EFFECT OF TOXIC URINARY EXTRACTS ON SPECIFIC ASSAYS FOR HUMAN CHORIONIC GONADOTROPHIN AND FOLLICLE-STIMULATING HORMONE

BY

L. J. Hipkin

ABSTRACT

Boiled urinary extracts, which had inhibited the activity of human chorionic gonadotrophin (HCG) in the mouse uterus assay, potentiated the effects of HCG and follicle-stimulating hormone (FSH) in the ovarian ascorbic acid depletion and rat ovarian augmentation assays respectively. This treatment inhibited responses to FSH when the ovarian augmentation assay was conducted in hypophysectomised animals. A toxic urinary extract did not affect the activity of HCG in the ventral prostate weight assay in either intact or hypophysectomised rats. Reasons for the different effects of boiled urinary extracts in these assays are discussed.

A boiled kaolin-alcohol extract of urine inhibited the activity of human chorionic gonadotrophin (HCG) in the immature mouse and rat uterine weight assays (Futterweit et al. 1963; Soffer et al. 1966). The presence of an HCG or lutenising hormone (LH) antagonist in urine was supported by the observation that LH activity was also suppressed by urinary extracts in the ventral prostate weight assay (VPW) in intact and hypophysectomised animals (Futterweit et al. 1963) and in the ovarian ascorbic acid depletion (OAAD) assay (Ota et al. 1967). Results in the mouse uterus assay were confirmed using boiled kaolin-acetone and tannic acid extracts of urine (Hipkin 1968a,b). On the other hand, these extracts potentiated responses to HCG in the rat uterus assay (Hipkin 1970). It was concluded that the urinary extracts were toxic and that the experimental animals responded to the stress of the injection by...
an alteration in endogenous gonadotrophin secretion. In view of the non-
specificity of uterine weight assays, the present report is concerned with the
effects of urinary extracts in specific assays for HCG and follicle-stimulating
hormone (FSH), namely the OAAD, the VPW and the rat ovarian augmen-
tation (OA) assays. The study was undertaken partly because of the contro-
versial nature of the urinary gonadotrophin inhibitor (a term abandoned for
this report because of its augmentation effects in certain assays), but also to
determine the effect of toxic extracts when the OAAD, the VPW and the OA
methods are used for gonadotrophin assay.

MATERIALS AND METHODS

a) Ovarian ascorbic acid depletion assay

Animals

Immature, female rats of the Wistar strain were obtained from Messrs. A. Tuck,
Rayleigh, Essex. They were 21- and 25-day-old and weighed 35-45 g.

Materials

Pregnant mares' serum gonadotrophin (PMS) and HCG were supplied by Organon
Laboratories (Gestyl® and Pregnyl® respectively).

Pooled urine from non-pregnant women and from male patients was extracted by
the kaolin-acetone procedure of Loraine & Brown (1959). The extracts were suspended
in saline (pH = 6) and boiled for 1 hour under reflux to destroy gonadotrophic ac-
tivity. Fifty ml urine equivalents of the boiled extract was inactive in the mouse
uterus assay.

Experimental procedure

The OAAD test was performed exactly as described by Bell et al. (1965). Each
animal was primed with 50 IU PMS followed 72 h later by 25 IU HCG. Both
gonadotrophins were administered subcutaneously in 0.5 ml distilled water. Six days
after the priming dose of HCG, the rats were divided into three groups containing
eight animals. These were injected intraperitoneally with either 0.5 ml saline, 0.5 IU
HCG or 1.5 IU HCG (both doses in 0.5 ml saline). Half the animals in each group
were then given an intraperitoneal injection of 0.5 ml saline while the remainder
were similarly treated with the boiled urinary extract, in a dose equivalent to 100 ml
urine. The intraperitoneal administration of the test materials was preferred because
of the toxic nature of the urinary extract. Even by this route, increasing the dose to
200 ml urine equivalents was associated with the death of some animals. The re-
mainder of the procedure was that used by Bell et al. (1965). The results were statis-
tically analysed by the method of Gaddum (1953).

b) Ventral prostate weight assay

Animals

Immature rats of the Wistar strain, from the colony of the Statens Seruminstitut,
were used when they were aged 20 to 22 days old and weighed 35 to 45 g. Hypophysectomies were performed by the parapharyngeal route. The experimental groups contained from 6 to 12 animals.

