A COMPARISON OF BIOLOGIC AND IMMUNOLOGIC POTENCY ESTIMATES OF HUMAN LUTEINIZING (LH) AND FOLLICLE STIMULATING (FSH) HORMONES

By
Robert J. Ryan

ABSTRACT

Published comparisons of biologic and immunologic potency estimates of human pituitary and urinary FSH and LH have been examined. In general, when pituitary fractions were assayed against pituitary standards and urinary fractions against urinary standards, there was good agreement between the two assay systems for both LH and FSH. However, when pituitary fractions were assayed against urinary standards, and vice versa, disparity occurred. This disparity was greater for LH than for FSH and was of such a nature as to suggest that the urinary hormone lost immunologic potency to a greater degree than biologic potency. A hypothesis, based on the formation of hybrid molecules, is presented to explain this immunologic deficiency of urinary LH. Evidence is also given to indicate that a dissociated, biologically inactive but immunologically recognizable form of LH, as well as the biologically active associated species of LH, is excreted in urine.

The purposes of this review are to compare biologic and immunologic potency estimates for human gonadotrophins, to point out where they agree and where they disagree, and to offer speculations concerning the causes for disagreements. The device used for comparing potency estimates is Gaddum's Index of Discrimination (ID), here expressed as the ratio of immunologic to biologic potency (I/B).

In all reviews it becomes necessary to set limits. Although some references
to human chorionic gonadotrophin (HCG) and LH of non-human origin were included for illustrative purposes, no attempt was made to review these subjects thoroughly. Data concerning immunologic end-points which are difficult to quantitate (i.e. biologic neutralization, immunodiffusion, etc.) and data derived from biologic assays which are not specific were excluded, unless there was a specific point of interest. Several published immunoassays were excluded because the specificity of the antibody was in doubt or no comparisons between biologic and immunologic potency estimates could be found.

One of the major sources of confusion in the literature concerning both biologic and immunologic potency estimates has been the use of several standards and therefore confusion concerning the conversion factors used to relate one standard with another. In particular, the factor 1538 for the conversion of NIH-LH-S1 units to International Units of the 2nd International Reference Preparation (IU 2nd IRP) using the ovarian ascorbic acid depletion assay (OAAD) has caused difficulty. In this review, 1 U NIH-LH-S1 is considered to be equivalent to 577 IU 2nd IRP (Albert 1968) in the OAAD assay and wherever possible, data published using the 1538 factor have been corrected to this value. I have regarded 1 NIH-FSH-S1 unit as being equivalent to 25 IU 2nd IRP (Albert 1968).

I. LUTEINIZING HORMONE

A. Pituitary Extracts Versus Pituitary Standards. – Data obtained from various published sources are presented in Table 1. The HCG agglutination inhibition assays, whether utilizing sheep red cells (HAI) or latex particles (LAI), have shown good agreement between biologic and immunologic potency estimates of LH when a pituitary standard was employed in both assay systems (Wide 1962; Wide & Gemzell 1962; Wide et al. 1961; Rizkallah et al. 1965). The results of the radioimmunoassays (RIA), however, require more detailed comment. The RIA, utilizing an HCG antibody, reported by Odell and his collaborators (Odell et al. 1966; Odell et al. 1967; Odell & Swerdloff 1968; Odell et al. 1969, 1968b) shows a systematic overestimation by the RIA since the mean ratio of immunologic to biologic potency (I/B) was 1.87 and all seven estimates were 1.0 or greater. A careful and independent appraisal of this same antiserum was made by the National Pituitary Agency (NPA) in a collaborative study (Albert et al. 1968) and the mean I/B was found to be 1.73 ± SE 0.035.

Two groups have reported potency comparisons with RIA using an anti-LH serum and labeled LH. The data of Faiman & Ryan (1967a) indicate a mean I/B of 1.01 for pituitary fractions containing less than than 50 NIH-S1 units
Table 1.
Indices of discrimination for immunologic and biologic potency estimates of luteinizing hormone when pituitary fractions are assayed against a pituitary standard in both assay systems.

<table>
<thead>
<tr>
<th>Method</th>
<th>Immunoassay</th>
<th>Bioassay</th>
<th>Index of discrimination</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Anti-serum</td>
<td>Label or coat</td>
<td>Std.</td>
<td>Method</td>
</tr>
<tr>
<td>HAI</td>
<td>HCG</td>
<td>HCG</td>
<td>pLH</td>
<td>SV</td>
</tr>
<tr>
<td>HAI</td>
<td>HCG</td>
<td>HCG</td>
<td>pLH</td>
<td>SV</td>
</tr>
<tr>
<td>LAI</td>
<td>HCG</td>
<td>HCG</td>
<td>pLH</td>
<td>VPW</td>
</tr>
<tr>
<td>RIA</td>
<td>HCG</td>
<td>pLH</td>
<td>pLH</td>
<td>OAAD</td>
</tr>
<tr>
<td>RIA</td>
<td>pLH</td>
<td>pLH</td>
<td>pLH</td>
<td>OAAD</td>
</tr>
<tr>
<td>RIA</td>
<td>pLH</td>
<td>pLH</td>
<td>pLH</td>
<td>OAAD</td>
</tr>
<tr>
<td>RIA</td>
<td>pLH</td>
<td>pLH</td>
<td>pLH</td>
<td>OAAD</td>
</tr>
</tbody>
</table>

* Fractions containing less than 50 NIH-S1 units of FSH/mg.
** Fractions containing more than 50 NIH-S1 units of FSH/mg.

In this and subsequent tables, the following abbreviations are employed:

HAI = haemagglutination inhibition assay
LAI = latexagglutination inhibition assay
RIA = radioimmunoassay
CF = complement fixation assay
Std. = standard

N = number of observations
SE = standard error of the mean
p = pituitary, as pituitary LH (pLH)
SV = seminal vesicle weight bioassay
VPW = ventral prostate weight bioassay
OAAD = ovarian ascorbic acid depletion assay
u = urinary
of FSH/mg but varying from 0.006 to 4.16 NIH-S1 units of LH/mg. They also noted that some, but not all (2 of 5), highly potent FSH fractions contained more immunologically recognizable than biologically assayable LH. The data of Schalch et al. (1968) indicated an I/B somewhat greater than unity, but the data are too sparse to speculate on any systemic differences. Indeed, if the potency of their immunoassay standard (hLH 5.1 NIH-S1 U/mg) was 30% too high, there would be no need to consider a systematic error.

