Regarding the validity of the endpoint response of the mouse (McKenzie) bioassay for thyrotrophin (TSH)

Melvin Ching

Department of Anatomy, Medical College of Virginia, Virginia Commonwealth University, Richmond, Virginia 23298, USA

Abstract. The release of radiolabelled thyroid hormone into the circulation in low iodine fed mice has been used extensively as a bioassay for thyroid stimulating hormone (TSH). However, the specificity of several bioassays of pituitary hormones have been subject to question. Consequently, the validity of the assay endpoint for TSH in the mouse was re-evaluated with respect to the effect of luteinizing hormone (LH) whose chemical composition closely resembles that of TSH. Mice, prepared for bioassay of TSH received injections of purified LH or α or β subunits of LH. Identical doses of LH and LH subunits were quantified by LH and TSH radioimmunoassays and the results compared with those obtained by the bioassay. Microgram quantities of LH and subunits of LH elicited appreciable responses in the TSH bioassay but produced only negligible effects in the TSH radioimmunoassay. The response of the TSH bioassay of LH and α or β subunits of LH was 40–56% that obtained with LH radioimmunoassay. However, the pituitary concentrations obtained by TSH bioassay when compared with those obtained by radioimmunoassays for LH, LH, or growth hormone (GH) paralleled closely the TSH radioimmunoassay data, although in terms of quantitative estimates, there was a 15-fold discrepancy between the TSH assays. Estimations of pituitary concentrations of LH lead to the conclusion that, at the doses normally employed, most crude rat pituitary extracts do not contain sufficient quantities of LH to alter significantly bioassayable (McKenzie) estimates of TSH.

The secretion of radiolabelled hormone from the thyroid gland into the blood stream of the low iodine fed mouse has been used extensively as an endpoint in a bioassay for thyrotrophin (TSH) (McKenzie 1958; Bakke 1965; van Rees 1966; Sinha & Meites 1966; Solomon & McKenzie 1966). The advent of the radioimmunoassay has largely supplanted the various bioassays for the quantitation of TSH in pituitary or blood. However, the McKenzie bioassay for TSH is still being used in cases where information concerning biological activity is deemed necessary (Rousset et al. 1977; Harada et al. 1979; Ochi et al. 1979). Past studies have explored the mechanisms of this bioassay (Florsheim et al. 1970) and examined the effect of TSH in plasma and pituitary extracts (Rerup & Melander 1965; Hershman 1970). However, a test of the specificity of this bioassay, particularly with respect to the effect of luteinizing hormone (LH) has not been performed. This is necessary since traces of LH are commonly associated with TSH prepared from pituitary extracts. Moreover, the primary structures of these two glycoprotein hormones are quite similar and it has been reported that their α and β chains can combine in vitro to form hybrid molecules which retain some TSH biological activity (Liao & Pierce 1970; Pierce 1971; Pierce et al. 1971a,b; Cornell & Pierce 1973). The present study, therefore, was conducted so as to determine the extent to which LH and its molecular subunits stimulate release of radiolabelled thyroid hormones into the circulation of the mouse. The response obtained were compared to those of a specific and sensitive radioimmunoassay that utilized highly purified bovine TSH as the reference preparation and antiserum to bovine TSH (Reichlin et al. 1970).
Materials and Methods

Preparation of hormone solutions for assay

The TSH contaminant was absorbed from an LH preparation (NIAMDD rat LH 1–2) with an antiserum to bovine TSH as previously described (Ching 1974). Standard and test solutions of rat LH (NIAMDD RP-1) and α and β subunits of ovine LH (NICHHD, WRR-1a/A and WRR-2β/B were prepared using phosphate buffered saline (PBS, 0.01 M PO₄ + 0.15 M NaCl), pH 7.5, as diluent. Concentrations of rat LH, ovine α LH and β LH were prepared for bioassay (20 μg/ml) and radioimmunoassay (50 μg/ml). Bovine TSH was used in the bioassay (NIAMDD B6, 2.54 U/mg) and radioimmunoassay (bioassay potency of 20–30 USP U/mg, chick thyroid ³²P uptake, Dr. John Pierce). It was dissolved in a NaCO₃ solution, pH 10, then diluted 20-fold with saline in order to reduce the pH and obtain the proper hormone concentration.

