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# Validation of coupled bioassay for inhibin by pituitary cell culture assay

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**Abstract.** Studies on the characterization of inhibin and inhibin-like factors have depended for the most part on the classical *in vitro* pituitary cell culture assay. A major drawback with this assay is the turn-around time which is in the order of two weeks and consequently slows down purification efforts. The 24 h bioassay for inhibin has been found to be sufficiently sensitive and also statistically valid. Unfortunately, based as it is on a secondary response, ambiguities arise in interpreting the results. By including a parallel assay in which the mice are primed with human menopausal gonadotropin rather than human chorionic gonadotropin, it was possible to device the coupled bioassay. This enables distinguishing inhibin-like factors acting to suppress pituitary follicle stimulating hormone output from those acting at the level of gonads. In this study the coupled assay for inhibin has been compared with the classical pituitary cell culture assay in order to assess its biological and statistical validity. The data validates the bioassay on both the above counts and when considered in conjunction with the short turn-around time suggests that this assay can be highly useful in studies on isolation of inhibin from various sources.

**Keywords.** Inhibin; bioassay; specificity; statistics.

## Introduction

The pioneering efforts of Baker *et al.* (1976) and de Jong *et al.* (1979) culminated in the adoption of the *in vitro* pituitary cell culture based assay as the method of choice for identifying and quantifying inhibin, the gonadal factor exerting feedback control on pituitary follicle stimulating hormone (FSH) output. The attractive features of this assay are its specificity, precision and sensitivity—factors that render it valuable in the clinical laboratory. However, a major drawback in its routine use in the research laboratory is the turn-around time which is of the order of 10–15 days. This militates against rapid progress in the fractionation and purification to homogeneity of inhibin/inhibin-like activity (ILA) from diverse sources—an objective still pursued in many laboratories around the world. Two other limitations that are serious enough for at least some laboratories are: (i) the cost and (ii) the hazard inherent to an assay dependent on the routine handling of radioactive iodine.

It would appear, therefore, that there is still a place for a simple, specific and rapid bioassay for inhibin that is at the same time economical. The bioassay developed by Ramasharma *et al.* (1979) appeared, at first sight to fulfill the above

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Abbreviations used: FSH, Follicle stimulating hormone; ILA, inhibin-like activity; hCG, human chorionic gonadotropin; hMG, human menopausal gonadotropin; SNK, Student's Newman-Keul; LH, luteinizing hoxmone.

criteria. However, it was soon realized that it gave ambiguous results as instanced by experience in this laboratory in the course of characterizing bovine seminal plasma inhibin (Mohapatra *et al.,* 1985). In light of this and based on the observations of Lamond and Emmens (1959), a modified assay, the so called coupled bioassay with a turn-around time of 24 h, has been used for some years with great advantage in this laboratory (Kharbanda *et al.,* 1985; Chari *et al.,* 1987). In order to further document the biological and statistical validity of this assay, preparations showing either ILA or, in the alternative, FSH-BI or LH-RBI activity have been compared in this as well as in the classical pituitary cell culture assay (Dhingra and Duraiswami, 1988a). The data reported in the present study suggest that the coupled assay can be usefully employed in studies aimed at the isolation and characterization of inhibin/ILA from various sources.

## Materials and methods

### *Coupled assay*

Swiss albino derived, colony bred, 24 ±1 day old, immature female mice weighing 12 to 13 g were used. Choice of animals younger than those used by Ramasharma *et al.* (1979) was necessitated by the fact that in our colony vaginal opening occurred on day 27. They were housed under the standard conditions of the animal house (temperature 24±1°C, 14 h light-l0h dark schedule, freely available commercial pelletted diet and water). The animals were randomly distributed to the various test groups and were assessed for uterine response to graded doses of test samples in two parallel assays, in one of which the control as well as treated animals received a total dose of 40 IU of human chorionic gonadotropin (hCG) (Pregnesine/Serono) while in the other they received a total dose of 5 IU of human menopausal gonadotropin (hMG) (Pergonal/Serono). Animals not receiving either gonadotropin but only saline at the scheduled times served as basal controls, while those receiving hCG or hMG and saline, in lieu of the test sample, served as gonadotropin-treated controls. The dose of hCG or hMG administered to the animals was such as to effect a tripling of uterine weight as compared to basal control. The injection schedule was as follows: Saline or test sample in appropriate dose was administered intraperitoneally at 09:00 h, followed 1 h later by hCG/hMG/saline *via* the subcutaneous route. The injections were repeated in the same order at 18:00 and 19:00h. The total dose of each of the injectibles was divided into two equal halves and administered in a total volume of 0·4 ml., *i.e.,* 0·2ml per injection. The animals were autopsied 15 h after the last injection. The uteri were dissected out, freed of adhering tissue and weighed to the nearest tenth of a mg in a torsion balance. In either case, the end point was a dose-dependent inhibition of gonadotropin (hCG/hMG) induced uterine weight gain.