It is of interest that Pelletier et al. (1968) using a radioimmunoassay for ovine LH and Niswender et al. (1968) using a radioimmunoassay for rat LH noted I/B’s of nearly 1.0 when comparing immunologic and biologic potency estimates on sheep and rat pituitary extracts.

B. Urinary Extracts Versus Pituitary Standards and Vice Versa. – Table 2A presents published comparisons of I/B ratios where urinary extracts were assayed against pituitary standards. Faiman & Ryan (1967a) called attention to a systematic discrepancy whereby the LH potencies of urinary extracts were underestimated by the RIA (I/B of 0.34) when a pituitary standard was employed. A similar result was obtained in 1 of 2 assays performed by Schalch et al. (1968). Three of 4 comparisons using HAI also showed I/B ratios less than 1 but the degree of discrepancy was less than that seen with RIA.

Table 2B presents published comparisons of I/B ratios where pituitary extracts were assayed against urinary standards. The single observations of Midgley (1966) and Schalch et al. (1968) again indicated an approximately 3-fold discrepancy, but in this instance the potency of the pituitary extract was overestimated. The data using the Odell HCG antisera, both from his own laboratory (Odell et al. 1966; Odell et al. 1967; Odell & Swerdloff 1968; Odell et al. 1969, 1968b) and from the NPA Study (Albert et al. 1968) indicated a 7–9 fold overestimation of potency by the RIA.

Mori (1968) using an antibody against urinary LH and a complement fixation assay discerned a distinct difference between urinary and pituitary LH (the immunologic index of dissimilarity was 6.01).

C. Urinary Extracts Versus Urinary Standards. – Published I/B ratios are given in Table 3. Odell et al. (1967) first called attention to the good correlation between immunologic and biologic potency estimates when urinary extracts were compared with urinary standards in both assay systems. Close inspection of these data revealed, however, that there was a systematic discrepancy, even though there was a good correlation. In 5 of the 6 comparisons the I/B ratio was less than 1.0 and the mean was 0.76. Nonetheless, subsequent reports by Kulik et al. (1968), Faiman et al. (1968), Donini et al. (1968) and Stevens (1968) indicated the I/B ratio was near unity for the RIA when urinary extracts were compared with urinary standards. Similar results were obtained by Mori (1968) using a complement fixation assay, and Kaivola et al. (1968) using HAI.

D. Immunoassays on Raw or Dialyzed Urine Versus Bioassays on Extracted
### Table 2.
Indices of discrimination for comparisons of LH in urinary extracts with pituitary standards and vice versa, in immunologic and biologic assays.

<table>
<thead>
<tr>
<th>Imunoassay</th>
<th>Bioassay</th>
<th>Index of discrimination</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Method</td>
<td>Anti-serum</td>
<td>Label or coat</td>
<td>Std.</td>
</tr>
<tr>
<td>A. Urinary extracts versus pituitary standard</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RIA</td>
<td>pLH</td>
<td>pLH</td>
<td>pLH</td>
</tr>
<tr>
<td>RIA</td>
<td>pLH</td>
<td>pLH</td>
<td>pLH</td>
</tr>
<tr>
<td>HAI</td>
<td>HCG</td>
<td>HCG</td>
<td>pLH</td>
</tr>
<tr>
<td>HAI</td>
<td>HCG</td>
<td>HCG</td>
<td>pLH</td>
</tr>
<tr>
<td>B. Pituitary extracts versus urinary standard</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RIA</td>
<td>HCG</td>
<td>HCG</td>
<td>uLH</td>
</tr>
<tr>
<td>RIA</td>
<td>pLH</td>
<td>pLH</td>
<td>uLH</td>
</tr>
<tr>
<td>RIA</td>
<td>HCG</td>
<td>pLH</td>
<td>uLH</td>
</tr>
<tr>
<td>RIA</td>
<td>HCG</td>
<td>pLH</td>
<td>uLH</td>
</tr>
</tbody>
</table>

* In some bioassays NIH ovine LH was used as a standard and the data converted to 1U 2nd IRP using the factor 577 as given in the text.
Table 3.
Indices of discrimination for comparisons of biologic and immunologic potency estimates when urinary extracts are assayed against urinary standards in both assay systems.

<table>
<thead>
<tr>
<th>Immunoassay</th>
<th>Bioassay</th>
<th>Index of discrimination</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Method</td>
<td>Anti-</td>
<td>Label or coat</td>
<td>Std.</td>
</tr>
<tr>
<td>RIA</td>
<td>HCG</td>
<td>pLH</td>
<td>uLH</td>
</tr>
<tr>
<td>RIA</td>
<td>HCG</td>
<td>pLH</td>
<td>uLH</td>
</tr>
<tr>
<td>RIA</td>
<td>HCG</td>
<td>HCG</td>
<td>uLH</td>
</tr>
<tr>
<td>RIA</td>
<td>HCG</td>
<td>HCG</td>
<td>uLH</td>
</tr>
<tr>
<td>RIA</td>
<td>HCG</td>
<td>pLH</td>
<td>uLH</td>
</tr>
<tr>
<td>RIA</td>
<td>pLH</td>
<td>pLH</td>
<td>uLH</td>
</tr>
<tr>
<td>CF</td>
<td>uLH</td>
<td>–</td>
<td>uLH</td>
</tr>
<tr>
<td>HAI</td>
<td>HCG</td>
<td>uLH</td>
<td>uLH</td>
</tr>
</tbody>
</table>

Urine With Urinary Standards in Both Assay Systems. – Comparisons of published I/B ratios are given in Table 4. Radioimmunoassays on raw or dialyzed urine grossly overestimated the amount of LH found by bioassays performed on kaolin extracts of the same urine. Similar data obtained from a single pool of postmenopausal urine are presented in Table 5 (Ryan, Northcutt & Albert, unpublished) and further indicate that this discrepancy also exists for ultrafiltered urine and alcohol precipitates of urine. It is of interest from the data in Table 4 and our own observations (unpublished) that the discrepancy is greater for raw urine than dialyzed urine and greater for urine of low biologic activity than that of high biologic activity.