**Materials**

HCG (Physex®, Leo) was dissolved in distilled water. The total doses used in hypophysectomised rats were 0.4, 1.6 and 6.4 IU while in intact animals they ranged from 0.4 to 3.2 IU.

The urinary material was obtained by pooling the residues of gonadotrophic extracts (Johnsen 1958) that had been used in routine assays. Small volumes (about 200 ml) were boiled for 1 hour to destroy gonadotrophic activity. The boiling also concentrated the material to near the required volume for the tests, but since the extracts had originally been dissolved in borate buffer (pH = 9.2), dialysis against an appropriate volume of distilled water was then necessary to restore the initial buffer concentration. The tannic acid extract being protein in nature would not be affected by the dialysis (Hipkin 1969a). After the solutions were pooled, a final adjustment in concentration was made by boiling for up to 15 min. The total dose given to each animal was equivalent to a 48 hour urine.

**Experimental procedure**

The VPW method was performed as described by Christiansen (1967). Injections were commenced on the 2nd day after hypophysectomy and were given daily, subcutaneously for 6 days. Animals were given HCG or water (0.5 ml) and either the urinary extract or borate buffer (0.25 ml). Because of the toxicity of the urinary extract, animals so treated were given the support of 200 µg cortisone acetate (Cortone®, Merck, Sharp and Dohme) in 0.1 ml water. All injections were at different sites. On the 7th day the animals were killed and weighed. The ventral lobes of the prostates and the adrenals (except in those given cortisone) were weighed and the sella turcica was inspected under a dissecting microscope. Animals with pituitary remnants were ignored. Results from the buffer and urinary extract treated groups were compared using the Student's t test.

c) **Rat ovarian augmentation assay**

**Animals**

Female Wistar rats for this assay were also obtained from the colony of the Statens Serum Institut. They were 26 to 28 days old and initially weighed 48–55 g. Responses were obtained in groups of intact and hypophysectomised animals, each group containing at least 6 animals.

**Materials**

FSH from the pituitary glands of domestic animals (FSH-P®, Armour) was dissolved in distilled water. The total doses ranged from 50 µg to 800 µg equivalents of the Armour standard. The doses have been given in terms of the Armour standard since the preparation used and its unitage are similar to those employed in the original Steelman & Pohley (1953) assay. Doses can be converted to the NIH-FSH-S1 standard since 1 mg of this material is equivalent to 550 µg of the Armour standard.
Each dose of FSH was mixed with 20 IU HCG (Physex®, Leo) in a total volume of 3 ml.

The urinary material was extracted from the urine of male and non-pregnant female patients by the kaolin-acetone method of Borth et al. (1961). The extracts were pooled, suspended in distilled water and boiled at pH 6 as before. A total dose of 800 ml urine equivalents was used for each animal. The uterine weight method of Johnsen (1958) failed to detect gonadotrophins in the boiled extract (< 3 MUU/l).

Experimental procedure

The rat ovarian augmentation assay was performed as described by Christiansen (in press). FSH was administered subcutaneously (0.5 ml), one injection being given on days 1 and 4 and two on days 2 and 3. Some groups of animals were only treated with 20 IU HCG. The boiled extract was also given daily (0.2 ml) at the same time as the first or only injection of FSH but at a different subcutaneous site. In hypophysectomised animals, treatment was started immediately after the operation. In this series, cortisone was necessary as for the VPW experiments. On the 5th day the animals were killed and weighed, and the paired ovarian weight obtained. The sella turcica from operated animals was examined and results from the groups were compared as in the VPW assay.

RESULTS

The effect of the boiled urinary extract on the depletion of ovarian ascorbic acid in response to HCG is shown in Fig. 1. Treatment with the extract and HCG induced a greater fall in ovarian ascorbic acid concentration than did HCG and saline. The potency of HCG in animals treated with the urinary extract was 1.76 (1.03–3.66) times greater than in those treated with saline (2 = 0.3). However, the boiled urinary extract itself caused a significant depletion of ovarian ascorbic acid (P < 0.001) and this was obviously the reason for its potentiation of HCG activity.