### *Pituitary cell culture assay*

All glassware used was siliconized and autoclaved. All fluids were sterilized by passing them through Millipore filters. Anterior pituitary glands were collected from adult male rats (Holtzman strain) after decapitation. Dispersed pituitary cells

were prepared by the method of Vale *et al.* (1972) with the difference that hyaluronidase was omitted from the dispersion medium. Furthermore, the medium used for the final culture was M-199 (GIBCO) supplemented with 10% fetal calf serum, freshly added 0·1 mM glutamine and antibiotics. Approximately $5 \times 10^5$ cells were added to each well of a 24-well tissue culture treated polystyrene plate (Cell Wells, TM Corning) and cultured under 95% air, 5% $CO_2$ at 37 ± 1°C in 2 ml of culture medium. The assay itself was set up according to de Jong *et al.* (1979). The end point used was suppression of GnRH-stimulated FSH release.

Radioimmunoassays for gonadotropins were performed by the double antibody method (Midgley, 1966) using the NIAMDD rat RIA kits. The results are expressed with reference to NIAMDD-FSH-RP-1 (sensitivity, 4 ng) and NIAMDD-LH-RP-2 (sensitivity, 5 ng). Intra and interassay variations were respectively, FSH: 5 to 6% and 8 to 9%; LH: 6 to 7% and 10 to 12%.

*Test preparations*: Details of the samples used as test preparations in this study are provided in table 1.

**Table 1.** Test preparations.

| Sample | Description | Biological property | Reference |
|---|---|---|---|
| oTI | Ovine testicular inhibin | Inhibin | Dhingra and Duraiswami (1988b) |
| oFFI | Ovine follicular fluid inhibin | Inhibin | Narendar Kumar (1985) |
| bSPI-DE2 | Preparation DE-2 from bull seminal plasma | Inhibin-like | Malhotra (1987) |
| bSPI-DE4 | Preparation DE-4 from bull seminal plasma | Inhibin-like | Sharma (1987) |
| LH-RBI | Luteinizing hormone receptor binding inhibitor, prepared from ovine corpora lutea | Inhibits binding of LH to its target cell receptors | Kumar, N., Dhir, R. N., Kumari, G. L. and Duraiswami, S. (unpublished results) |
| FSH-BI | Follicle stimulating hormone binding inhibitor, prepared from ovine follicular fluid | Inhibits binding of FSH to its target cell receptors | Dhir, R. N., Narender Kumar, Dhingra, S. R., Kumari, G. L. and Duraiswami, S. (unpublished results) |

*Statistical analysis*:

The following statistics were performed: one way analysis of variance (ANOVA), standard correlation and regression analyses; differences among individual treatment means, after significant ANOVA main effects, were compared by the Student's Newman-Keul's (SNK) post hoc test (Sokal and Rohlf, 1969). A $P < 0{\cdot}05$ was considered statistically significant. The level of significance from the control is denoted by asterisks ($*P < 0{\cdot}05$, $**P < 0{\cdot}01$, $***P<0{\cdot}001$).