Many reports have appeared in which LH or HCG was measured immunologically in raw urine or alcohol and acetone precipitates of urine without direct comparisons made with biologic assays. These immunologic assays have generally given higher values than would be expected from other studies where biologic assays were employed.

A number of non-specific factors such as pH, salt concentration, specific ions and organic solvents, among others, are known to interfere with binding of antigens to antibody and thus could lead to the discrepancies seen in Tables 4 and 5 (Margoulies 1969). Dialysis or ultrafiltration, and adjustment of pH and molarity, however, do not completely resolve these discrepancies.

It was previously noted (Ryan 1969a) that human pituitary LH tended to dissociate into a biologically inactive, immunologically active form upon extreme dilution, particularly in the presence of high concentrations of salt. Since LH is present in urine at extreme dilutions (nanograms/ml) and since urine is briny, experiments were performed to see if a dissociated form of LH was present in ultrafiltered urine but not in kaolin extracted urine. This was found to be the case. Fig. 1 illustrates experiments (Ryan, Northcutt & Albert, unpublished) in which a kaolin extract or an ultrafiltrate of postmenopausal urine was put through a Sephadex G-100 column as previously described (Ryan 1969a). The elution of LH from the column was determined by radioimmunoassay. The bulk of material that emerged when a kaolin extract was applied to the column was in a position corresponding to the large species and the biologically active form of pituitary LH. When urinary ultrafiltrate was applied to the column, two species were seen to emerge, one in a position corresponding to large species pituitary LH and the second in a position corresponding to the dissociated biologically inactive form of pituitary LH.

E. Discussion. – Each antiserum must be regarded as a unique reagent. Although cross-reactivity has been noted between HCG and LH, some antisera have been found to cross-react identically and others only partially. The presence of parallelism in the radioimmunoassay, although a necessary criterion, is not alone a sufficient criterion for judging identity. The most discriminating test comes in comparing biologic and immunologic potency estimates over a
Table 4.
Comparisons of immunoassays on raw or dialyzed urine with bioassays on kaolin extracts of urine using urinary standards in both assays.

<table>
<thead>
<tr>
<th>Type of urine extract and bio-potency</th>
<th>Immunoassay</th>
<th>Bioassay</th>
<th>Index of discrimination</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Method</td>
<td>Antiserum</td>
<td>Label</td>
</tr>
<tr>
<td>Raw &lt; 1.1 IU/d</td>
<td>RIA</td>
<td>HCG</td>
<td>pLH</td>
</tr>
<tr>
<td>Raw &gt; 1.1 &lt; 10 IU/d</td>
<td>RIA</td>
<td>HCG</td>
<td>pLH</td>
</tr>
<tr>
<td>Raw &gt; 10 IU/d</td>
<td>RIA</td>
<td>HCG</td>
<td>pLH</td>
</tr>
<tr>
<td>Dialyzed &gt; 10 IU/d</td>
<td>RIA</td>
<td>HCG</td>
<td>pLH</td>
</tr>
</tbody>
</table>

References
- Kulin et al. (1968)
- Stevens (1968)
Table 5.
A comparison of LH RIA potency estimates on various types of extracts of postmenopausal urine with bioassay potency of a kaolin extract of the same urine.

<table>
<thead>
<tr>
<th>Type of urine*</th>
<th>Bioassay Method</th>
<th>Immunoassay IU/d ± SE</th>
<th>I/B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kaolin extract</td>
<td>VPW 40.8</td>
<td>44.3 ± 3.0</td>
<td>1.09</td>
</tr>
<tr>
<td>Raw urine</td>
<td></td>
<td>97.1 ± 5.7</td>
<td>2.38</td>
</tr>
<tr>
<td>Dialyzed urine</td>
<td></td>
<td>58.4 ± 2.4</td>
<td>1.43</td>
</tr>
<tr>
<td>Ultrafiltered urine</td>
<td></td>
<td>57.5 ± 5.6</td>
<td>1.41</td>
</tr>
<tr>
<td>80% alcohol ppt.</td>
<td></td>
<td>83.3 ± 2.6</td>
<td>2.04</td>
</tr>
</tbody>
</table>

* All urine and extracts were adjusted to the pH (7.5) of the RIA procedure before assay.

Fig. 1.
Gel filtration, on Sephadex G-100, of kaolin acetone (KA) extracted and ultrafiltered (UF) urine. The 2.5 × 92 cm column was developed with 0.2 M NaCl–0.01 M phosphate buffer pH 7.5. The arrows and abbreviations indicate the elution position of Blue Dextran (BD), bovine serum albumin (BSA), native pituitary LH (pLH₁), dissociated pituitary LH (pLH₂), chymotrypsinogen (chy) and cytochrome C (cyto).
wide range. Unfortunately, this last criterion has not been applied to many of
the antisera presently in use.

Despite some variations, which probably can be ascribed to differing anti-
sera and uncertainties in the bioassays, some conclusions can be drawn from the
data presented above:

1. When pituitary extracts are compared in biologic and immunologic assays
using pituitary standards, good agreement is obtained between the two assay
systems.

2. When urinary extracts are compared in biologic and immunologic assays
using urinary standards, good agreement is obtained between the two assay
systems.

3. When immunoassays on raw or dialyzed urine are compared with bio-
logic assays on kaolin extracted urine, using urinary standards in both assays,
the immunoassay results are higher than the bioassay results. This discrepancy
may be explained by the presence in urine of factors which non-specifically
interfere with antigen-antibody binding, and of an immunologically active,
biologically inert form of LH present in significant amounts in ultrafiltered
urine but not in kaolin extracts of urine.

