THE SPECIFICITY OF THE MOUSE
OVARIAN AUGMENTATION ASSAY FOR FOLLICLE
STIMULATING HORMONE

By

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ABSTRACT

The specificity of the mouse ovarian augmentation assay for gonadotrophins has been examined. Variations of the augmenting dose of HCG from 12.5 to 100 IU per mouse had no significant influence on the assay characteristics. However, the addition of ovine luteinising hormone to standard gonadotrophins produced a depression of the expected response in all standards tested. Similarly, the assay of mixtures of standard gonadotrophins did not produce the additive response expected from the simultaneous assay of the standards alone. These effects were less marked when pure human pituitary preparations were assayed.

It is concluded that although this assay is sensitive and precise it cannot be regarded as specific for follicle stimulating hormone.

Assays of gonadotrophic substances are usually defined as being specific for luteinising hormone (LH) or follicle stimulating hormone (FSH) or non-specific and measuring «total» gonadotrophic activity (Loraine & Bell 1966). Recent publications have indicated that two tests commonly believed to be specific for LH, the ovarian ascorbic acid depletion assay and the hypophysectomized rat ventral prostate assay, apparently measure different parameters, neither of which may be completely specific (Rosenberg et al. 1964, 1965; Reichert 1966). The present report describes our experience with the mouse ovarian augmentation assay (Brown 1955) which is usually believed to be relatively specific for FSH. In particular, the specificity of this assay has been examined, both by the addition of pure ovine luteinising hormone to FSH preparations of high and low specific activity and by the assessment
of the effect of mixtures of various gonadotrophin extracts upon each other in the assay system.

MATERIALS AND METHODS

Method of Assay

The method of assay was routinely as follows: The extract of standard was dissolved in borate buffer (pH 9.0) and a similarly prepared solution of human chorionic gonadotrophin (HCG) was added. Mice were injected over a 48 hour period and killed 72 hours after the first injection. The volume of material to be injected was so arranged that the total volume of mixed HCG and gonadotrophin extract was 1.0 ml. Both ovaries from each mouse were dissected under a strong light, carefully freed from loose connective tissue without incising the ovarian capsule and weighed wet on a torsion balance accurate to 0.1 mg. The mice used were from the same colony as previously described (Martin 1965) and were used aged 21 to 24 days, 3 days after weaning. They were kept in a constant temperature room at 21°C with liberal food and water for the period of assay. The mice usually gained between 0.4 and 1.5 g during the assay so that the final weight was between 8.5 and 12.5 g. Toxicity as assessed by death of animals or by apparent disease was low with the preparations used.

The human chorionic gonadotrophin (HCG) used was commercially available Pregnyl® – Organon. In the experiments concerning the specificity of the assay the same batch of material was used throughout. Standard gonadotrophins used were: Ovine, pituitary follicle stimulating hormone and luteinising hormone (NIH-FSH-S2 and NIH-LH-S8); human urine extracts, 2nd IRP, NIH-HPG-UE and HMG-24. Human pituitary »FSH« prepared by Dr. J. B. Brown from acetone dried pituitary powder by extraction with acetic acid buffer at pH 5.6 (Brown et al. 1966) and extracts of pooled, post-menopausal urine prepared as laboratory standards by the method of Johnsen (1958). The potency of the preparations used as determined by the mouse ovarian augmentation assay in terms of the second international reference preparation (2nd IRP) were: NIH-FSH-S2 = 1.9, NIH-HPG-UE = 2.4, pituitary extracts = 10.0 to 24.0, HMG-24 = 0.04, laboratory standard gonadotrophin = 0.04. The working range of mean combined ovarian weights was from 4.0 to 10.0 mg and the results of calculations were routinely expressed in arithmetic terms. Computation of assays was by the methods of Gaddum (1958) and Borth (1960).

RESULTS

Assay Characteristics

The dose response curve of the combined ovarian weights in the strain of mice used in our laboratory to increasing doses of HCG up to 200 IU per assay is shown in Fig. 1. The plateau of response lies between 3.125 IU and 100 IU per assay. The effect of using augmenting doses of HCG of 12.5, 50 and 100 IU per assay is shown in Fig. 2. There was no evidence of any effect on either the slope or sensitivity of the assay of the standards NIH-FSH-S2 and 2nd IRP by this variation in HCG dose. With further experience it has now
The response of mouse ovarian weight to human chorionic gonadotrophin (HCG). Each point represents the mean with one standard deviation.