For the statistical validity of the assays, a simplified computation of the statistics of multiple parallel line assays, based on a symmetrical design in which the number of animals per dose as well as the log dose interval are kept constant for all dose levels as described by Borth (1960), was used. In our assays, we have used 3 doses

with a log dose interval of 2, with 4 mice per dose in the case of the coupled assay and 3 wells per dose in the cell culture assay. The following criteria were applied for Statistical validity: (i) index of significance of slope ($g_c$); (ii) variation of slope ($G_c^2$) and its $F$ value ($F_v$); (iii) significance ($t$) of the differences of mean response and (iv) the index of precision ($\lambda$). Following Borth (1960) the level of statistical significance was set at 5%. Under these conditions, the combined slope for the assay is considered significant if the computed value for $\lambda$ is < 1. Variation in the slope and differences of mean responses are considered negligible if the calculated values are smaller than the table values for $P = 0{\cdot}05$. An index of precision of 0·2 is generally considered acceptable for a bioassay. Since parallelism in the slopes of the log dose-responses were observed, the data was used for calculating relative potencies.

## Results

### *Coupled assay*

Inhibin/inhibin-like preparations (oTI, oFFI, bSPI-DE2 and bSPI-DE4) significantly inhibited uterine weight gain ($P<0{\cdot}001$, $F$-test) in a dose-dependent manner (figure 1) in the hCG-treated animals. The data exhibited highly significant linear regression ($P < 0{\cdot}001$). The dosages required for 50% suppression of uterine weight as read off from the log dose-response curves (figure 2), are as follows: oTI = 7 $\mu$g; oFFI = 6·2 $\mu$g; bSPI–DE2 = 21 $\mu$g; bSPI–DE4 = 31 $\mu$g. Thus oFFI is slightly more active than oTI and the bSPI preparations. When these preparations were checked for their effect in hMG-treated immature mice, no significant suppression of uterine weight was observed even at as high a dose as 200 $\mu$g ($P >0{\cdot}75$, $F$-test, figure 3).

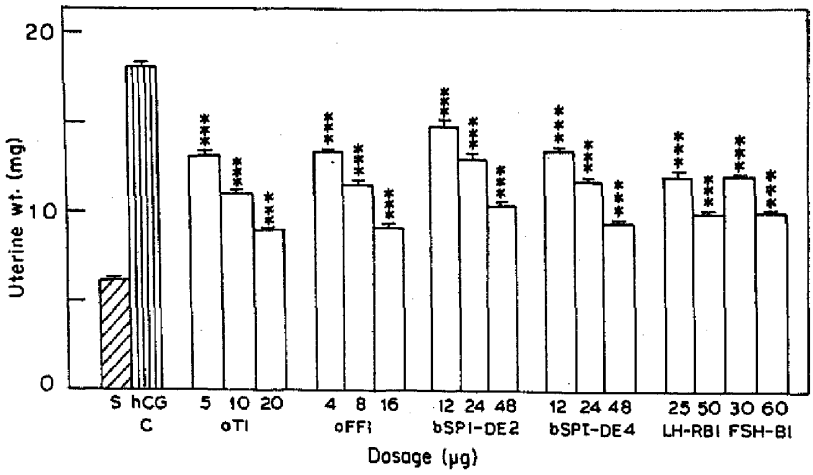


**Figure 1.** Effect of different preparations on hCG-induced uterine weight gain in the coupled assay. Bar chart represents mean ± SEM, n = 4. All the preparations inhibited the uterine weight gain significantly ($P < 0{\cdot}001$, $F$-test) in a dose-dependent manner. In this and in all subsequent histograms the mean differences among the test preparations were individually compared for the statistical significance by an a-posteriori SNK test and are indicated by the number of asterisks, *$P < 0{\cdot}05$; **$P < 0{\cdot}01$; ***$P < 0{\cdot}001$.

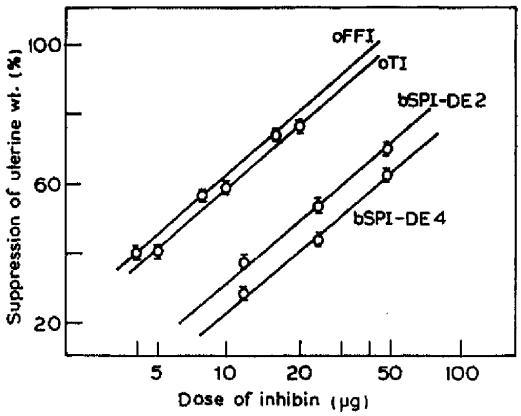


**Figure 2.** Log dose-response curves for the inhibin activity of different preparations tested at 3 dose levels in the hCG assay. The regression lines are based on statistical analysis as detailed in the text. The individual points however, represent percentage suppression of hCG-induced uterine weight increase (mean ± SEM, n = 4) for specified doses of the given preparation calculated from actual assay data. Percentage suppression was calculated by the following formula:

$$\% \text{ suppression} = \frac{(\text{Uterine weight of hCG control}) - (\text{Uterine weight of inhibin group})}{(\text{Uterine weight of hCG control}) - (\text{Uterine weight of saline control})} \times 100.$$

