could be used for the bioassay data, using the linear portion of the log % response-log TSH plots.

(1) The asterisk denotes the I* labeled preparation.

Results

Standard Displacement curves. Figure 1 shows typical displacement curves simultaneously obtained with serial dilutions of different TSH preparations. Parallelism of curves prepared with different samples obtained from rats was excellent. On the basis of displacement curves obtained with the R-TSH and the NIAMDD-Rat-TSH-RP-1 preparations in the same RIA, it appears that 1 of the 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_5 preparation appeared parallel to those obtained with R-TSH. One of the 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 %

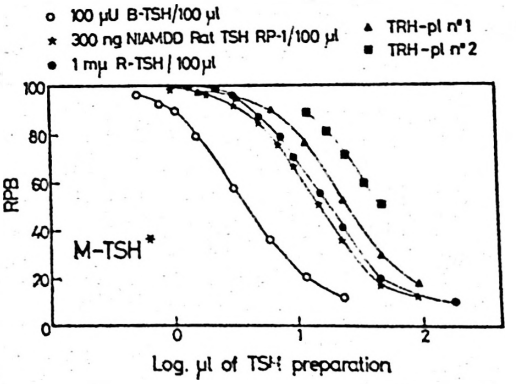

Fig. 1. *Displacement curves obtained simultaneously with M-TSH* and serial dilutions of different preparations contaning bovine or rat TSH.*

The final Ab dilutions was 1:120000 and conditions of the RIA were the standard ones. TRH-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.

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, a degree of displacement compatible with the known TSH content of this B-LH preparation was then found.

Possible interference from high gonadotropin levels. A possible interference by *rat* LH or FSH also appeared to be unlikely on the basis of previous preliminary tests using plasma from male rats gonadectomized for two months (11). To further assess the possible influence of high gonadotropin levels, female rats were ovariectomized ($\overline{O}$) for one and a half months and then subdivided into three main groups (fig. 2). The circulating TSH levels of the $\overline{O}$, $\overline{O}$ + PE, and $\overline{O}$ + PE + LH-RH rats were higher than those of the intact females sacrificed on the same day, though within limits often found for intact male an female rats. Previous injection with T_4 influenced these TSH values. This treatment did *not*, however, decrease the circulating LH levels, which in $\overline{O}$, $\overline{O}$ + PE and $\overline{O}$ + PE + LH-RH rats had increased to 16; 6 and 44 times normal, respectively, as determined by using the NIAMDD rat LH immunoreactants.

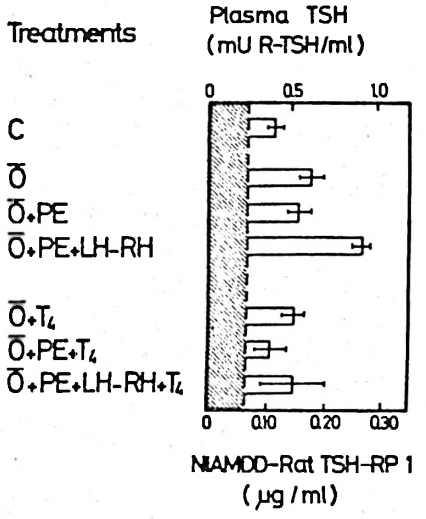

Fig. 2. *Mean plasma TSH levels found in plasma pools from rats which were ovariectomized ($\overline{O}$), treated with progesterone and estrogen ($\overline{O}$+PE) (20) and injected with 17 ng LH-RH iv. 15 min before bleeding ($\overline{O}$+PE+LH-RH).*

Five of the 10 animals undergoing each of these treatments were injected ip with 50 μg T_4 the day before bleeding. A group of age-paired control female rats (C) were also bled. Plasmas from the rats of each group were pooled. The bars represent the SD of the TSH values obtained for these pools on 3 different occasions. Data were obtained against R-TSH as standard and are shown on the upper abscissa; these were recalculated in terms of the NIAMDD-Rat-TSH-RP 1 preparation on the basis of the equivalence between both preparations as determined later, and are shown on the lower abscissa. The limit of sensitivity of these assays was 0.25 mU R-TSH/ml. Several of the plasmas for group III fell below this limit and were assigned this value for the calculation of the means ± SD.