Fig. 1.

The dose-response curves of three assays of two standard preparations (NIH-FSH-S2 and 2nd IRP) at different augmenting doses of HCG.

Fig. 2.
become our routine practice to use 12.5 IU as the augmenting dose of HCG in this assay.

The precision and slope of the assay were assessed in a similar manner to that used by Brown & Wells (1966) by studying the figures obtained for all assays of standards and clinical extracts over an eighteen month period. The variation in the slope (b) and the index of precision (Lambda, $\lambda$) in 94 assays is shown in Fig. 3. In only 4 assays was lambda above 0.2 and in none above 0.3. The sensitivity of the assay was such that the minimal effective dose, that is, the response greater than two standard deviations above the HCG control varied from 9.5 to 20 $\mu$g of NIH-FSH-S2 and from 25 to 45 $\mu$g of the 2nd IRP. Thirty-five per cent of the assays were non-parallel. However, if the ovarian weights were converted to logarithms (Brown 1959) the number of non-parallel assays was reduced to 15%. Using the 2nd IRP as standard a linear response was observed over the dose range 50 to 800 $\mu$g. (Ovarian weights 4.8 to 15.1 mg).

**Specificity of Assay**

This was tested by the addition of NIH-LH to solutions of the gonadotrophin assayed. The effect of NIH-LH on the response of HCG alone was tested first at the three doses of HCG used. At doses of NIH-LH above 12 $\mu$g there was an inconsistent increase in ovarian weight above control, but

![Fig. 3.](attachment:image.png)

The slopes (b) and indices of precision ($\lambda$) in 94 assays of gonadotrophin extracts with the mouse ovarian augmentation assay.

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below this dose this was not observed. Assays of mixtures of NIH-LH and other gonadotrophin standards were then performed directly against each other with four or five mice per dose and the difference between the mean responses was determined by the t test. In Table 1 the number of assays in which the response of the standard preparations to which LH had been added was significantly depressed (P < 0.01) below the response of the gonadotrophin standards alone is shown, for different ratios weight for weight of added NIH-LH to standard gonadotrophin in micrograms. This effect was studied at different augmenting doses of HCG (12.5, 50 and 100 IU respectively). The actual weight of NIH-LH added varied from 0.625 μg to 50 μg in different assays and no allowance was made in calculating the ratio of standard gonadotrophin to LH for any intrinsic LH content of the standard used. From the results in Table 1 it will be seen that there was a significant depression in the expected response in from 60 to 100% of assays when ovine luteinising hormone was added to ovine pituitary FSH or human pituitary gonadotrophin obtained from urine at all levels of HCG augmentation. In contrast, when NIH-LH was added to gonadotrophin of high specific activity obtained from human pituitary glands only between 14 and 22% of the assays were significantly depressed.

The effect of added NIH-LH to NIH-FSH as studied in 4 point assays at augmenting doses of 12.5 and 100 IU HCG and ratios of FSH to LH from 4:1 to 32:1 is shown in Table 2. Significant depression of the response of NIH-FSH occurred in the four assays studied. Further, this depression of the response of the mouse ovary to ovine FSH was produced without loss of parallelism even in the untransformed response. The possibility that the mixtures of pure gonadotrophins used in this assay system may have given a different response when injected together than if they were injected separately at different sites was also investigated. Assays were conducted in which either each of the gonadotrophins used was injected separately or the LH was injected separately to the mixed FSH and HCG preparation. No difference in the assay response was noted with these manoeuvres. In addition it was found that dissolving the standards in solutions of bovine serum albumen and saline instead of borate buffer did not affect the response.

The performance of the assay system when different FSH-containing gonadotrophin preparations were mixed together was next examined. It was thought that if the assay system was specific for FSH then the capacity of a mixture of gonadotrophins to increase the ovarian weight in the assay system (»FSH« response) may be equal to the sum of the simultaneously determined »FSH« content of the two preparations alone, either as an arithmetical or logarithmic function. In preliminary assays to test this idea it became obvious using the preparations NIH-FSH and 2nd IRP that this was not so and the combined response was often depressed below that expected and, in some
Table 1.
Ratio standard to added NIH-LH in microgrammes.
The effect of added ovine luteinising hormone (NIH-LH-S8) on the expected response of the mouse ovary to standard gonadotrophins at augmenting doses of 12.5, 50 and 100 IU HCG. The number of assays depressed and the number performed at each dose level are shown together with the totals for each standard used. Depression was said to occur when the difference between the mean response was such that $P < 0.01$.