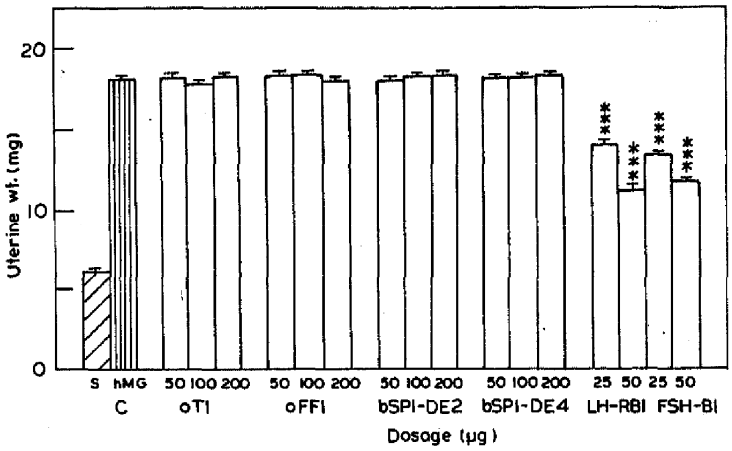


**Figure 3.** Effect of different preparations on hMG-induced uterine weight gain in the coupled assay. Bar chart represents mean ± SEM, n = 4. The inhibin-like preparations did not inhibit uterine response in the hMG-treated animals ($P > 0{\cdot}75$, $F$-test). On the other hand, preparations with LH-RBI/FSH-BI activity suppressed in a dose-dependent and statistically significant ($P < 0{\cdot}001$) manner the hMG-induced uterine weight gain.

In contrast, the receptor binding inhibitors (LH-RBI and FSH-BI) suppressed uterine weight gain in a dose-dependent manner not only in the hCG-treated but also in the hMG-treated animals ($P < 0{\cdot}001$, $F$-test, figures 1 and 3), when tested in the same dose ranges.

*Pituitary cell culture assay*

All inhibin-like preparations (oTI, oFFI, bSPI-DE2 and bSPI-DE4) also suppressed GnRH-stimulated FSH secretion into the medium significantly ($P < 0{\cdot}001$, *F*-test, figure 4). FSH levels showed a highly significant linear regression with the dose ($P < 0{\cdot}01$), deviation from linear regression being non significant ($P > 0{\cdot}75$). On the other hand, receptor binding inhibitors (LH-RBI/FSH-BI) did not suppress GnRH-stimulated FSH secretion into the medium significantly ($P > 0{\cdot}75$, *F*-test, figure 4). While the inhibin-like preparations sometimes suppressed GnRH-stimulated release of luteinizing hormone (LH) into the medium, no statistically significant dose-dependent effect could be observed ($P > 0{\cdot}75$, *F*-test, figure 5).