4. When comparisons are made between pituitary extracts and urinary
standards, the immunoassay overestimates the potency of pituitary LH relative
to its biologic potency. Conversely, when urinary extracts are assayed against
pituitary standards, the immunoassay underestimates the potency of urinary
LH relative to its biological potency.

Odell et al. (1968b) have correctly pointed out that this discrepancy (con-
clusion 4), as opposed to the one stated in conclusion 3, cannot be accounted
for by the presence of an immunologically active, biologically inactive form
of LH present in urinary extracts. They speculate that this discrepancy may be
accounted for by the radioimmunoassay measuring something in the pituitary
not measured by bioassay or the bioassay measuring something in urine not
measured by the immunoassay. If these speculations were correct, then one
would expect I/B ratios to be continuously variable as either urinary or pitui-
tary LH were purified. The data in the literature do not support continuous
variability of I/B ratios.

A speculation can be made that an alteration has occurred in the LH mole-
cule as a result of metabolism or extraction from urine that results in a loss
of immunologic reactivity out of proportion to a loss in biologic activity. A
variety of chemical alterations have been made to the LH and HCG molecules
and comparisons made between biologic and immunologic activity. Oxidation
with hydrogen peroxide (Midgley 1969), periodate (Trenkle et al. 1962) or
performic acid (Trenkle et al. 1962); denaturation with heat (Wide & Gemzell
1962), or urea (Reichert & Midgley 1968; Mori, personal communication);
digestion with chymotrypsin (Reichert & Midgley 1968), trypsin (Mori, personal

309
communication); neuraminidase (Barr & Collee 1967; Mori, personal communication), or β-glucosidase (Mori, personal communication) have all been studied. All appear to result in a greater loss of biologic activity than immunologic activity. In a way this seems logical since the radiation inactivation data of Odell & Paul (1964) suggest that a larger portion of the molecule is required for biologic activity than immunologic activity. I have yet to find data showing that an in vitro alteration to the LH or HCG molecule results in a greater loss of immunologic than biologic activity.

Since these simple explanations seem lacking, I would like to offer a more complicated speculation. There is good evidence that ovine, bovine and human LH exist in a biologically active associated form and a biologically inactive dissociated form (Ward et al. 1966; Papkoff & Samy 1967; De la Llosa & Jutisz 1969; Ryan 1969a; Reichert & Midgley 1968). There is also evidence (Papkoff & Samy 1967) that the dissociated form of ovine LH (at least that obtained by counter current distribution) consists of two different forms (here called A and B) that have nearly identical size but different amino acid composition. It would, therefore, be hypothetically possible for the associated form to be AB, BA, AA and BB. Let it be that all of these associated forms are biologically active, but perhaps of different potencies, and let the major antigenic determinant be B. Let us further suppose that this situation applied to human LH and that human pituitary LH consists of 100% AB. Let us further speculate that after secretion, the hormone dissociates to A and B and reassociates randomly as a spontaneous event or partially as a result of the extraction procedure. An explanation for the discrepancy would then be available, if the kaolin extracted LH consisted of 25% AB and 75% AA, Fig. 1 suggests that kaolin extracts contain mainly associated LH). Since only a small portion of secreted LH is recovered in urine, it would appear quantitatively possible to have this mixture of A’s and B’s in urinary extracts. Furthermore, there is some evidence to suggest that the A’s and B’s of ovine LH are antigenically different (Papkoff et al. 1968). This hypothesis, which has been stated in its simplest form, is subject to test if the two forms of human LH can be isolated and antibodies can be prepared against them.

II. FOLLICLE STIMULATING HORMONE

A. Pituitary Extracts Versus Pituitary Standards. – Table 6 presents comparisons of published I/B ratios. The data have been subdivided (Table 6) so
Table 6.
Indices of discrimination for comparisons of pituitary FSH extracts with pituitary standards in immunologic and biologic assays.

<table>
<thead>
<tr>
<th>Radioimmunoassay</th>
<th>Bioassay**</th>
<th>Index of discrimination</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Preparations with high FSH/LH ratios</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pFSH</td>
<td>HCG</td>
<td>pFSH</td>
<td>S. P.</td>
</tr>
<tr>
<td>pFSH</td>
<td>HCG</td>
<td>pFSH</td>
<td>S. P.</td>
</tr>
<tr>
<td>pFSH</td>
<td>HCG</td>
<td>pFSH</td>
<td>S. P.</td>
</tr>
<tr>
<td><strong>Preparations with low FSH/LH ratios</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pFSH</td>
<td>HCG</td>
<td>pFSH</td>
<td>S. P.</td>
</tr>
<tr>
<td>pFSH</td>
<td>HCG</td>
<td>pFSH</td>
<td>S. P.</td>
</tr>
</tbody>
</table>

* Standard was a human pituitary FSH extract bioassayed in terms of NIH-S1 Units/mg.
** S. P. = Steelman-Pohley augmentation assay. The standards were in NIH-FSH-S1 U/mg.
that preparations with a bioassay FSH/LH ratio* greater than 1.0 in NIH-S1 U or 0.04 in IU 2nd IRP are considered as one group and those with lower ratios as a separate group. It is apparent that there is good agreement between immunologic and biologic potency estimates when the FSH/LH ratio is high. In LH-rich fractions, the immunoassay detects more FSH than is measured by the bioassay.

B. Urinary Extracts Versus Pituitary Standards and Vice Versa. – Published I/B ratios are presented in Table 7 and are again subdivided by the bioassay FSH/LH ratio. Two conclusions can be made from the data. First, the immunologic potencies of urinary extracts relative to biologic potencies were underestimated when a pituitary standard was employed in the RIA or conversely the potencies of pituitary extracts were overestimated when a urinary standard was employed in the RIA. Secondly, the discrepancy was even greater when preparations rich in LH were considered.