Comparison of RIA and bioassay results. Plasmas were obtained from rats

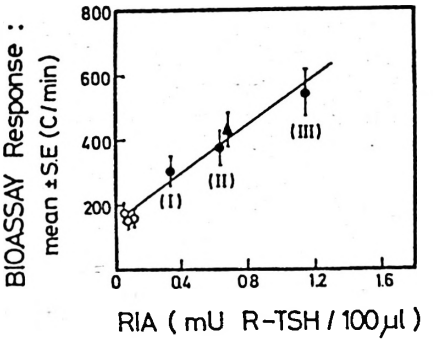

Fig. 3. *Comparison of the TSH levels obtained by RIA with the responses of mice prepared for the McKenzie bioassay.*

For the RIA, 70 μl of each of the control (O) plasma pools were used in triplicate, and 10 μl of the plasmas from rats on 5 mg PTU/day (●) for 3 (I), 6 (II) and 10 (III) days and on PTU for 11 days, injected with 50 μg T_4 18 h before bleeding (▲). There were 6 rats/pool. For the bioassay 300 μl/mouse of each of the plasma pools were injected into 5-6 mice.

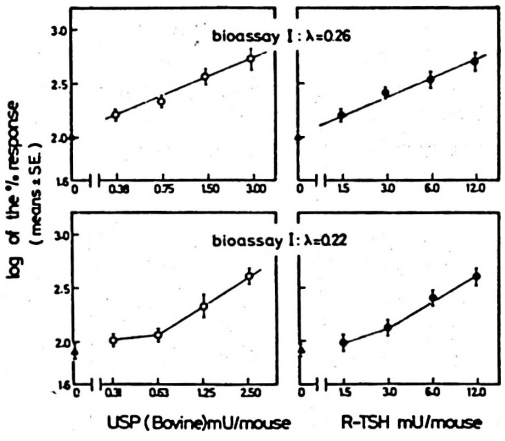

Fig. 4. *Log of the % response plotted against log TSH concentration, using data obtained in two bioassays performed with the -NIH TSH-B_5 preparation* (○) *and our internal rat standard* (●).

The triangles correspond to the log of the % response to saline. The amounts of TSH indicated in the abscissa were injected in 300 µl/mouse, with 6-7 mice/assay point. Only the linear portion of the curves corresponding to bioassay II were used to assess the potency of the R-TSH preparation.

fed 6-propyl-2-thiouracil (PTU) (fig. 3) and assayed both by the present RIA and the McKenzie bioassay. In either case the R-TSH preparation was used as internal standard, but the TSH concentrations could not be calculated for the bioassay, because most of the plasmas from PTU-treated rats induced a response which exceeded that of the highest dose of the standard. Despite this, it may be seen that the variations in TSH concentration detected with the RIA were consistent with the responses observed in the bioassay.

Several pituitary preparations and plasma pools obtained from rats with high circulating TSH levels were also assayed both by RIA and bioassay. For the RIA, serial dilutions comprising 6-7 different concentrations of each preparation were carried out using the (T_4 + T_3)-Pl as diluent. This involved two different RIA.

Table I. *TSH content of 1 R-TSH Unit in terms of USP (bovine) units, as determined in two different RIA and two different bioassays.*

Numbers in brackets are the 95 % fiducial limits.

Assay	RIA data	Bioassay data
I	0.026 (0.022; 0.030)	0.23 (0.17; 0.31)
II	0.026 (0.023; 0.029)	0.23 (0.16; 0.31)

The same samples were injected into mice prepared for the McKenzie assay, dilutions being carried out in 0.9 % NaCl containing 2 % rat plasma. The R-TSH and NIH-TSH-B_5 preparations were injected at 4 dilutions, the other pituitary preparations or plasmas pools at 2 or 3 dilutions. Two different bioassays were carried out. Both for the RIA and bioassay data tests of linearity and parallelism were carried out prior to calculations of potencies and 95 % fiducial limits. Dilution curves obtained with rat an bovine preparations were parallel both in the RIA system (fig. 1) and in the bioassay (fig. 4). Thus, in each case the TSH potencies could be estimated against both the internal R-TSH preparation and the NIH-TSH-B_5 standard (table I). There is a very good coincidence of the results obtained in each of the two RIAs, or the two bioassay. But there is almost a nine-fold differences between the values obtained by RIA, and those found by bioassay. Figure 5 shows the results obtained with different preparations, and expressed in the units indicated in the legend. In all three panels the dotted line represents a 1:1 ratio of the bioassay to RIA data. As may be observed in panels A and C, the values of several of the plasma samples obtained by bioassay were higher than the RIA data. In these cases the 1:1 value was found to be below the 95 % fiducial limits of the potency estimates (not shown in the figure). Taking all the data as a whole, the regression coefficients for the