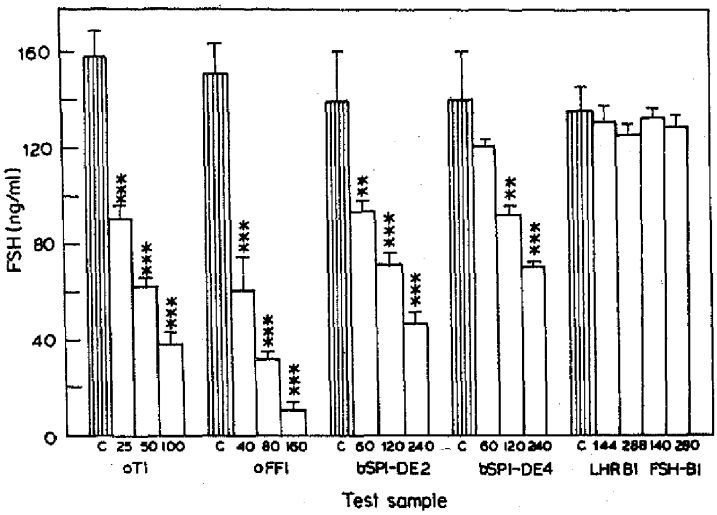


**Figure 4.** FSH levels in media of pituitary cells cultured for 6 days followed by 6 h of GnRH-stimulation. The bar chart represents mean ± SEM, n = 3. All inhibin-like preparations suppressed FSH in the medium significantly ($P< 0{\cdot}001$, *F*-test) in a dose-dependent manner. On the other hand preparations with LH-RBI/FSH-BI activity did not suppress FSH in the medium ($P > 0{\cdot}75$).

Figure 6 presents the log dose-response curves for the 4 inhibin preparations based on which the amount of protein required for 50% suppression by oFFI, oTI, bSPI-DE2 and bSPI-DE4 are found to be 28, 34, 120 and 190 ng respectively.

The possibility that a given test material might permanently damage the cells and thus reduce the secretion of FSH, was examined by replacing the medium containing test substances at the end of assay with fresh medium and assessing subsequent FSH output. The data presented in figure 7 are typical and indicate that the cells do recover their ability to secrete FSH into the medium after exposure to varying doses of the inhibin used *i.e.* bSPI. The phase contrast photomicrograph (figure 8) also suggests continued viability of the cultured cells as evidenced by the confluent appearance of cells till the end of assay (Vale *et al.* 1972; Baker *et al.,* 1981).

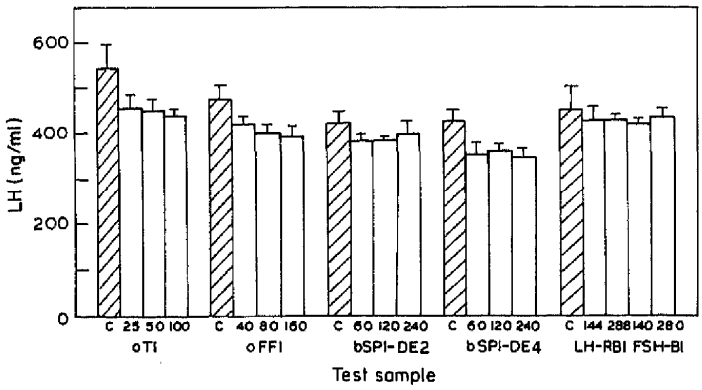


**Figure 5.** LH levels in the media of pituitary cells cultured for 6 days followed by 6 h of GnRH-stimulation. The bar chart represents mean ± SEM, n = 3. In no case was a statistically significant suppression observed ( $P > 0{\cdot}75$).

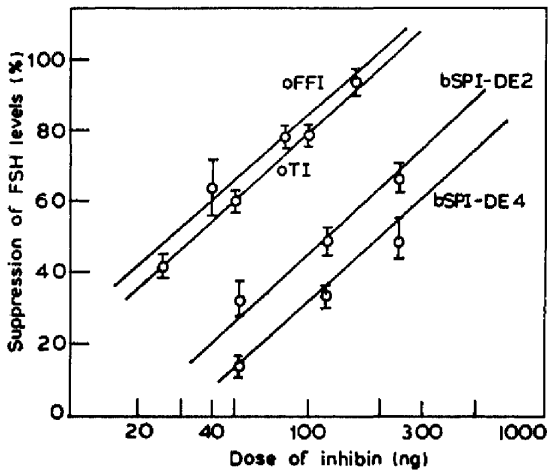


**Figure 6.** Log dose-response curves for ILA of different preparations tested at 3 dose levels in the pituitary cell culture assay. The regression lines are based on statistical analysis as detailed in the text. The individual points however, represent percentage suppression of GnRH-stimulated FSH levels in the medium (mean±SEM, n = 3) for specified doses of the given preparation calculated from actual assay data. The percentage suppression was calculated as follows:

$$\% \text{ suppression} = \frac{(\text{FSH level of control}) - (\text{FSH level of inhibin group})}{(\text{FSH level of control})} \times 100.$$