C. Urine Versus Urinary Standards. – Published I/B ratios are presented in Table 8. When kaolin extracts of urine were assayed against urinary standards in the biologic and RIA assays, the ID was unity. This was also true for the complement fixation assay which utilized an antibody against urinary FSH.

As was the case with LH, the radioimmunoassay of FSH in raw or dialyzed urine gave higher values than those obtained by the biologic assay of kaolin extracts of the same urine.

D. Discussion. – Similar conclusions can be drawn from the comparisons of biopotency and immunopotency estimates for FSH as were drawn for LH. When pituitary fractions are assayed against pituitary standards and urinary fractions against urinary standard, the indices of discrimination are unity. When urinary materials are assayed against pituitary materials, the ID deviates significantly from unity. Radioimmunoassays for FSH performed on raw urine are unreliable and those on dialyzed urine are at best of questionable validity.

There is no doubt, from the data presented, that the radioimmunoassay for FSH grossly overestimates the amount found by bioassay when LH-rich fractions are analyzed. It appears, however, that this only pertains when the bioassay FSH/LH ratio is less than 1.0 in terms of NIH-S1 units or 0.04 in 2nd IRP IU. These ratios are far below those found in urinary extracts or serum. When this overestimation of FSH in LH-rich fractions was first noted (Faiman & Ryan 1967b), it was postulated that these LH preparations were contaminated with immunologically recognizable, but biologically inert FSH. The observation that this immunologically recognizable contaminant could be separated from LH by gel electrophoresis lends credence to this postulate (Midgley & Reichert 1969).

* S.P./OAAD.
<table>
<thead>
<tr>
<th>Anti-</th>
<th>Adsorption</th>
<th>Label</th>
<th>Std.</th>
<th>Method</th>
<th>Std.</th>
<th>N</th>
<th>Mean I/B</th>
<th>SE</th>
<th>Range</th>
<th>References</th>
</tr>
</thead>
</table>

**A. Urinary extracts versus pituitary standards:**

I. Preparations with high FSH/LH ratios

<table>
<thead>
<tr>
<th>pFSH</th>
<th>HCG</th>
<th>pFSH</th>
<th>pFSH</th>
<th>S. P.</th>
<th>pFSH</th>
<th>3</th>
<th>0.73</th>
<th>0.07</th>
<th>0.63–0.85</th>
<th>Midgley (1967)</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>pFSH</th>
<th>HCG</th>
<th>pFSH</th>
<th>pFSH</th>
<th>S. P.</th>
<th>pFSH</th>
<th>4</th>
<th>0.63</th>
<th>0.05</th>
<th>0.49–0.71</th>
<th>Ryan &amp; Faiman (1969)</th>
</tr>
</thead>
</table>

**B. Pituitary extracts versus urinary standards:**

I. Preparations with high FSH/LH ratios

1. Preparations with high FSI1/LH ratios

<table>
<thead>
<tr>
<th>pFSH</th>
<th>None</th>
<th>pFSH</th>
<th>uFSH</th>
<th>S. P.</th>
<th>uFSH</th>
<th>3</th>
<th>1.43</th>
<th>0.05</th>
<th>1.3–1.5</th>
<th>Odell et al. (1968a)</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>pFSH</th>
<th>HCG</th>
<th>pFSH</th>
<th>uFSH</th>
<th>S. P.</th>
<th>uFSH</th>
<th>4</th>
<th>1.86</th>
<th>0.57</th>
<th>0.77–3.4</th>
<th>NPA Report (1968)</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>pFSH</th>
<th>HCG</th>
<th>pFSH</th>
<th>uFSH</th>
<th>S. P.</th>
<th>uFSH</th>
<th>8</th>
<th>1.56</th>
<th>0.12</th>
<th>1.23–2.18</th>
<th>Midgley &amp; Reichert (1969)</th>
</tr>
</thead>
</table>

II. Preparations with low FSH/LH ratios

<table>
<thead>
<tr>
<th>pFSH</th>
<th>None</th>
<th>pFSH</th>
<th>uFSH</th>
<th>S. P.</th>
<th>uFSH</th>
<th>1</th>
<th>24.0</th>
<th>–</th>
<th>–</th>
<th>Odell et al. (1968a)</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>pFSH</th>
<th>HCG</th>
<th>pFSH</th>
<th>uFSH</th>
<th>S. P.</th>
<th>uFSH</th>
<th>3</th>
<th>6.6</th>
<th>0.9</th>
<th>5.2–8.2</th>
<th>NPA Report (1968)</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>pFSH</th>
<th>HCG</th>
<th>pFSH</th>
<th>uFSH</th>
<th>S. P.</th>
<th>uFSH</th>
<th>4</th>
<th>14.3</th>
<th>3.3</th>
<th>8.1–20.6</th>
<th>Midgley &amp; Reichert (1969)</th>
</tr>
</thead>
</table>
**Table 8.**

Indices of discrimination for comparisons of urinary FSH extracts with urinary standards in biologic and immunologic assays.