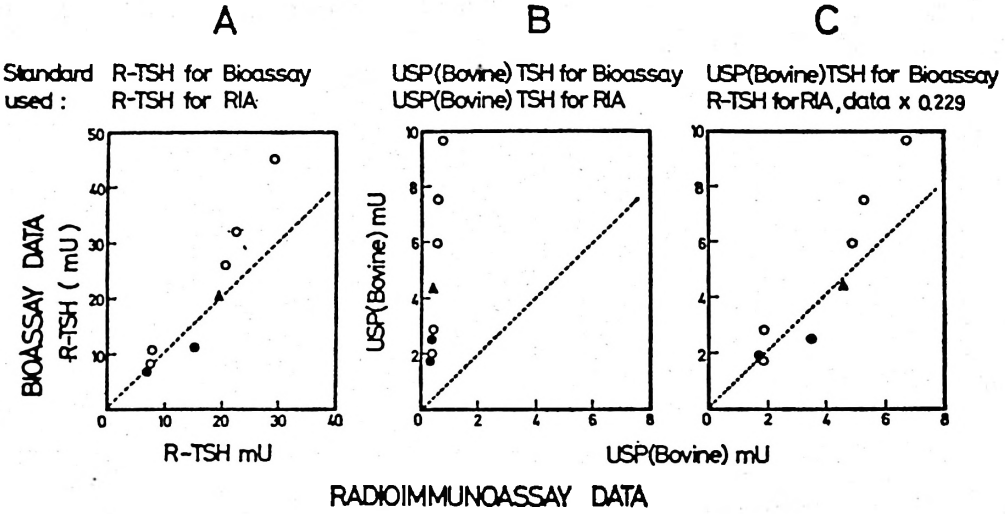

Fig. 5. *Comparison of THS data obtained by bioassay and RIA with different plasma and pituitary preparations.*

Plasmas (O) were obtained from TRH-injected rats (2 μg iv 20 min before bleeding) or from animals thyroidectomized for a month or longer. The rat pituitary preparation used (●) was NIAMDD-Rat TSH-RP 1 and NIH-LH-B, is represented by ▲. For panel A, both bioassay and RIA data are given in terms of our arbitrary R-TSH units, as read off the bioassay and the RIA dose-response curves obtained with the internal rat pituitary extract standard; for panel B data are given in terms of USP (bovine) units, as read off the bioassay and RIA dose-response curves obtained with NIH-TSH-B_5. For panel C, however, the data obtained in the RIA were read off the R-TSH dilution curve, and then transformed into USP (bovine) potency units on the basis of the potency of this preparation in the two bioassays (see table I). The regression coefficients ($b \pm S_b$) of the bioassay values over the RIA values were: 1.57 ± 0.18 for A; 10.89 ± 1.41 for B and 1.48 ± 0.19 for C.

plots in panels A and C were about one and a half the theoretical value of 1, with $P < 0.025$ and < 0.05, respectively. In the case of the plot in panel B the data fall on a line which has a regression coefficient which is not one and a half, but *ten* times the theoretical value of 1.

Discussion

Data published elsewhere in detail (11) have validated this heterologous RIA system using M-TSH*, on the basis that it permitted the detection of predicted changes in circulating TSH levels of rats submitted to different experimental procedures. It has also been used successfully for studies of rat physiology (8, 12, 14, 15). Present data again confirm the validity of the heterologous assay: it is not appreciable influenced by very high circulating levels of pituitary gonadotropins. Even in the $\overline{O}$ + PE + LH-RH rats, with LH levels 44 times normal, the measured TSH concentration showed values which may be found in normal rats. It is quite likely that the values found for rats with high circulating gonadotropin levels (fig. 2) represent TSH concentrations: Thus, the TSH levels were affected by the prior administration of a T_4 dose, whereas the LH levels were not. Moreover, the LH-RH preparation was of porcine origin and might have contained some TRH activity.