*Statistical evaluation*

The data of the hCG-based and the *in vitro* pituitary cell culture assay was subjected to Borth's analysis. The results are summarized in table 2. Both the assays were found to be statistically valid. The relative potencies of the test preparations, taking oTI as the standard, are summarized in table 3. oFFI was found to be approximately 1·3 to 1·4 times more potent than oTI, whereas bSPI-DE2 and bSPI-

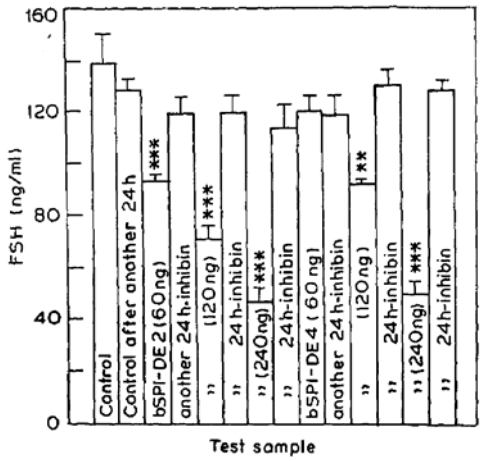


**Figure 7.** Demonstration of rebound FSH secretion by pituitary cells in culture following withdrawal of inhibin treatment. Controls, for this purpose, were the original controls (cells exposed to the 6-day culture + 6 h. GnRH exposure schedule), but which were then returned to the normal medium without additions and incubated for another 24 h. The bars labelled 'another 24 h-inhibin' represent those wells incubated for the prescribed 6 h in the presence of GnRH and inhibin, but returned for a final 24 h to normal medium not containing any other additions. Note that in all cases the cells have fully recovered their ability to secrete the 'control' levels of FSH.

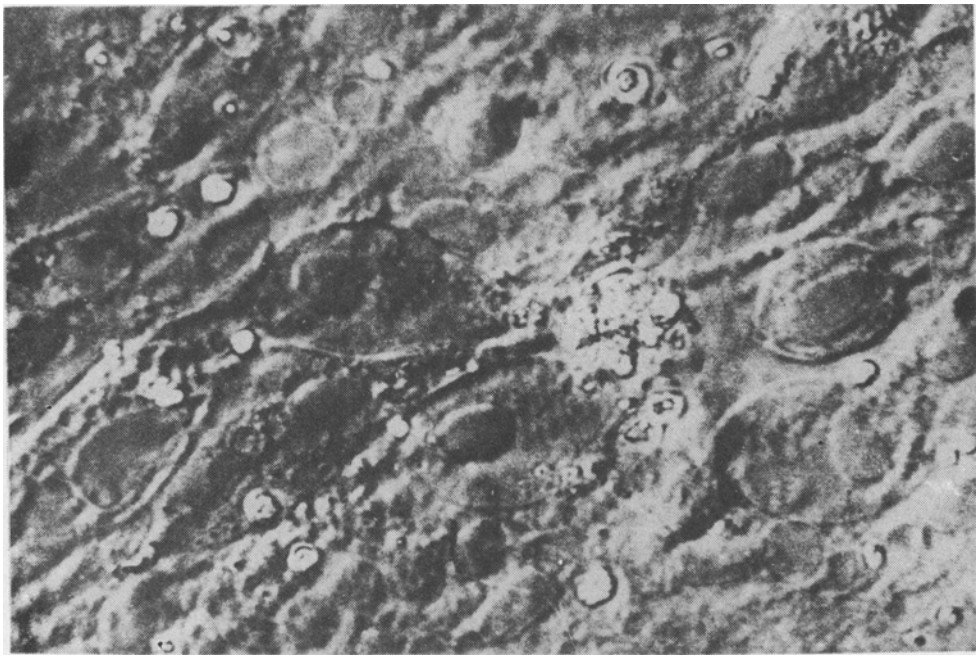

**Figure 8.** Phase contrast photomicrograph of pituitary cells at the end of 6 days of culture (× 500). Note the extension of cytoplasm of cells in an elongated form indicative of the viability of cells.