Anterior lobes, obtained from rats 4 weeks after ovarioctomy, between the 18th–20th day of gestation or after the first week of lactation, were weighed then homogenized individually in 1 ml of 0.05 M phosphate buffer, pH 7.5 containing 1% bovine serum albumin. Following refrigerated centrifugation at 1500 x g, the clear pituitary supernatants were stored at –20°C.

Preparation of bioassay recipients

The TSH and LH preparations and the subunits of ovine LH were tested for their effects on a bioassay for TSH (McKenzie 1958, 1960) that utilized as recipients female Swiss-Webster mice weighing 13–15 g at onset. Pellets of a low iodine diet (Nutritional Biochemicals Corporation (NBC), Cleveland, Ohio) and distilled water were provided ad libitum. After 10 days the mice were each injected ip with 3 μCi ¹²⁵I followed 1 h later with a sc injection (nuchal region) of 10 μg of the sodium salt of l-thyroxine (T₄, NBC). Two days later 10 μg T₄ was again administered to maintain the suppression of endogenous TSH release. The following day the animals were used as bioassay recipients.

Bioassay protocol

Five min before the removal of the first blood sample and injection of hormone or test substance, mice, serving as recipients in the TSH bioassay, were warmed under an incandescent lamp in order to dilate their tail veins. Animals were removed one by one from the warming chamber and 50 µl blood obtained by puncture of a retro-ocular sinus using a Drummond micropipette; the blood was diluted in 0.5 ml distilled water contained in a 10 x 75 mm disposable glass tube. The mouse was placed in a restraining chamber and 0.25 ml of saline, hormone or test solution injected into a tail vein. Hormone solutions were administered in a volume of 0.25 ml at doses depicted in Tables 1 and 4, whereas pituitary extracts were diluted with PBS so that the dose administered was 1/250 of the gland per 0.25 ml. The time that elapsed between the collection of the blood and the iv injection was 1 min. After 2 h a second sample of 50 µl blood was removed from either the same or the opposite retro-ocular sinus. The release of radiolabelled thyroid hormone elicited by administration of the test substance was determined by measuring the increase in blood radioactivity which was then related to a standard curve and equivalence in bovine TSH estimated.

TSH, LH and GH radioimmunoassays

Test doses of purified LH and LH subunits were quantified by radioimmunoassays for LH (Niswender et al. 1968, 1969) and TSH (Reichlin et al. 1970) to determine the extent to which they cross-reacted with TSH antiserum. Radioimmunoassays were also employed to measure concentrations of TSH, LH and GH (Schalch & Reichlin 1966) in crude pituitary extracts of Sprague Dawley female rats. The reference preparation of bovine TSH was from Dr. John Pierce as previously mentioned. The antiserum to bovine TSH from Dr. Seymour Reichlin was used at a final dilution of 1:210 000. The [¹²⁵I]TSH tracer was adjusted to 10 000 cpm per tube. The mixture containing 50 µl reference standard, 100 µl antiserum and 50 µl tracer was incubated for 4 days at 6 to 8°C after which time the antigen-antibody complex was precipitated by the addition of 1 ml BSA-coated charcoal suspension (0.5 ml 10% norit A + 0.5 ml 2% fraction V bovine serum albumin (Sigma Chemical Co., St. Louis, Missouri) in 0.05 M PO₄ buffer, pH 7.5) per tube. Following refrigerated centrifugation at 1500 x g for 30 min, the supernatant radioactive counts were obtained and related to the total counts per tube. The GH radioimmunoassay employed a rabbit antiserum to porcine GH and was used at a final dilution of 1:42 000 against the NIAMDD RP-1 standard. The same volumes of reference standard, antiserum, and ¹²⁵Irat hormone was used as in the TSH radioimmunoassay. The reaction mixture was incubated at 6–8°C for 3 days. The antigen-antibody complex was then precipitated over the next 24 h by the addition of 100 µl each of 1:8 rabbit anti-guinea pig gamma globulin and 1:200 normal guinea pig serum followed by centrifugation. The LH radioimmunoassay utilized the NIAMDD RP-1 standard which had a biological potency (OAAD) assay of approximately 0.03 X NIH-LH-S1. It was reacted against the anti-ovine No. 15 LH serum obtained from Dr. Gordon D. Niswender, and ¹²⁵I labelled NIAMDD LH-1-4. The final dilution of the O-LH antiserum was 1:160 000. The antigen-antibody complex was precipitated by the addition of 100 µl aliquots of 1:40 goat antirabbit gamma globulin (Antibodies Inc., Davis, California) and 1:200 normal rabbit serum. The fraction of pituitary assayed per tube were as follows: TSH, 50 µl of 1/100 anterior pituitary (AP); LH, 50 µl of 1/150 AP; GH, 50 µl of 1/4000 AP. These data were compared with quantitative estimates obtained by the TSH bioassay.
**Evaluation of the bioassay and radioimmunoassays**