One of the principal difficulties encountered by different investigators measuring

TSH in rat samples has arisen from the lack of an International Standard preparation, similar to the one in use for the determinations of TSH in human samples. Therefore, it was initially attempted to express results obtained by a rat TSH RIA in terms of USP TSH potency units. The USP standard is, however, composed of *bovine* TSH. WILBER and UTIGER (24) read data directly off the RIA curve prepared with a B-TSH standard and expressed the results obtained with rat samples in international USP (bovine) potency units. They believed this could be done because in their RIA system (also using M-TSH* as labeled antigen) the standard curve obtained by serial dilutions of the USP B-TSH preparation was parallel to that obtained by serial dilutions of rat plasmas and pituitaries. They did not validate their results by bioassay of the rat samples against the USP B-TSH preparation. When we followed their procedure, however, the data were completely different from those obtained by direct bioassay against the bovine standard (table I and fig. 5B), being lower by a factor of 10. This merely stresses the fact that, although lack of parallelism clearly indicates lack of identity of preparations or the presence of interfering factors, *parallelism does not necessarily show identity of equivalence.* Though our antiserum and the one employed by WILBER and UTIGER (24) are different ones, there is no evidence that

Table II. *Circulating TSH values given for normal rats by different authors, obtained with different heterologous and a homologous RIA systems.*

Reference	Type of RIA	Reference preparation used in the RIA	Plasma TSH concentrations [a]	
			In terms of µg/ml NIAMDD-Rat-TSH-RP 1	USP (bovine) mU/ml
Wilber & Utiger (24)	Heterologous M-TSH*	USP (bovine) standard		0.015-0.066
Reichlin *et al.* (21)	Heterologous B-TSH*	Rat pituitary extract		0.47
Azizi *et al.* (1)	» »	NIAMDD-Rat-TSH-RP 1	(2.7-5.0)	0.60-1.10
Azizi *et al.* (2)	» »			0.26
Griessen *et al.* (13)	» »	USP (bovine) standard		0.26
García *et al.* (11)	Heterologous M-TSH*	NIAMDD-Rat-TSH-RP 1	0.13-0.29	0.029-0.064
Bakke *et al.* (3)	Homologous NIAMDD	NIAMDD-Rat-TSH-RP 1	0.46	0.10
Kojima *et al.* (18)	» »	»	0.08-0.15	(0.018-0.033)
Blake 1974 (4)	» »	»	0.40-0.60	(0.088-0.132)
Rivier & Vale (22)	» »	»	0.55-0.80 [b]	(0.12-0.18)
Brown & Hedge (6)	» »	»	0.60-0.75	(0.13-0.17)
Chen & Meites (7)	» »	»	0.31-0.35	(0.068-0.077)
			ng/ml NIAMDD-Rat-TSH I 1	
Kieffer *et al.* (16)	» »	NIAMDD-Rat-TSH I 1	2.06	(0.074)
Kieffer *et al.* (17)	» »	»	1.1-2.3	(0.039-0.071)

[a] Values shown outside brackets are those reported or shown in graphs by the authors. Those given inside brackets have been calculated on the basis of the reported potency of NIAMDD-Rat-TSH-RP 1 and NIAMDD-Rat-TSH I 1. Though this procedure might be incorrect, as discussed above, these calculations have been carried out to permit rough comparison of results.

[b] PE-treated rats.

the procedure was correct with the antiserum employed by these authors. REICHLIN *et al.* (21) used B-TSH* as labeled antigen and found no parallelism between the serial dilution curves obtained with rat and bovine preparations. We have confirmed this finding (11). However, using B-TSH* with our antiserum, there was also *no* parallelism between different *rat* TSH preparations, in contrast to the report by REICHLIN *et al.* (21). For this reason REICHLIN *et al.* (21) could not read the data directly off a RIA curve obtained by serial dilutions of the USP B-TSH preparation, as had been done by WILBER and UTIGER (24). Therefore they used a rat pituitary extract as internal standard and then converted the data to international USP (bovine) potency units on the basis of the potency of their rat standard in a bioassay. This procedure ought to be correct and is the one that has been applied to the present data (fig. 5, panel C). It resulted in data which were closer to those obtained by direct bioassay (fig. 5, panel B) than those obtained following the procedure used by WILBER and UTIGER (24). But even so, results as a whole showed some deviation from a 1:1 ratio. Whether this is due to a discrepancy between the biological and immunological potency of TSH in a given sample, or to differences between circulating and pituitary TSH is not known. The possibility that the bioassay is overestimating the TSH content of plasma samples has also not been ruled out. Thus, the injection of some human sera into the test mice may result in the release into the bloodstream of labeled compounds derived from extrathyroidal pools, and falsely high responses migth be obtained (23). These possibilities, are currently under study.

