A STUDY OF THE IMMUNOLOGICAL DETERMINATION
OF PITUITARY GONADOTROPHIN IN URINE

By
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ABSTRACT

An antiserum to chorionic gonadotrophin (HCG) has been studied in agar diffusion, haemagglutination and haemagglutination inhibition experiments. The anti-HCG serum contained antibodies to inert urinary proteins and serum proteins as well as a hormone-specific antibody. Absorption of the anti-HCG serum with small amounts of inert urinary protein removed precipitins to non-hormonal protein, without altering the haemagglutinating titre of the antiserum. There was no difference between absorbed and unabsorbed anti-HCG serum in haemagglutination inhibition reactions with inert and gonadotrophic urinary extracts. When larger amounts of inert protein were used for absorption there was a fall in the haemagglutinating titre of the anti-serum but it was shown that this was due to removal of hormone specific antibodies. Urinary extracts were obtained from patients with various endocrine disorders, from patients with normal pituitary and gonadal function and from menopausal subjects. The results of bioassay and immunoassay of these extracts were compared. It was found that many of the extracts, although potent in the immunoassay, were biologically inactive. It is concluded that a specific or non-specific reaction can occur between non-hormonal urinary proteins and the antihormone. The anti-HCG serum used in the present investigation, although successfully used for pregnancy diagnosis and HCG assay, was unsuitable for urinary gonadotrophin estimation in non-pregnant individuals.

The discovery of the antigenic nature of human chorionic gonadotrophin (HCG) led to the development of several immunological methods for pregnancy diagnosis and HCG assay. These included precipitation (McKean 1960), haemagglutination inhibition (Wide & Gemzell 1960) and complement fixation.
(Brody & Carlström 1961). The immunological assay of gonadotrophins of pituitary origin is not so well established. Wide et al. (1961) noted that concentrated urine from non-pregnant individuals inhibited the reaction between anti-HCG serum and HCG sensitised erythrocytes. They ascribed this result to a cross-reaction between pituitary luteinising hormone (LH) and anti-HCG. An antigenic relationship between HCG and LH might be expected in view of their biological similarity and in fact the authors used this cross reaction for the quantitative assay of LH in urine (Wide & Gemzell 1962). An anti-HCG serum, similar to that used for LH assay by Wide & Gemzell (1962), has been successfully used in this laboratory for HCG estimation. This paper is concerned with the use of this antiserum for the immunological assay of gonadotrophin in urinary extracts from non-pregnant individuals. The results are compared with those obtained by routine bioassay.

MATERIALS AND METHODS

Urine extraction

Twenty four or 48 hour urine collections were obtained from patients with a variety of endocrine disorders, from post-menopausal subjects and from a group with normal pituitary and gonadal function.

Each urine was extracted by the kaolin-acetone procedure described by Loraine & Brown (1959). To avoid toxic effects, further purification with 10% ammonium acetate/ethanol (59:41) was carried out if a low gonadotrophin excretion was anticipated. Each extract was homogenised in saline and after centrifuging, the supernatant fluid was examined by immunoassay and bioassay as described below.

Inert urinary protein (IUP) was extracted from the urine of a hypophysectomised patient and purified by the procedure above. Gonadotrophins could not be detected in this extract even when the equivalent of a 12 hour urine sample was injected into each test animal.

Production of antiserum

A commercial preparation of HCG (Pregnyl) with an approximate activity of 2000 IU/mg was obtained from Organon Laboratories Ltd. Antiserum to this HCG was produced in rabbits by the method described by Wide & Gemzell (1960). After 2 to 3 months' immunisation, antibody titres by haemagglutination reaction reached an average of 16 000.

Absorption of antiserum

This was carried out by incubating the anti-HCG serum either with an equal volume of IUP in saline (5 mg/ml) at 37°C overnight or with 20 mg IUP/ml for 3 days. In a further series of experiments absorption was carried out with pooled normal human sera (NHS) and with menopausal gonadotrophin (HMG). The absorbed sera were centrifuged before comparing them with the unabsorbed antiserum (treated in the same way but only with saline) in agar gel diffusion studies and haemagglutination reactions.
Agar gel diffusion studies

These were performed by the technique described by Ouchterlony (1958). Absorbed or unabsorbed anti-HCG sera were placed in the central agar cups and HCG (3000 IU/ml), IUP (5 mg/ml), HMG (5 mg/ml) and NHS peripherally. The plates were inspected at 72 hours for the presence of precipitin lines.

Immunoassay procedures

The haemagglutination of HCG sensitised erythrocytes by absorbed and unabsorbed antisera was investigated using the method of Wide & Gemzell (1960). Perspex agglutination trays (MRC pattern) rather than tubes were used.