The bioassay and radioimmunoassay standard curves were linearized by stepwise multiple regression utilizing the method of least squares (Stanley 1963). The equation used was \( y = b + m \log x \) where \( b \) is the intercept, \( m \) is the slope and \( x \) is the dose of hormone standard in nanograms. The inherent index of precision, \( \lambda \), was calculated at each dose level using the formula \( \lambda = \text{SD}/m \) where SD is the standard deviation at each dose and \( m \) the value of the slope (Cornfield 1970). Only the \( \lambda \) of the \( x \) and \( y \) values falling within the steep rectilinear portion of the curve were used to compute the average \( \lambda \). Moreover, the coefficient of determination, \( r^2 \), was computed by a Texas Instruments SR-52 calculator utilizing the same ST1-08 applied statistics programme.

**Table 1.**

Bioassay standard curve for thyrotrophin (TSH) (NIAMDD B6) expressed as increase in blood radioactivity per dose of TSH.

<table>
<thead>
<tr>
<th>Dose of TSH (ng)</th>
<th>Change in blood radioactivity (cpm) (mean ± sd)</th>
<th>( y )</th>
<th>( \lambda )</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>-6 ± 28</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>80</td>
<td>94 ± 36</td>
<td>55.9</td>
<td>0.024</td>
</tr>
<tr>
<td>160</td>
<td>427 ± 160</td>
<td>498.8</td>
<td>0.108</td>
</tr>
<tr>
<td>320</td>
<td>946 ± 391</td>
<td>941.7</td>
<td>0.264</td>
</tr>
<tr>
<td>640</td>
<td>1440 ± 437</td>
<td>1384.6</td>
<td>0.296</td>
</tr>
<tr>
<td>1280</td>
<td>1802 ± 483</td>
<td>1827.5</td>
<td>0.327</td>
</tr>
</tbody>
</table>

The data represent the means ± sd of 5 recipients at each point. \( y = -2749 + 1476.3 \log x \). \( r^2 = 0.994 \). The average \( \lambda \) of the valid portion of the curve is 0.204.

**Results**

**Comparison of the TSH bioassay and radioimmunoassay**

The release of radioactive thyroid hormone into the circulation of the mouse followed a characteristic rectilinear log-dose response to TSH. However, the vagary of the bioassay was evident when the index of inherent precision (\( \lambda \)) test (Cornfield 1970) was applied (Table 1). The average \( \lambda \) for all doses was 0.204 which is within the limits of acceptability for bioassays. It is to be noted that in this bioassay the \( \lambda \) diminished as the dose of TSH increased.

In the TSH radioimmunoassay, increasing log doses of non-radioactive TSH progressively displaced radioactive TSH tracer from the antibody binding sites (Table 2). In contrast to the bioassay, the \( \lambda \) at each dose was minute, the average \( \lambda \) being only 0.045 which is well within the range of acceptability. This measure of precision illustrates the superiority of a radioimmunoassay over a bioassay in terms of ability in measuring minute differences in hormone concentration. It should be noted also that the sensitivity of the TSH radioimmunoassay was 25 times that of the bioassay (Tables 1 and 2).