**Table 2.** Statistical evaluation of the validity of the hCG-based and pituitary cell culture assay by Borth's system of computation.

| | Parameter | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | $\bar{n}$ | $J$ | $I$ | $b_c$ | $S_c$ | $S_c^2$ | $V$ | $g_c$* | $G_c^2$ | $F_v$† | $\lambda$** |
| | | | | | | hCG-based assay | | | | | |
| Value | 4 | 2 | 0·301 | 6·87 | 0·396 | 0·157 | 0·039 | 0·005 | 0·036 | 0·923 | 0·057 |
| | | | | | | Pituitary cell culture assay | | | | | |
| Value | 3 | 2 | 0·301 | 76·83 | 8·91 | 79·3 | 26·4 | 0·026 | 27·13 | 1·03 | 0·116 |

fi, Average number of animals per dose level; *J,* ratio of contiguous doses; *I,* log *J; $b_c$,* combined slope; $S_c$ = standard deviation of response; $S_c^2$, residual variance; *V,* variance of the mean effects; $g_c$, index of significance of slope; $G_c^2$, departure from parallelism; $F_v$, *F* value for departure from parallelism. λ, index of precision.

*, The computed value of $g_c$ is less than 1 and therefore the combined slope for the assay can be considered significant.

†, The computed value of $F_v$ is less than the table value of 3·01. Therefore departure from parallelism is negligible.

**, The value of $\lambda$ is less than 0·2 and therefore the index of precision of the assay is acceptable.

**Table 3.** Assessment of relative potencies of inhibin preparations.

| | Preparation | | |
|---|---|---|---|
| | oFFI | bSPI-DE2 | bSPI-DE4 |
| hCG based assay | | | |
| Relative potency (oTI as standard) | 1·24 | 0·240 | 0·35 |
| Fiducial limits | 0·825–1·85 | 0·16–0·48 | 0·24–0·53 |
| Pituitary cell culture assay | | | |
| Relative potency (oTI as standard) | 1·46 | 0·340 | 0·17 |
| Fiducial limits | 1·13–2·1 | 0·26–0·46 | 0·15–0·22 |

DE4 had potencies of 0·2 to 0·4. These are in good agreement with the ratios calculated from log dose-response curves.

The above results in addition to validating the coupled assay design, also signify that gonadal inhibins are far more active than inhibin from extragonadal sources.

## Discussion

### *Rationale of the coupled assay*

Using intact as well as hypophysectomized immature female mice, Lamond and Emmens (1959) observed that the effect of hCG on uterine weight gain was virtually abolished by hypophysectomy, suggesting the need for endogenously secreted gonadotropins. On the other hand hMG was able to elicit its response in both the intact as well as the hypophysectomized mouse. This is in line with the observations of Lamond and Braden (1959) who demonstrated a diurnal variation in the uterine

response of the immature mouse to hCG and pregnant mare serum gonadotropin but did not find it with hMG. Thus the response to hMG is not dependent on endogenous gonadotropins in the intact mouse. Using a more direct approach Ramasharma *et al.* (1979) demonstrated that hCG evokes an acute discharge of FSH which, by stimulating follicular maturation and synthesis of estrogen, causes an increment in uterine weight. Neutralization *in vivo* of the hCG induced FSH by a highly specific antiserum was found to abolish the hCG effect.

Based on these observations the assay of Ramasharma *et al.* (1979) was modified by the inclusion of a parallel assay in which hMG was injected instead of hCG so as to differentiate between inhibin-like preparations and those acting at the level of the gonads.

*Validity of the coupled assay*

From the foregoing it would be evident that if a test preparation suppresses uterine weight gain in hCG-treated animals but not in those treated with hMG, it is clearly inhibin in nature. On the other hand, if the hMG treated animals also respond by a suppression of uterine weight gain the preparation can be taken to act at the gonadal level. The data summarized in table 4, validates the argument on which the coupled assay design is based. Animals treated with oTI, oFFI, bSPI showed suppression of uterine weight gain only if they had been exposed to hCG. In contrast, animals treated with FSH-BI and LH-RBI preparations suppress uterine weight gain in both hCG and hMG-treated animals. These preparations have been shown already to be receptor binding inhibitors by appropriate radioreceptor assays (Kumari *et al.* 1984; Kumar, N., Dhir, R. N., Kumari, G. L. and Duraiswami, S., unpublished results).