Each urinary extract was tested for its ability to inhibit a 1/6000 dilution of anti-HCG serum. The inhibition produced by the urinary extract was compared with that produced by a standard amount of HCG and the approximate potency of the extract in terms of IU HCG per 24 hour urine calculated (Wide & Gemzell 1960). The biological activity of the urinary extracts was measured in terms of mg equivalents of the international reference preparation for menopausal gonadotrophin (IRP-HMG) per 24 hour urine. To determine the immunological potency of HCG in terms of IRP-HMG the quantitative assay of Wide (1962) was employed. (Eight IU HCG were equivalent to 1 mm HMG-IRP in this assay). It was then possible to express the results of the immunoassay as mg equivalents IRP-HMG per 24 hour urine.

Biological assay

The gonadotrophin content of the urinary extracts was assayed by the mouse uterine weight method described by Loraine & Brown (1959). Most of the assays were of a 3 point design using a substandard assayed against IRP-HMG. A number of 4 point assays were carried out and in these there was no significant deviation from parallelism between the standard and unknown dose-responses. The results of the bioassays were expressed as mg equivalents IRP-HMG per 24 hour urine.

RESULTS

Agar gel diffusion studies

Several precipitin lines (arcs) developed between anti-HCG serum and HCG (Fig. 1). An almost identical pattern was observed between anti-HCG and IUP. More lines developed between these two reactions than between anti-HCG and HMG or between anti-HCG and NHS. Because there were so many antigenic components in the peripheral cups, it was impossible to attribute »hormone specificity« to any of the lines between anti-HCG and HCG. This could only be done when many of the antibodies to inert proteins present in the HCG extract were removed by absorption procedures. The results of the diffusion experiments with absorbed sera are shown in Table 1.

By employing the »reaction of identity« to the precipitin lines formed between absorbed anti-HCG serum and HCG, an »HCG specific« line was demonstrated. Absorption with NHS removed serum protein but not all inert urinary protein antibodies from anti-HCG. HMG removed serum proteins and
Interaction of anti-HCG (centre well) with HCG, IUP, HMG and NHS in diffusion experiments in agar gel.

Table 1.
Number of precipitin lines formed by absorbed anti-HCG serum in agar diffusion experiments.

<table>
<thead>
<tr>
<th>Anti-HCG serum absorbed with:</th>
<th>Peripheral cups containing:</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>HCG</td>
</tr>
<tr>
<td>HMG (5 mg/ml)</td>
<td>2</td>
</tr>
<tr>
<td>IUP (5 mg/ml)</td>
<td>1</td>
</tr>
<tr>
<td>NHS (equal vol)</td>
<td>2</td>
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</tbody>
</table>

most of the urinary protein antibodies. IUP, on the other hand, was most effective since it removed all precipitins to contaminating non-hormonal proteins.

Haemagglutination reactions of absorbed sera

The haemagglutination titre of anti-HCG serum was unaffected by absorption with IUP (5 mg/ml), HMG (5 mg/ml) or NHS. Furthermore, inhibition reactions with HCG and several urinary extracts (including some which were inert) gave the same results with both absorbed and unabsorbed anti-HCG
sera. Therefore, although the specificity of the anti-HCG serum was increased by absorption in the gel diffusion experiments, the presence of inert protein antibodies in the unabsorbed serum did not apparently interfere with the haemagglutination inhibition reaction. Absorption of anti-HCG serum prior to use in the immuno-assay was therefore considered unnecessary.

When IUP was used for absorbing anti-HCG serum at a concentration of 20 mg per ml saline (equal volume), there was a fall in the haemagglutinating titre of the antisera from 8000 to 1000. (There was also less visible precipitate formed in agar between this absorbed antisera and HCG.) However, when haemagglutination inhibition reactions with the same dilution of absorbed anti-HCG serum were compared, a standard amount of HCG was eight times more potent in the inhibition of the absorbed anti-HCG sera. Therefore, the use of excessive amounts of IUP for absorption of anti-HCG serum not only removed antibodies to inert protein but also reduced the titre of »hormone-specific« antibodies.

Correlation of biological and immunological results

The 24 hour excretion of gonadotrophins measured biologically is compared with the results of immunoassay in Figs. 2 and 3. The most significant finding was that many biologically inert urinary extracts were active in inhibition reactions with anti-HCG serum. On the other hand, a few extracts containing

Fig 2.
Comparison of biological and immunological results (expressed as mg equivalents HMG-IRP per 24 hours) for kaolin-acetone extracts of urine. Gonadotrophin was not detected on the occasions indicated by arrows.
Comparison of biological and immunological results for purified urinary extracts. Gonadotrophin was not detected by either method in 16 extracts (<3 mg equivalents HMG-IRP/24 h).

gonadotrophin were inactive in the immunoassay. In 16 extracts, gonadotrophin could not be detected by either method. The random scatter of results in the remaining assays showed that there was no correlation between immunoassay and bioassay.

**DISCUSSION**

A haemagglutination inhibition reaction for the determination of urinary HCG levels was described by Wide & Gemzell (1960). Subsequently it was found that the reaction between HCG-coated cells and rabbit anti-HCG sera was inhibited by concentrates of human hypophyses or of urine from non-pregnant women, provided they contained LH (Wide et al. 1961). This was confirmed by Butt et al. (1961) for pituitary LH. Most of the evidence in the literature supports the hypothesis that HCG and LH are related antigens.