<table>
<thead>
<tr>
<th>Type of urine</th>
<th>Method</th>
<th>Anti-serum</th>
<th>Adsorption</th>
<th>Label</th>
<th>Std.</th>
<th>Bioassay Method</th>
<th>Std.</th>
<th>N</th>
<th>Mean I/B</th>
<th>SE</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>KA</td>
<td>RIA</td>
<td>pFSH</td>
<td>HCG</td>
<td>pFSH</td>
<td>uFSH</td>
<td>S. P.</td>
<td>uFSH</td>
<td>38</td>
<td>0.98</td>
<td>0.03</td>
<td>0.5–1.4</td>
</tr>
<tr>
<td>KA</td>
<td>RIA</td>
<td>pFSH</td>
<td>None</td>
<td>pFSH</td>
<td>uFSH</td>
<td>S. P.</td>
<td>uFSH</td>
<td>6</td>
<td>0.98</td>
<td>0.06</td>
<td>0.8–1.3</td>
</tr>
<tr>
<td>KA</td>
<td>RIA</td>
<td>pFSH</td>
<td>HCG</td>
<td>pFSH</td>
<td>uFSH</td>
<td>S. P.</td>
<td>uFSH</td>
<td>10</td>
<td>1.38</td>
<td>0.21</td>
<td>0.6–2.4</td>
</tr>
<tr>
<td>KA</td>
<td>CF</td>
<td>uFSH</td>
<td>uLH serum</td>
<td>–</td>
<td>uFSH</td>
<td>S. P.</td>
<td>uFSH</td>
<td>10</td>
<td>0.94</td>
<td>0.05</td>
<td>0.7–1.2</td>
</tr>
<tr>
<td>Raw*</td>
<td>RIA</td>
<td>pFSH</td>
<td>HCG</td>
<td>pFSH</td>
<td>uFSH</td>
<td>S. P.</td>
<td>uFSH</td>
<td>10</td>
<td>6.49</td>
<td>1.69</td>
<td>1.8–18.0</td>
</tr>
<tr>
<td>Dialyzed*</td>
<td>RIA</td>
<td>pFSH</td>
<td>HCG</td>
<td>pFSH</td>
<td>uFSH</td>
<td>S. P.</td>
<td>uFSH</td>
<td>10</td>
<td>2.93</td>
<td>0.75</td>
<td>1.2–4.2</td>
</tr>
</tbody>
</table>

* Immunoassays were performed on raw or dialyzed urine and the bioassays on kaolin acetone (KA) extracts of the same urine.
An immunologic distinction between urinary and pituitary FSH was first pointed out by Taymor et al. (1967) when they noted spur formation in immunoaffinity studies of human pituitary and urinary FSH and departure of ID from 1.0 when comparing the two in a LAI assay. Faiman & Ryan (1967b) first noted discrepancy in bio-immuno potency using RIA and this has subsequently been confirmed by others (see Table 7) using RAI. Mori (1968) using an antibody against urinary FSH in a complement fixation assay, found that the index of dissimilarity for pituitary FSH was $> 3.2$. Robyn & Diczfalussy (1968) studying neutralisation of biologic activity, also noted a difference between pituitary and urinary FSH.

The cause of the discrepancy is unknown. It should be pointed out that the degree of discrepancy is much less for FSH (approximately 1.5-fold) than for LH (3–8 fold). It has been suggested (Taymor et al. 1967) that a portion of the FSH molecule concerned with immunologic reactivity is lost during metabolism and excretion. The available data suggest that a portion of the molecule is lost as the molecular weight of urinary FSH has been reported to be 27 000 (Roos 1967) as compared to 36 000 for pituitary FSH (Ryan 1969b). The radius of urinary FSH is approximately the same as that of pituitary FSH (Roos 1967; Ryan 1969b), however, and, therefore, the molecule must be extended and the modest loss of immunologic potency could result from this change in tertiary structure. On the other hand, Mori (personal communication) has data suggesting that a D-galactopyranosyl unit may be a constituent of the antigenic determinant of FSH. Perhaps loss of this moiety or change in its steric relationship could account for a disproportionate loss of immunologic activity. Although FSH can be dissociated with guanidine or urea (Ryan 1969b; Reichert & Midgley 1968), there is no evidence at present that subunits exist in urine or that the subunits have different chemical compositions.

III. IMMUNOASSAY OF SERUM GONADOTROPHINS

The data presented above indicate the necessity for pituitary standards when assaying pituitary extracts and urinary standards for assaying urinary extracts. The question of a suitable standard for the RIA of LH and FSH in serum has been discussed in great detail (Ryan 1969; Albert et al. 1968; Albert 1968; Rosemberg 1968) and the general conclusion has been reached that a serum standard would be desirable. This conclusion was not based on hard data but on the general principle that in any assay system the standard and unknown should be as like as possible. There are now some data to indicate that the general principle is once again correct. Mori (1967, 1968) using antibodies
against urinary LH and FSH, in complement fixation assays, has shown that
urinary LH and FSH are not only immunologically different from pituitary
LH and FSH, but that serum LH and FSH have different immunologic indices
of dissimilarity than either the urinary or pituitary hormones.

ACKNOWLEDGMENTS

I am grateful to K. F. Mori for allowing me to read and cite from two manu-
scripts that have been submitted for publication but are not yet in print. I am
particularly grateful to my friend and colleague, A. Albert, for many discus-
sions and criticisms. Parts of this work were supported by the USPHS (HD
03726) and the Mayo Foundation.

REFERENCES

28 (1968) 1214.
Midgley A. R. & Reichert L. E. In: Margoulies M., Ed. Protein and Polypeptide Hor-
Mori K. F.: Endocrinology 82 (1968) 945.
(N. Y.) 128 (1968) 807.

DISCUSSION

Hunter: I would like to suggest the following criteria for selection of antisera for FSH radioimmunoassay.
For all assays:
1. No cross reaction with plasma proteins.
2. No or small cross reaction with LH, TSH (or HCG), at least of some antibodies present.
The requirements for assays on plasma and urine then differ.
For assays on plasma:
Correlation with bioassay values on pituitary material.
High avidity (sensitivity).
Detect only biologically active forms of FSH and not inactive fragments.
Test by (a) correlation with bioassays on blood, (b) parallel immunological and biologival half life studies following iv injection of exogenous FSH or immediately following hypophysectomy in post-menopausal women, (c) in the absence of information on (a) or (b), choose antiserum giving the shortest half life.
For assays on urine:
Correlation between immunological determinations on urine and dose of iv injected FSH.
Correlation between plasma and urinary levels in steady state situations.

Ryan: I think that for the moment we have to continue to rely upon standards that reflect biological activity. Perhaps in the long haul we shall want to measure the subunits as well, but this is going to be a complicated problem. First of all, we need to have documentation of the existence of the human subunits in biologic fluids. It would then be necessary to have assays specific for the subunits. We could then quantitate them separately and independently, and at that time we may need to change to standards that do not reflect biologic activity.