<table>
<thead>
<tr>
<th>Dose HCG IU</th>
<th>Standard</th>
<th>1</th>
<th>2</th>
<th>4</th>
<th>8</th>
<th>16</th>
<th>32</th>
<th>64</th>
<th>128</th>
<th>256</th>
<th>256</th>
<th>Total depressed</th>
<th>Total assays</th>
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<tr>
<td>100</td>
<td>NIH-FSH</td>
<td>1/1</td>
<td>1/1</td>
<td>3/5</td>
<td>6/7</td>
<td>4/7</td>
<td>7/10</td>
<td>3/5</td>
<td>1/1</td>
<td></td>
<td></td>
<td>26/37</td>
<td>13/22</td>
</tr>
<tr>
<td></td>
<td>IRP-2</td>
<td>1/1</td>
<td>1/1</td>
<td>2/3</td>
<td>1/4</td>
<td>2/4</td>
<td>5/7</td>
<td>0/1</td>
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<td></td>
<td></td>
<td></td>
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</tr>
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<td></td>
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<td></td>
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<td>1/1</td>
<td></td>
<td></td>
<td></td>
<td>1/1</td>
<td>3/3</td>
<td>4/4</td>
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<td></td>
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<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td>1/1</td>
<td>3/3</td>
<td></td>
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<tr>
<td></td>
<td>FSH-human</td>
<td>0/1</td>
<td>1/1</td>
<td>0/1</td>
<td>0/2</td>
<td>0/1</td>
<td>0/1</td>
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<td>1/7</td>
<td>7/8</td>
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<tr>
<td>50 + 25</td>
<td>NIH-FSH</td>
<td>2/2</td>
<td>2/2</td>
<td>1/1</td>
<td>1/1</td>
<td>1/2</td>
<td></td>
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<td>7/8</td>
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<tr>
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<td>NIH-FSH</td>
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<td></td>
<td></td>
<td></td>
<td>4/7</td>
<td></td>
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<tr>
<td>12.5</td>
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<td>1/2</td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td>FSH-human</td>
<td>0/1</td>
<td>1/2</td>
<td>1/2</td>
<td>0/1</td>
<td>0/2</td>
<td>0/2</td>
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Table 2.
The assay of NIH-FSH-S2 against mixtures of NIH-FSH-S2 and NIH-LH-S8 at two doses of HCG, with a 2 X 2 design.

<table>
<thead>
<tr>
<th>HCG (IU)</th>
<th>FSH (µg)</th>
<th>LH (µg)</th>
<th>b</th>
<th>S</th>
<th>λ</th>
<th>Relative potency against FSH alone (95% confidence limits)</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>6.25</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.72</td>
</tr>
<tr>
<td>50</td>
<td>12.5</td>
<td>6.98</td>
<td>0.42</td>
<td>0.06</td>
<td></td>
<td>(0.65–0.79)</td>
</tr>
<tr>
<td>25</td>
<td>1.57</td>
<td>4.95</td>
<td>0.34</td>
<td>0.07</td>
<td></td>
<td>(0.41–0.62)</td>
</tr>
<tr>
<td>50</td>
<td>3.13</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.50</td>
</tr>
<tr>
<td>25</td>
<td>6.25</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(0.35–0.74)</td>
</tr>
<tr>
<td>50</td>
<td>1.57</td>
<td>2.82</td>
<td>0.79</td>
<td>0.18</td>
<td></td>
<td>(0.30–0.78)</td>
</tr>
<tr>
<td>12.5</td>
<td>0.78</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.50</td>
</tr>
<tr>
<td>25</td>
<td>1.57</td>
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<td></td>
<td></td>
<td></td>
<td>(0.35–0.74)</td>
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<tr>
<td>50</td>
<td>1.57</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(0.30–0.78)</td>
</tr>
</tbody>
</table>