Goss & Lewis (1964) demonstrated by micro-gel diffusion techniques that purified preparations of pituitary LH and HCG had an antigen in common and similar conclusions were reached by Shahani & Rao (1964) using immunoelectrophoresis. Butt et al. (1960) produced antisera to relatively crude menopausal gonadotrophin (HMG) and showed that it inhibited the biological ac-
tivity of HCG. Furthermore, Moudgal & Li (1961) found that a human pituitary LH antiserum inhibited HCG and this was confirmed by Wide (1962) and by Goss & Lewis (1964). On the other hand, anti-HMG serum had no effect on the biological activity of HCG in the experiments of Moritz & Illei (1963) while Lunenfeld et al. (1962) proved that anti-HMG serum neutralised only the activity of HMG in a mixture of HMG and HCG. Although many of these authors demonstrated that serum prepared against HCG inactivated either HMG or pituitary LH, it can be shown that the small amounts of pituitary gonadotrophin present as a contaminant of HCG can also give rise to antigonadotrophin (Hipkin 1961) and therefore these results lose much of their significance. Since most of the evidence is in favour of an antigenic relationship between HCG and LH, there have been several attempts to assay LH in pituitary and urinary extracts using anti-HCG serum. These attempts have not always been successful.

There seems little doubt that LH obtained from human pituitaries can be assayed immunologically. Wide et al. (1961) found good agreement between the results of immunological and biological determinations of LH in concentrates of human hypophyses. These results were confirmed with selected anti-HCG sera by Butt et al. (1964) and using latex-HCG agglutination inhibition by Goss & Taymor (1962). Such consistent findings have not been obtained when the method has been applied to the assay of LH in urine.

Wide et al. (1961) found that urinary LH could be detected and estimated by an immunological method. Furthermore, changes in urinary gonadotrophin excretion throughout the menstrual cycle measured immunologically were of the same general order as those obtained using biological methods (Wide & Gemzell 1962). On the other hand, Goss & Taymor (1962) did not obtain close agreement between the results of bioassay and immunoassay of urinary LH. Butt et al. (1964) found reasonable agreement between the results of bioassay and immunoassay except for urinary extracts from young women in whom the immunological results were considerably higher than the biological. The greatest differences occurred with extracts of lowest potency. It is clear that further research into the immunological assay of urinary gonadotrophin is required.

In the present investigation, routine gonadotrophin estimations using the mouse uterus test have been carried out on patients with various endocrine disorders. Although the uterine weight method is alleged to measure the combined action of both follicle stimulating and luteinising hormones, the results using this procedure agree closely with those for LH alone (Lorraine 1958; van Hell et al. 1964). For this reason and for its reliability and practicability, the uterine weight assay was chosen for the present work.

In a high proportion of patients, gonadotrophins could not be detected by bioassay. However, when these urinary extracts were assayed immunologically,
the excretion levels were similar to those obtained with extracts from normal or menopausal subjects. The anti-HCG serum used in the present investigation is obviously unsuitable for use in urinary LH assays.

One possible explanation for the results reported here and by other workers is that inert urinary proteins, present in the gonadotrophic extracts, may react with non-hormonal or even with »hormone specific« antibodies in the anti-HCG serum. To investigate this problem absorption studies were carried out.

In gel diffusion experiments several antigenic components could be identified in the HCG preparation. These consisted of inert urinary proteins and serum proteins as well as the hormone. Absorption of the anti-HCG serum with inert urinary protein (IUP) removed all precipitins except »hormone-specific« antibodies. Similar results were reported by Midgley et al. (1961) using a male urine extract for absorption. Although the specificity of the anti-HCG serum used in the present investigation was improved by absorption with IUP, there was no fall in the haemagglutination titre of the antiserum. There was no difference between absorbed and unabsorbed serum in the immunoassay. When larger amounts of IUP were used for absorption there was a fall in the haemagglutination titre of the antiserum but this was accompanied by a loss of »hormone specific« antibodies. It appears from these results that »hormone-specific« antibodies in the anti-HCG serum either react with inert urinary protein or are absorbed during non-specific antigen-antibody formation. In either case urinary LH immunoassay is invalidated.

Biological activity does not appear to be necessary for the antigen specificity of HCG. Reduction of the biological activity of HCG by heating does not reduce its immunological activity (Wide 1962; Connon 1964). Furthermore, when levels of HCG are estimated immunologically throughout pregnancy, much of it appears to be in a biologically inactive form (Wide 1962; Fulthorpe et al. 1963). The problem of whether inert urinary antigens can react with »hormone-specific« antibodies or not will only be resolved when immunologically pure preparations of HCG are available.

Although an anti-HCG serum gives accurate results for HCG and pituitary LH assay (in these cases interfering antigens are in relatively small amounts or even absent), and appears to be »hormone specific« on agar diffusion testing, it does not necessarily follow that the same antiserum is suitable for urinary LH assay.

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