**LH radioimmunoassay**

The precision and sensitivity of the radioimmunoassay was illustrated once more in the standard curve for LH (Table 3). The average inherent \( \lambda \) of all dose standards was 0.034 which is similar to that of the TSH radioimmunoassay. In contrast to the
greater and partially TSH binding radioimmunoassay of test doses of rat LH (NIAMDD LH 1–2) and ovine LH α and β subunits (NICHHD WRR-1 α/A and WRR-1B/B.

<table>
<thead>
<tr>
<th>Dose (ng)</th>
<th>TSH cross-reactivity (ng)</th>
<th>LH immunoactivity (ng)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bioassay</td>
<td>Radioimmunoassay</td>
</tr>
<tr>
<td>0</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>450</td>
<td>69 ± 87</td>
<td>ND</td>
</tr>
<tr>
<td>750</td>
<td>79 ± 58</td>
<td>ND</td>
</tr>
<tr>
<td>5000</td>
<td>1625 ± 308</td>
<td>ND</td>
</tr>
<tr>
<td>5000 α LH</td>
<td>81 ± 59</td>
<td>3 ± 1</td>
</tr>
<tr>
<td>5000 β LH</td>
<td>110 ± 141</td>
<td>1 ± 0*</td>
</tr>
</tbody>
</table>

* SD < 0.05. Shown are the means ± SD of 4–6 determinations per point.
ND = non-detectable.
Table 5.
Concentration of bioassayable thyrotrophin (TSH) and radioimmunoassayable TSH, luteinizing hormone (LH), and growth hormone (GH) in pituitary extracts of intact normal control (NC), ovariectomized (O), pregnant (P) and lactating (L) Sprague-Dawley rats.

<table>
<thead>
<tr>
<th>Group</th>
<th>Bioassay TSH</th>
<th>Radioimmunoassay</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>TSH</td>
</tr>
<tr>
<td>NC</td>
<td>4100 ± 3300</td>
<td>300 ± 100</td>
</tr>
<tr>
<td></td>
<td>(100%)</td>
<td>(100%)</td>
</tr>
<tr>
<td>O</td>
<td>3600 ± 1300</td>
<td>240 ± 30</td>
</tr>
<tr>
<td></td>
<td>(88%)</td>
<td>(80%)</td>
</tr>
<tr>
<td>P</td>
<td>2200 ± 800</td>
<td>150 ± 20</td>
</tr>
<tr>
<td></td>
<td>(54%)</td>
<td>(50%)</td>
</tr>
<tr>
<td>L</td>
<td>3600 ± 1600</td>
<td>80 ± 10</td>
</tr>
<tr>
<td></td>
<td>(88%)</td>
<td>(27%)</td>
</tr>
</tbody>
</table>

* P < 0.05 when compared to its NC value.

1 ng/mg anterior pituitary gland wet weight. 2 10–12 pups per liter.
Shown are the means ± SD of 6 determinations per point. the per cent of change from normal are shown within parentheses.