The effect of a boiled Johnsen (1958) extract (48 h urine equivalent/rat) in the VPW assay in intact rats is shown in Fig. 2. The basal ventral prostate weight and the responses to HCG were not significantly different from those in buffer treated controls. The same conclusion was reached when the assay was conducted in hypophysectomised animals (Fig. 3). There was no significant difference in mean final body weight between buffer and extract treated animals in either the intact (49.2 ± 2.6 g and 48.5 ± 1.9 g respectively) or hypophysectomised groups (38.1 ± 1.7 g and 35.1 ± 0.9 g respectively). However, the material was toxic judged by the significantly higher (P < 0.001) adrenal weight resulting from injections of extract into intact animals. The mean weight of both glands in those given buffer was 12.3 ± 0.5 mg compared with 15.5 ± 0.5 mg for those treated with urinary material. The completeness of hypophysectomy, at least in the buffer group was supported by a mean adrenal weight of 5.5 ± 0.2 mg.
The effect of the urinary extract on FSH activity in the ovarian augmentation assay is compared with water treated controls in Fig. 4. The urinary extract did not affect responses to 20 IU HCG given alone or to 400 µg or 800 µg equivalents (Armour standard) FSH. On the other hand, doses of 100 µg and 200 µg equivalents were significantly augmented when rats were given the urinary material (P = 0.05 and P < 0.005 respectively). This occurred in spite of the mean final body weight of the extract treated groups (53.3 ± 1.4 g) being significantly less (P < 0.05) than that from animals treated with water (57.7 ± 1.3 g). The response to 50 µg equivalents FSH (57.3 ± 6.0 mg) was no different from that obtained using only HCG and was therefore not plotted. The results of experiments in hypophysectomised animals were completely different (Fig. 5). The urinary extract inhibited responses to HCG given alone (P < 0.025), and to 200 µg, 400 µg and 800 µg equivalents FSH (P < 0.005, P = 0.05 and P < 0.05 respectively). In the hypophysectomised series there was no difference in mean final body weight between the extract (48.0 ± 1.6 g) and water treated groups (48.5 ± 0.7 g).
VENTRAL PROSTATE

Fig. 2.
Prostatic weight responses to HCG in intact rats treated with urinary extract (---) or buffer (----). se of means indicated by vertical lines.

DISCUSSION

Boiled kaolin-acetone and tannic acid extracts of urine have previously been shown to inhibit HCG activity in the uterus assay in mice (Hipkin 1968a,b) while responses in the rat uterus assay were augmented (Hipkin 1970). In the present investigation, boiled urinary material also augmented responses to HCG in the OAAD assay but was without effect on HCG activity in the VPW assay in intact and hypophysectomised rats. FSH was augmented in the OA assay unless the animals were hypophysectomised when urinary extracts inhibited responses.

One problem has been to decide whether or not augmented gonadotrophin responses are caused by residual activity in the boiled extracts. Bell (1967) has shown that up to 10% of the original activity may be retained after boiling gonadotrophin preparations for 1 hour. On the other hand, Hamburger & Johnsen (1957) completely inactivated a urinary pituitary gonadotrophin.
solution (pH 8.9) by heating at 80°C for 1 hour. Boiling for 1 hour is also recommended for the preparation of the urinary gonadotrophin inhibitor from urinary gonadotrophic extracts (Futterweit et al. 1963). In the present study care was taken that HCG from pregnancy urine did not contaminate the extracts. Furthermore, two of the boiled extracts were devoid of gonadotrophic activity when tested in the mouse uterus assay, while the third was inactive in the VPW assay.