The differential diagnosis effected by the coupled assay has been fully borne out by the results of the *in vitro* pituitary cell culture assay performed on the same preparations. Those preparations which suppressed hCG-induced but not hMG-induced uterine weight gain in the coupled assay were alone effective in suppressing GnRH-induced FSH secretion (see table 4). While inhibin-like preparations sometimes suppressed GnRH-stimulated release of LH into the medium, no statistically significant dose-dependent effect was observed. These results are in conformity with the observations of de Jong *et al.* (1979), Scott *et al.* (1982), Baker *et al.* (1981), LeGac and deKretser (1982) and Robertson *et al.* (1986).

**Table 4.** Statistical significance of the response as calculated by *F*-test.

| Test sample | Coupled assay | | Pituitary cell culture assay |
|---|---|---|---|
| | hCG | hMG | |
| oTI | *** | ns | *** |
| oFFI | *** | ns | *** |
| bSPI-DE2 | *** | ns | *** |
| bSPI-DE4 | *** | ns | *** |
| LH-RBI | *** | *** | ns |
| FSH-BI | *** | *** | ns |

***$P < 0{\cdot}001$; ns, nonsignificant.

*Statistical evaluation*

Parallel dose-dependent responses have been obtained in the hCG-treated animals and in the pituitary cell culture assay. The statistical data shows that the linearity of the dose response curve, the lack of deviation from parallelism of the dose response curves and the precision are within the acceptable range (table 2). The index of precision ($\lambda$) was found to be 0·057 for the coupled assay and 0·116 for the pituitary cell culture assay. The good agreement between the potencies of the different preparations by the two assays permits the conclusion that the physiological basis of the two assays must be the same, namely action of the active principle at the level of pituitary to suppress FSH secretion.

*Performance and practicability*

Inhibin bioassays based on the reversed Steelman-Pohley design have come in for criticism on grounds of lack of specificity and reproducibility (Baker *et al.,* 1981; Grady *et al.,* 1982). The coupled assay design has been shown to be specific as it is able to discriminate between true ILA and other interfering activities.

Based on the experience in this laboratory, several features were found to be important for the reproducibility of the assay. Proper care in the maintenance of the mouse colony and control over the litter size and weight were crucial to reproducibility. In addition reference to figure 9 would enable an appreciation of the importance in this context of the temporal span of the assay. A preparation that showed highly significant dose-dependent suppression of uterine weight gain in the 24 h assay design turned out to have no significant effect when assessed by the 3 day injection schedule as used by Setchell and Sirinathsinghji (1972). Thus by this one single modification a major source of variance could be avoided.

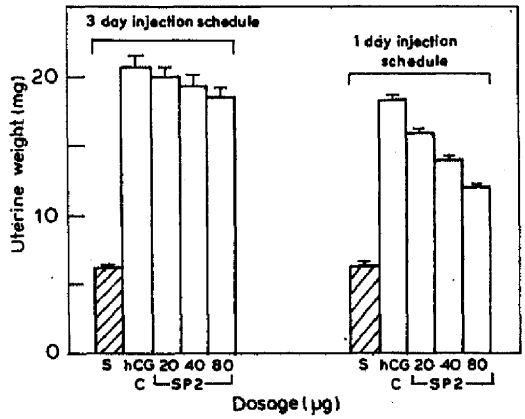


**Figure 9.** A comparison of the 4 day reversed Steelman–Pohley assay design for inhibin activity with the 24 h design used in this laboratory. Note that the preparation which gives a highly significant dose-dependent response in the 24 h schedule turns out to be marginally effective in the 4 day schedule.

The important shortcoming, namely, low sensitivity, is none too serious in a research laboratory, whereas it can be a handicap in the clinical laboratory.

Therefore for the purpose for which it is designed it would appear to be eminently suitable, even though it is not anticipated that the coupled assay will replace the classical pituitary cell culture assay when plasma samples have to be assayed for their inhibin activity.

## Acknowledgements

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