Discussion
The results of Table 4 suggests a dose of 5000 ng LH has the same effect as 1625 ng TSH in eliciting release of radiolabelled thyroid hormone in the mouse. This is not particularly surprising in view of the finding that TSH and LH molecules, of human and cattle, consist of 2 chemically similar polypeptide chains (Pierce 1971; Pierce et al. 1971b; Cornell & Pierce 1973). Indeed biochemical studies suggest that the CI or α chains of LH and TSH of cattle possess identical amino acid sequence and 3-dimensional configurations (Pierce 1971; Pierce et al. 1971a) and that the primary, secondary, and tertiary structures of the β chains, while not identical, were similar (Pierce 1971). It was not surprising, therefore, that restoration of bioassayable thyrotrophin activity occurred when the β chain of TSH was combined with the α chain of LH as compared with activity elicited by either polypeptide chain (Pierce 1971; Pierce et al. 1971b). Thus, this introduced the possibility that, because of the close relationship of the chemical nature of LH and TSH, either glycoprotein hormone could be recognized by the other’s biological receptors. Recent studies have demonstrated the presence of gonadotrophic hormone α and β subunits within pituitary extracts and sera and further that an LH preparation, free from TSH, stimulated the endpoint response of a bioassay for TSH (Hagen & McNeilly 1975; Ching 1974). The present study also suggests that microgram quantities of LH are able to elicit a significant endpoint response in the mouse thus demonstrating the non-specificity of this TSH bioassay (Table 4). Although FSH possesses a common α subunit with that of TSH and LH and a hormone-specific β chain (Pierce 1971), the testing of rat FSH subunit cross-reactivity was not included in this study simply because these materials were not readily available.

In view of these findings the more appropriate concern should be whether or not pituitary extracts, at dilutions normally employed in the McKenzie bioassay, contain sufficient concentrations of LH to stimulate enough secretion of radio-labelled thyroxine to impair the reliability of the bioassay. To answer this question, bioassay and radioimmunoassay were employed to quantitate TSH, LH and GH in the same crude pituitary extracts of female rats physiologically manipulated to bring about changes in pituitary contents of LH (Table 5). GH was included for study because, although it is chemically unlike TSH or LH, it is abundant in the pituitary gland and has been
shown to possess some potentiating ability on another TSH bioassay whose endpoint is the thyroid gland's uptake of $^{131}$I (Evans et al. 1958; Ching et al. 1975).

Calculations reveal, utilizing the data provided in Table 5, that the dose of LH administered to recipient mice would not exceed 200 ng LH even at the higher anterior lobe weights of 15 mg. According to Table 4, this dose of LH is not able to stimulate an endpoint response in the mouse. With the exception of group L, the close parallelism of the radioimmunoassay and bioassay of TSH in the same rat pituitary, in which LH was quantified, lends support to the notion that the estimation of TSH concentrations by the McKenzie bioassay evidently were not altered by LH present in the pituitary extracts.

Whereas the absolute values of the data in Table 5 may differ from those of other investigators, the trends were nevertheless similar when comparing the various experimental groups. For example, earlier studies showed pituitary GH content decreased in lactating rats (Tucker & Thatcher 1968; Sar & Meites 1969). Pituitary LH concentrations were elevated in ovariecimized and pregnant rats but declined to control values during lactation (Nagai & Kunii 1972; Smith & Neill 1977; Steger & Peluso 1978), although lower than normal values have been reported for suckled rats (Minaguchi & Meites 1967) and monkeys (Weiss et al. 1976). Orsini & Schwartz (1973) reported pituitary LH content increased in the pregnant hamster and decreased to post-ovulation levels at 48 h post-partum. The normal concentration of bioassayable TSH in the pituitary of lactating rats (Table 5) lends support to the original data of Sar & Meites (1969). The discrepancy between the TSH bioassay and radioimmunoassay trend data of the lactating rat pituitary (Table 5) is difficult to explain even if the assertion is made that the immunoassay was more specific for endogenous TSH. The quantity of LH in the pituitary extracts administered to the mouse recipient is estimated to be less than 37 ng. According to Table 4, this amount of LH would have negligible influence in the McKenzie bioassay unless consideration is given that FSH, which reportedly is increased in the pituitaries of pregnant or lactating rats (Nagai & Kunii 1972; Smith & Neill 1977) exerts similar effects in the bioassay. The possibility also exists that in the suckled rats the presumed increased prolactin secretion from the pituitary could be attributable in part to an enhanced release of hypothalamic TRH which in turn would stimulate pituitary secretion of TSH (Burnet & Wakerley 1976; Wakerley & ter Haar 1978). If true, this could serve to explain the lower mean concentrations of bioassayable and immunoassayable TSH in Table 5 although in both instances the values were not significantly different from normal.