Although different extracts and different doses have been employed in the above studies, they have had in common a toxic effect on the experimental animals used. In the uterine weight assays (Hipkin 1968a, 1970), and in the OA test in intact animals reported here, animals did not gain weight normally. In the OAAD assay, the treated rats became lethargic and exhibited piloerection while intact animals in the VPW assay had increased adrenocortical function judged by the significantly higher adrenal weight in these animals. It should be pointed out that although urinary gonadotrophic extracts

Fig. 3.
Prostatic weight responses to HCG in hypophysectomised rats treated with urinary extract (---) or buffer (——). se of means indicated by vertical lines.
may contain corticotrophin, the amounts are very low (Hamburger & Johnsen 1957) and 12 h urine equivalents of Johnsen (1958) extract has no adrenal weight stimulating activity in hypophysectomised animals (Christiansen, personal communication). It is the toxicity of the various urinary extracts which is thought to be responsible for their gonadotrophin-inhibitory activity in the mouse uterus assay (Hipkin 1969a). There seemed little advantage in purifying the extracts since fractionation of urinary material using gel filtration yielded several fractions, all with toxic and gonadotrophin-inhibitory properties (Davis et al. 1966).

When animals are stressed by the injection of toxic urinary extracts or by other injurious procedures, gonadotrophin assay may be affected in at least two ways. Firstly, endogenous gonadotrophin secretion may be altered (Hipkin 1969b, 1970) and secondly, the growth of gonadotrophin stimulated tissues may be suppressed as part of a generalized interference with growth (Davis et al. 1966; Hipkin 1966; McArthur et al. 1967a). The effect of toxic extracts
on gonadotrophin assay will therefore depend on whether growth is impaired, on the sensitivity of the assay procedure and on the functional state of the animal's pituitary with respect to gonadotrophins. A further problem is that hypophysectomised animals may not gain weight in short term experiments and this makes the assessment of the toxicity of extracts in such animals difficult (McArthur et al. 1967b). However, presumably the same dose of extract inhibiting growth in intact rats will also inhibit gonadotrophin responses in hypophysectomized animals. These factors may account for some of the differences between assays in the effects of toxic extracts in this and other investigations.

**Ovarian ascorbic acid depletion assay**

In the rat uterus assay, the boiled urinary extract stimulated some uterine growth when given alone, and for this reason it potentiated HCG activity in this assay (Hipkin 1970). An identical situation exists in the OAAD assay reported here since the extract itself produced significant ovarian ascorbic acid depletion. As pointed out earlier, it is most unlikely that the ovarian ascorbic acid depletion results from residual gonadotrophin in the boiled extract. Even if 10% of the activity remained, it is improbable that the response obtained with 100 ml urine equivalents of heated extract would be reproduced by 10 ml equivalents of unboiled extract containing only pituitary gonadotrophin. Fur-
thermore, 200 ml urine equivalents of boiled extract produced the same percentage fall in ovarian ascorbic acid concentration as did 100 ml equivalents. In any case, the results of the present investigation support the finding of Koed & Hamburger (1968). They noted that urinary extracts from children and hypophysectomised patients produced a significant response in the OAAD assay, although proven to be devoid of gonadotrophic activity by other methods. The authors concluded that inert urinary proteins deplete ovarian ascorbic acid non-specifically and that the effect was similar to that produced by a heterogeneous group of substances reviewed by Gibson et al. (1965). Of the materials listed, it is interesting to note that adrenaline and serotonin (Hipkin 1969b), pitressin (Hipkin 1969a), arginine vasotocin (Pavel & Petrescu 1966) and propylene glycol (unpublished observations) all inhibit gonadotrophic activity in the mouse uterus assay.

Although it is possible that the ovarian ascorbic acid depleting activity of the urinary extract results from a direct toxic effect on the ovary, a more likely explanation is that it causes secretion of endogenous luteinising hormone as part of a stress reaction. This hypothesis is based on the similarity between the results obtained in this investigation and those from the rat uterus assay (Hipkin 1970). Apart from the toxic urinary extract, such stressing procedures as starvation and carbon tetrachloride injection also stimulated uterine growth in the rat. Stressful procedures cause non-specific inhibition of HCG activity in the mouse uterus assay (Hipkin 1969b). This would explain why the materials listed above produce different effects depending on whether the mouse uterus assay or OAAD assay is used. The effect of the toxic extract on the rat uterus assay was studied using intact and hypophysectomised animals (Hipkin 1970). To prove that the extract causes endogenous LH secretion in the OAAD assay, ideally the same approach should have been used. This was not attempted since Guillemin & Sakiz (1962) have shown that hypophysectomy causes a marked fall in ovarian ascorbic acid, and the depleting effect of exogenous LH is lost.