cases, below one or both of the individual responses. Mixtures of the standard preparations NIH-FSH-S2, 2nd IRP, NIH-HPG-UE and human pituitary extracts were then assayed at the same time as the standards alone. Twenty assays were performed and in the majority the expected additive effect was not observed. A typical result is shown in Fig. 4. The apparent suppressive effect of mixing different standard preparations appeared to be greatest with the two urine extracts as when this was done no assay was obtained which was both parallel and not suppressed. Even relatively pure pituitary gonadotrophin was affected by mixing with the 2nd IRP and the only four point assay which appeared completely additive was obtained when 12.5 µg of NIH-FSH was added to both 4 µg and 8 µg of a pituitary extract.

DISCUSSION

The present investigation throws doubt on the specificity of the mouse ovarian augmentation assay for follicle stimulating hormone. The principle of this assay was first defined by Bates & Schooly (1942) using rats. They concluded that as human chorionic gonadotrophin has a luteinising action and is present in excess, then any further increase in ovarian weight over the plateau of response must be due to follicle stimulating hormone. Subsequently, Bates (1961) in discussion has stated that the assay is relatively specific for FSH, but may be influenced by the relative amounts of FSH-LH in a preparation.
particular if there is not a large excess of FSH. Steelman & Pohley (1953) adapted Bates’ and Schooly’s work (Bates & Schooly 1942) and standardised an ovarian augmentation assay system for FSH in rats using purified pituitary preparations. They found that intact immature rats were more satisfactory than hypophysectomised rats and that the assay was relatively specific for FSH although there was some depression of response if the ratio of added ovine LH to FSH was above 1:4. Subsequently, Simpson (1961) showed that with pure pituitary preparations, parallel responses were obtained when the ovarian follicular growth assay in hypophysectomised rats and the ovarian augmentation assay in immature rats were conducted simultaneously. Simpson (1961) concluded that the latter assay was relatively specific for FSH. Brown (1955) adapted the Steelman-Pohley assay to mice (Steelman & Pohley 1953), but published no data concerning the effect of luteinising hormone on the specificity. Subsequent workers have also published little concerning this aspect, although Brown & Wells (1966) concluded from the assay of mixtures of puri-
fied human pituitary FSH and human pituitary LH assayed against FSH alone that there was no evidence that the assay system used was not specific for FSH. However, only two assays were performed, one of which was disregarded as non parallel, and both had wide fiducial limits. Schmidt-Elmendorf et al. (1962) quote Simpson (1961) that the mouse ovarian response is specific for FSH, but they did not find a significant variation in the index of discrimination from unity when human FSH and Pergonal were assayed by both the mouse augmentation and uterus assays. In this laboratory a similar lack of significant change in the index of discrimination from unity between results of the mouse ovarian assay and the mouse uterus assay has been noted with human gonadotrophin extracts.

The results of this investigation indicate that the addition of ovine LH to standard gonadotrophin preparations of both sheep and human origin will result very often in a depression of the augmentation response expected from the standard gonadotrophin alone, and that this can occur even when the augmenting dose of HCG is varied from 12.5 to 100 IU. Further, the conduct of 4 point assays for parallelism will not eliminate the possibility of such depression being recognised and the assay result consequently rejected. These results are essentially similar to those of Riley (1961) who showed that there was a depressant effect when NIH-LH was added to Pergonal using the mouse uterus assay. The failure of the expected ovarian augmentation response to occur when mixtures of gonadotrophin preparations were assayed also makes it unlikely that the assay is specific for FSH and such an effect could be due to the presence of various amounts of LH in the extracts assayed. It would seem that there is less chance of this depression occurring when human gonadotrophin preparations of high specific activity are assayed.

The overall performance of this assay in our hands compares favourably with the mouse uterus assay (Martin 1965) and, in particular, it is as sensitive as the latter assay and much more precise at the level of dose response used. It should be noted that the level of the dose-response curve that we have used (4.0–10.0 mg combined ovarian weight) is below that used by others (Brown, personal communications). We have chosen to work at this lower limit of response because we were concerned with obtaining a sensitive assay for clinical use. We conclude that although the mouse ovarian augmentation assay is precise and sensitive at this level of response it cannot be regarded as specific for FSH.

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