Other investigators, employing radioimmunoassay (RIA) or bioassay (OAAD), also report concentrations of LH in pituitary that are too low to sufficiently stimulate the TSH bioassay. Calculations from data of Gay & Midgley (1969) reveal that no more than 172 ng LH (RIA male rat pituitaries) would be administered to mouse recipients. Similarly only 112 ng LH (OAAD) from pro-oestrous rat pituitaries would be injected into mice (Bradshaw & Critchlow 1966). The study of Anderson & McShan (1966) shows that a mere 128 ng LH (OAAD) would be administered, based on equivalent fractions of anterior pituitaries from ovariecimized rats; even smaller doses of LH would be obtained in rats in oestrus or dioestrus.

The experiments of Niswender et al. (1968) show some pituitary concentrations of LH to be of sufficient intensity (20 μg–64 μg, RIA or OAAD), even after a 250-fold dilution and theoretically would stimulate thyroidal release of thyroxine in the mouse. However, LH concentration in rat sera (Niswender et al. 1968) or ovine sera (Niswender et al. 1969) are too minute (< 90 ng/0.25 ml) to stimulate the TSH bioassay. Thus it appears that although some confusion exists as to whether or not pituitary concentration of LH are sufficiently high to significantly alter true estimates of TSH when assayed in the radiiodine-primed mouse, the majority of evidence suggests that the hormone levels are too low to be of much consequence. Certainly, this is true of the circulating levels of LH in rats and sheep. As Table 5 suggests, glandular changes in GH concentration do not appear to alter in obvious fashion the estimates of the TSH bioassay. However, interpretations of this kind are not without risk since crude pituitary extracts contain other hormones, any number of which could stimulate or inhibit the endpoint response of the McKenzie bioassay for TSH.

Although an attempt was made to minimize the stressing of the mice through undue restraint, surgery or warming, it was impossible to eliminate these insults entirely nor was it desirable to do so since stress of the recipients are an unavoidable
part of the McKenzie bioassay. After all, this was an inquiry into the reliability of this bioassay's end-point response to test substances which indeed is thought to be influenced by stress (Sowers et al. 1977; Sterling & Lazarus 1977). There is ample evidence that short term immobilization, surgery, ether anaesthesia or other stresses decrease pituitary secretion of TSH in animals and man (Sowers et al. 1977; Sterling & Lazarus 1977; DuRuisseau et al. 1978) as well as alter the secretion of GH and LH (Schalch & Reichlin 1966; Krulich et al. 1974; Sowers et al. 1977; DuRuisseau et al. 1978) which indirectly influence thyroid gland activity (Evans et al. 1958; Ching 1974; Ching et al. 1975). However, the reduction of TSH secretion by the pituitary of the mouse recipient as a result of stress would be of little consequence in the McKenzie assay since supranormal doses of T₄ are administered in order to suppress endogenous TSH release. Rather, the more appropriate concern should be what modulatory effect stress has on thyroid activity. Sowers et al. (1977) reported that serum T₃ levels were depressed in humans 10 min after surgery and presumably stress does suppress some thyroid hormone release in the mouse recipient in the McKenzie TSH bioassay. However, judging by the good correlation of radiolabelled thyroid hormones released into the blood in response to stepwise increments of administered TSH (Table 1) it would appear that the stresses to which the mice had been subjected, did not alter the endpoint of the assay sufficiently to compromise its veracity.

Acknowledgments

This work was supported by NIH Grant 1 RO1 HD 10912, and by a grant from the Human Growth Foundation. The bovine TSH and some reagents for the TSH, LH and GH radioimmunoassays were gifts from NIAMDD. The α and β subunits of ovine LH were the gifts of NICHHD. The highly purified bovine TSH, from Dr. John G. Pierce of UCLA, and the antiserum to bovine TSH from Dr. Seymour Reichlin of Tufts University, Boston, were utilized in the TSH radioimmunoassay. Thanks are also extended to Dr. Gordon Niswender of Colorado State University, Fort Collins, for generously providing the ovine LH No. 15 antiserum used in the LH radioimmunoassay.

References


Received on March 19th, 1980.