When the OAAD assay was investigated for its routine clinical usefulness, there was a low degree of precision and a proportion of assays were invalid because of lack of parallelism and the toxicity of the urinary extracts employed (Schmidt-Elmendorff & Loraine 1962; Hutchinson & Worden 1964). Even the standard, the second IRP-HMG, is toxic and produces a flat dose-response curve at certain dose levels (Rosemberg 1967). These results can be explained on the basis of a non-specific release of endogenous LH. If the toxic effects of the urinary extracts can be reduced by altering the route of administration, then this should improve the OAAD assay. This is the basis of a modified test described by Mondina & Polvani (1970).

The demonstration of a urinary gonadotrophin inhibitor reported by Ota et al. (1967) was not confirmed.
Ventral prostate weight assay

Two problems arise from the failure of the urinary extract to influence HCG activity in the VPW assays in the present investigation. Firstly, why do the results differ from those of Futterweit et al. (1963) who reported that a urinary extract inhibited gonadotrophic activity in VPW assays and secondly, why is there no augmentation of HCG activity in the VPW assay in intact rats? The former question was also posed by Hahn & Albert (1965) following the failure of their extract to inhibit LH and urinary pituitary gonadotrophin in the VPW assay in hypophysectomised animals.

An important difference between the results of Futterweit et al. (1963) and those reported here is that in the former, growth was retarded in intact rats treated with urinary extract. As pointed out earlier, this would not only account for the gonadotrophin inhibition in intact rats, but also for that in hypophysectomised animals.

Failure of the urinary extract to augment HCG activity by increasing endogenous gonadotrophin secretion is certainly not due to inadequate dosage. Augmentation of HCG activity was noted with a dose approximately equivalent to a 24 h urine (1.5 l) in the rat uterus assay (Hipkin 1970). In fact, the dose chosen for the present investigation was double that used in the earlier study because in the VPW assay, 6 rather than 3 daily injections are given. As pointed out earlier, this dose was toxic judged by the adrenal weight stimulation in the test animals. The most likely explanation for the failure of the extract to augment HCG activity is that the VPW assay is relatively insensitive. In the uterine weight assay a dose of 0.2 IU HCG produced a significant response (Hipkin 1970), while in the VPW method a dose between 0.4 and 0.8 IU is necessary (Fig. 2). It would therefore be possible for increased endogenous gonadotrophin secretion to escape detection in the VPW assay.

Rat ovarian augmentation assay

Christiansen (in press) has modified the original Steelman & Pohley (1953) assay so that 26 to 28 day-old (compared with 21 to 22-day-old) rats are used, and animals are given 6 injections in 4 days instead of 3 injections in 3 days. For routine assays, Steelman & Pohley (1953) recommended that responses should fall between those produced by 100 µg and 200 µg Armour standard. The effect of the modification suggested by Christiansen is to increase the working range to include responses up to 400 µg equivalents (Fig. 4).

In addition to the enhanced endogenous LH secretion produced by toxic extracts referred to earlier, the results in the OA assay suggest that endogenous FSH secretion is also increased (Fig. 4). The augmentation of HCG activity by stressful procedures in the rat uterine weight assay (Hipkin 1970) is therefore due to the secretion of both gonadotrophins.

The effect of the toxic urinary extract in the present investigation differs
from the findings of McArthur et al. (1967a,b). Using the Steelman & Pohley (1953) assay, they noted that extracts inhibited the FSH activity of menopausal gonadotrophin if they were sufficiently toxic to interfere with normal body weight gain. A similar conclusion was reached when the ovarian augmentation assay was conducted in mice (Hipkin 1969a). As pointed out earlier, one difference between the Steelman & Pohley (1953) assay and the modification described by Christiansen (in press) is the greater maturity of the animals in the latter method. The pituitaries of such rats would be expected to contain greater amounts of FSH and this probably accounts for the different effects in the two assays. However, when the OA assay is conducted in hypophysectomised animals this source of gonadotrophin is removed, and the responses are inhibited by the toxic extract (Fig. 5) through its effect on tissue growth. This result is then similar to that of McArthur et al. (1967a) in the younger immature, intact rats they used, and also agrees with findings in the uterine augmentation assay in hypophysectomised mice (Stevens 1967).

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