Butt: I would like to say how interested I was in Dr. Ryan's speculations on the subunits of LH and the associated forms that may occur in urine. I wonder if carbohydrates are involved? It is known, for instance, whether the carbohydrate contents of the A's and B's of ovine LH account for their antigenic differences? You may remember that I reported that the carbohydrates were not really involved in the binding between FSH and our antiserum. This may not be universal, but on the other hand it may be significant. We all know how steroids are conjugated, and how they pass into the urine; could something like this be happening with gonadotrophins? Could they be taking on more carbohydrates or other groups, which are blocking immunologically active sites? Could this also explain why HMG is such a poor antigen for raising antibodies?

Ryan: I do not know the carbohydrate composition of the ovine and bovine subunits. I will make a prediction. Since carbohydrate units tend to be greatly hydrated, I expect that they would be present in the more polar subunit (the one I referred to as A) in greater quantity than in the more nonpolar subunit. I do not know of any evidence, aside from an observation of K. F. Mori (personal communication), that the carbohydrate is the immunologic determinant. Mori's data suggest that a galactose residue is involved in the antigenic site of urinary FSH. I do not know what accounts for the lower immunologic potency of urinary FSH relative to pituitary FSH. I suspect that it may be due to a change in tertiary structure although a loss of a fragment is also possible.

Donini: According to the study of Odell et al. (1964) on the determination of molecular weight by radioinactivation, it seems that pituitary FSH and urinary FSH have the same molecular weight.

Ryan: You are correct, Dr. Donini; the discrepancy between those data and Dr. Roos'
data concerning the molecular weight of urinary FSH needs to be resolved. I would again like to emphasize that immunologic measurements of FSH and LH in raw urine are fraught with difficulties, since pH, molarity and specific salts may create artifacts by altering the binding of antigen to antibody. Dialyzed and ultrafiltered urine may be more reliable, but there is still discrepancy when values are compared with those obtained from kaolin extracts. It may be desirable to use a standard obtained from dialyzed urine when assaying dialyzed or ultrafiltered urine.

**Odell:** There is some possibility that there are some antisera which are less affected by raw urine than others.

**Ryan:** You probably will approach reliability if you have very high titers of hormone in the urine and therefore need to add less urine to the assay mixture.

**Wide:** There are differences between different antisera, and it seems to me that you can use raw urine if you dilute it enough. In our assay system, we usually dilute it 12 and 24 times: we take 50 and 100 µl to a total volume of 1.2 ml, and the buffer solution is rather strong. With these precautions, it is possible to use even raw urine with some antisera.

**Donini:** For the determination of FSH and LH in urine, or in urinary extracts, I believe that a urinary system should be used. For that we need urinary hormones for labelling and antisera raised in the animals by injecting urinary material.

**Robyn:** The conclusions as presented by Dr. Ryan and derived from radioimmunoassay data indicate the presence of biologically inert immunologically active material in gonadotrophin preparations and the existence of differences in antigenic properties between urinary and pituitary gonadotrophins. These two observations fully agree with the data obtained by bioassays for gonadotrophin and antigonadotrophin neutralizing potencies. Therefore it seems that similarities exist between what is implied in both types of assays. The characterization of gonadotrophins based on the antigenic sites carrying the biological activity could be more useful in the definition of specificity and immunological activities. These frequently misleading ratios could profitably be replaced by ratios between the immunological activity estimated in a immunoassay and the immunological activity estimated in a bioassay of the type presented by Dr. Petrusz.

**Stevens:** When we use a pituitary system and measure pituitary extracts or urinary materials, using appropriate standards, we get discrimination indices remarkably close to 1, indicating that you can use this system to measure pituitary extracts or kaolin extracts of urine and reflect biological activity. However, when one uses a totally urinary system to measure urinary extracts, one gets significantly higher values than in bioassay, indicating that there is more immunoreactive material than biologically active material in this system for the urine, or even urinary extract. The same situation is true when using a truly urinary system for measuring serum. We have significantly higher values using a urinary system than when we use a pituitary system to measure serum. So, I agree with Dr. Ryan that there are different antigenic groups present in urine and serum. Whether or not there are subunits, and whether or not these data will fit into Dr. Ryan's theory, remains to be explained. Concerning standards, you must come back to the question: What is the real objective of our studies? Do we want to measure, in urine or serum, the biologically active component, or that component that may reflect more truly the production rate?
Diczfalusy: I would like to answer Dr. Stevens by recalling what Sir Henry Dale said many years ago: «The ultimate purpose of bioassays is self-extinction.» If you look back into the history of the International Standards, it clearly indicates that as soon as a steroid has been isolated and identified, there was no longer any need for International Standards. Still, nobody would question that the biologically inactive steroid does in many respects reflect what you were asking about, important biological processes. Therefore it would not be very wise to limit ourselves only to the biologically active material, because then most of what we know today about certain feedback mechanisms would be very incomplete. Now, why do we need standards? Obviously, we hope that we shall know more about the exact relationship between immunologically active and biologically active molecules. But you need standards, because you have to carry out clinical assays with which you cannot wait until the day when these materials will be available in a chemically pure form. So, for the time being, it would be very important to establish at least a working standard in large enough quantity, so that it should be available to many centers, to be tested against many antisera, and I personally would say that, for the time being, you would need an immunological working standard, at least for serum and for urine, and you may find yourselves soon in a position when you may need it also for tissues.

Bangham: All the data presented during these three days make convincing evidence that like should be compared with like, so that the test substance acts in the assay system as a dilution of the standard. This is a primary assumption in comparative assays where consistent and valid potency ratios are being sought. Departure from this axiom inevitably leads to non-validity in one form or another. This may be overt, as non-parallelism, or it may be expressed in the form of anomalous and apparently irreconcilable differences in potency estimates obtained between laboratories or methods.

Strictly, test and standard should be identical at a molecular level, but this ideal is seldom achieved; one cannot for example expect to assay all individual forms of the variety of HCG molecules that seem to exist in the urine. Since we cannot set up standards for each and every form we should constantly be aware of this heterogeneity as a possible cause of otherwise apparently inexplicable discrepancies in potency estimates. Setting up a standard properly is a laborious process and with the resources available can only be done for the more distinctly different forms of the »important« hormones.