Present data indicate that TSH data obtained by RIA should not be converted into international USP (bovine) potency units, either in the manner indicated by WILBER and UTIGER (24) or that used by REICHLIN *et al.* (21). Now that the NIAMDD is distributing a Rat TSH reference preparation, results from different laboratories ought to be expressed merely in terms of *weight* equivalents of this NIAMDD Rat-TSH-RP 1 preparation, even if they have been obtained with the homologous NIAMDD rat TSH immunoreactants. To our knowledge, TSH data obtained with the NIAMDD homologous RIA system hace not been confirmed by bioassay, and therefore it is not known whether a 1:1 ratio would be obtained with the NIAMDD immunoreactants. It might be even better, if it were possible to use the highly purified NIAMDD Rat-TSH-RP I 1 as standard as proposed by KIEFFER *et al.* (16), since the results might be a more meaningful approximation of true TSH values.

Table II summarizes data regarding circulating TSH levels of normal rats reported by different authors. Even if we consider only those data which have been obtained against the NIAMDD-Rat-TSH-RP 1, there is considerable variability. This might be due in part to differences in the rats and manners in which the serum or plasma were obtained. They might also be due to the fact that plasma from hypophysectomized rats or rats pretreated with thyroid hormones is not always added to the standard curve. Some data (16) suggest that this might be necessary even in the case of the homologous NIAMDD RIA system. Despite these differences, data obtained with the NIAMDD immunoreactants and heterologous systems employing M-TSH* as labeled antigen appear to fall within a comparable range. On the contrary, those obtained with a heterologous RIA system employing B-TSH* as labeled antigen stand out as much higher. It has been remarked in connection with this by GRIESSEN and LEMARCHAND-BÉRAUD (13) that, using different antisera raised against B-TSH, the TSH values obtained for the same sample were quite different, even

when the same labeled B-TSH* and the same standard were used.

In conclusion, a heterologous RIA system can be developed for the determination of rat TSH, which is both sensitive and specific. Results obtained with this RIA for normal rats fall within the same order of magnitude as results obtained with the NIAMDD immunoreactants. However, when rat plasmas were used, the TSH values obtained by RIA were somewhat lower than those obtained by bioassay. Therefore, until it is shown that results obtained by RIA with the NIAMDD immunoreactants are the same as those obtained by bioassay, it appears highly desirable that at present all laboratories give results in terms of *amounts* of the same stable and, if possible, highly purified rat TSH reference preparation without converting them to potency units. Further studies comparing data obtained by RIA and by bioassay might help to solve some of the discrepancies described here and encountered in existing data.

Acknowledgements

We are extremely grateful to Dr. R. W. Bates for his gift of highly purified mouse tumour TSH, used for radioiodination, and to Drs. A. Aranda and F. Escobar del Rey of this Department for LH determinations and the preparation of the ovariectomized, PE and LH-RH treated rats, respectively. We are grateful to Dr. A. Parlow for supplying us with NIAMDD-Rat-THS-RP 1 preparation before it became more widely available, to NIAMDD for the supply of B-TSH and B-LH standard preparations, and to Dr. A. V. Schally for the highly purified porcine LH-RH/FSH-RH preparation. The technical and secretarial assistance of Ms. B. Sánchez, M. Ruiz and M. de la Fuente is also gratefully aknowledged.

Resumen

Los autores describieron con anterioridad un radioinmunoensayo (RIA) heterólogo desarrollado por ellos para la valoración de hormona tirotropa (TSH) en suero e hipófisis de ratas. Dicho RIA utiliza un antisuero obtenido en el cobaya contra TSH bovino, y un preparado de TSH murino altamente purificado como antígeno para marcar. Con dichos inmunorreactivos se pueden detectar los niveles de TSH circulante de ratas normales, y diferenciarlos de los de ratas hipofisectomizadas o tratadas con hormonas tiroideas.

En el presente trabajo se confirma que el sistema está libre de interferencias por parte de las gonadotropinas hipofisarias. Asimismo se comparan los datos obtenidos por RIA con los obtenidos por el bioensayo de McKenzie, usando plasmas e hipófisis de rata. Los valores obtenidos por bioensayo tendían a ser más altos que los obtenidos por RIA. Para el número limitado de muestras ensayadas hasta ahora, el cociente medio del valor obtenido en el bioensayo dividido por el obtenido en el RIA es de 1,5 ± 0,2. Por tanto, no deben expresarse los datos de TSH de rata en unidades (que reflejan potencia biológica) cuando los valores se han obtenido por RIA; deben expresarse en unidades ponderales, referidas a una preparación de referencia internacional.

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