Perhaps it is useful, however, to avoid talking too much about non-specific activity, such as »LH activity«, and instead to talk of the activity of an individual hormone. One may talk of »antibiotic activity« of tetracyclines in general, but the potencies of tetracycline, oxytetracycline and chlortetracycline are each measured in terms of an individual standard for each of the three substances.

While minor differences between test and standard are likely to pass undetected, submerged in the relative imprecision of most hormone bioassays, the extraordinary specificity of the antibodies used in immunoassay systems may magnify what appear to be very slight differences. One has only to think of the extent to which the tertiary structure of a molecule may be altered by changing one constituent amino acid to appreciate why this should be. These differences may then be expressed as potency ratios that differ depending on the antiserum used. One should constantly be on the look out for apparently anomalous figures, and I suggest that one would have a much greater chance to spot them if duplicate immunoassays were routinely run with at least two different antisera, side by side.
On the question of «units», in expressing results of assays clearly it is necessary to distinguish between results obtained by immunoassay from those obtained by bioassay. The World Health Organization has recommended that results should always state whether the estimate was by bioassay or by immunoassay and specify the standard used.

In this connection it should be remembered that the majority of existing international hormone standards and reference preparations were set up for bioassay and their suitability for use with immunoassay has not been validated.

There is a tendency for research workers to state the relative potency of two materials in terms of the mass of one equivalent to a mass of another. Many people have wondered why this is not extended to biological and immunoassay standards. There is nothing inherently wrong in this, although in practice some preparations exist in such small quantities that accurate weighing is difficult and moisture content and buffer salts are not always accounted for.

Statements of «pure» hormone simply beg the question: what is «the pure hormone»? A preparation may be homogeneous by a number of criteria but who knows if it is «the» pure hormone? However, it is the transfer of «equivalent weights» (in this context weights containing the same amount of activity) from one preparation to another, and thence to another, that leads to successive and cumulative tables of weight ratio figures – which give rise to appalling confusion. Whereas the unit notation system – that has been in use for insulin, for example, for 45 years, enables the simple direct transfer of «units of activity» from one preparation to another; providing the method of assay is stated; WHO recommends the use of a similar unit notation for immunoassays and their standards.

The relationship of the immunological unit to the biological unit (the imm and the bion, respectively, are the shorthand versions suggested by Dr. R. Borth 1969) should be determined where possible for any standard used. So that the two units should at least start off meaning the same thing, it is intended that an international material should have its immunological unit assigned to it on the basis of its biological potency. In other words, the number of imms assigned to a given mass of a standard should be by definition made equal to the number of bions in it and determined by bioassay. This is a logical and straightforward step for a first standard for immunoassay. The unitage assigned to a succeeding replacement standard would be on the basis of immunoassays with a number of different antisera. Because of possible differences between preparations it cannot be assumed that the same procedure applied to a number of different house standards will mean that the imms of each will bear a predictable relation to each other especially where different antisera are used. Still the best way to achieve the international currency of a common imm for a hormone is by relating it to a common internationally used standard. Only thus would it be possible to enable results of immunoassays of complex hormones to be expressed in uniform terms meaningful throughout the world; the facilitation of biological standardization is one of WHO’s aims of its constitution.

Replacement of an international standard should be carried out by calibration with several different antisera in different assay systems. To keep differences between the materials to a minimum and thus avoid markedly disparate estimations of relative potency, standards should consist of native hormone that can be as nearly as possible reproduced; nature makes the most accurate reproductions of these complex molecules, and so the safest materials are probably simple extracts in which there is a minimum of denaturation.

When one is working with a type of material which does not give valid parallel line
assays with an accepted standard, it is usually best to set up a laboratory working standard for routine potency estimations. If it can or has to be related to an existing standard this can be done in matching comparisons, preferably with more than one method, and the results reported with details of the method(s) used.

One of the drawbacks of many papers describing immunoassay systems is the lack of evidence of specificity. Again, better international agreement would be obtained by having preparations of highly purified hormones available in stable and homogeneous form for testing the specificity of assay systems. While the value of such preparations (and of a standard) reflects the care taken in selecting, ampouling and characterizing it, one should never forget that the substance in the extract may in fact be different from the hormone itself in the body. A hormone tends to become defined by what gets used as a standard, and one should always remember that a standard is really only a practical working hypothesis. Like a conceptual hypothesis it can be replaced should it be proven unsuitable, or a better one demonstrated. The value of a standard is related closely to the care taken in ampouling it in homogeneous and stable form, and in characterizing it and showing its stability. This work takes time, and while »all things may be possible«, I do think the question of priorities should be discussed: which are the more important gonadotrophins in clinical practice and research?

Standards and Reference Preparations

The following biological standards and reference materials for gonadotrophins and other human pituitary hormones are available on request from the Division of Biological Standards, National Institute for Medical Research, London N. W. 7.

These materials are provided in limited quantities and are intended for assay and comparison purposes only. Further information about their availability, and details of the preparations and of their biological characterization can be obtained on request.

**WHO International standard preparations**

2nd International Standard for Chorionic Gonadotrophins, Human, for Bioassay.

2nd International Reference Preparation of Human Menopausal Gonadotrophins (FSH and ICSH), Urinary, for Bioassay.

2nd International Standard for Serum Gonadotrophin (P. M. S.), Equine, for Bioassay.

1st International Standard for Thyrotrophin (TSH), Bovine, for Bioassay.

1st International Standard for Prolactin, Ovine, for Bioassay.

1st International Standard for Growth Hormone, Bovine, for Bioassay.

1st International Reference Preparation of Growth Hormone, Human, for Immunoassay.

**Research Standards issued by the Division**

MRC Human Pituitary ICSH

Human Pituitary FSH

Human Pituitary TSH

Postmenopausal plasma

Postmenopausal serum
References: