2. Deacetylcephalosporin C has been isolated as a crystalline sodium salt. It resembles cephalosporin C in a number of its properties, including its ultraviolet-absorption spectrum, but is more readily converted into the lactone, cephalosporin C₆, in acid solution.

3. Deacetylcephalosporin C shows about 20 % of the activity of cephalosporin C against *Staphylococcus aureus* (Oxford strain) and *Salmonella typhi*. It is highly resistant to hydrolysis by purified preparations of penicillinase from *Bacillus cereus* strains 569/H and 5/B.

4. Deacetylcephalosporin C and cephalosporin C may be distinguished by paper chromatography.

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Studies on Human Urinary and Pituitary Gonadotrophins

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The interest in human gonadotrophic hormones derives from the necessity for their accurate measurement in body fluids and from the prospect of their therapeutic usefulness. The separation of these hormones from urine and from pituitary glands and the comparison of certain of their biological, physical and immunological properties form the subjects of this paper.

METHODS

Preparation of gonadotrophins

From urine. Urinary gonadotrophins have been prepared by two different methods.

(i) Pooled urine of postmenopausal women was extracted by the benzoic acid-tungstic acid method described by

Butt (1958). Material soluble in water was adsorbed on diethylaminoethylcellulose and was then eluted in 0.5M-ammonium acetate. After precipitation from 3 vol. of ethanol the material was redissolved in water and fractionated by chromatography on a column of diethylaminoethylcellulose. Material eluted in 0.2M-ammonium acetate was collected and reprecipitated from 3 vol. of ethanol, washed with ethanol and ether and dried (Butt, Crooke & Cunningham, 1959).

(ii) A generous supply of urinary gonadotrophin from postmenopausal women (HMG-J5) was given to us by Dr S. G. Johnsen (Statens Seruminstitut, Copenhagen). It had been prepared by Johnsen's (1955) method and was further fractionated by chromatography on a column of calcium phosphate by the procedure described by Butt, Ingram & Round (1956), except that the calcium phosphate was prepared by a method described by Main, Wilkins & Cole (1959). Calcium phosphate was precipitated by

adding 100 ml. of 0.5M-calcium chloride solution dropwise with stirring to 120 ml. of 0.5M-phosphate buffer, pH 6.7. Stirring was continued for 1 hr. and then the precipitate was washed by decantation with water. Water (600 ml.) was then added and the mixture was transferred to a large conical flask. A few drops of phenolphthalein were added and sufficient ammonia (sp.gr. 0.88) to turn the indicator pink. The suspension was boiled for 30 min., more ammonia being added if necessary to maintain the pink colour. Finally the precipitate was thoroughly washed with water and suspended in 50 ml. of 0.01M-disodium phosphate.

From human pituitary glands. Three human pituitary-gland preparations were kindly supplied by Dr A. Korner (University of Cambridge, Department of Biochemistry). These were (i) an acetone-dried extract of pituitary glands, (ii) an extract of the powder in potassium chloride at pH 5.5, and (iii) a by-product from the process used for extracting growth hormone. This latter material, HGH.1/3, was prepared as previously described (Butt, Crooke, Cunningham & Evans, 1961).

The powders were subsequently treated in this Laboratory. The first steps were essentially the same as those described by Steelman, Segaloff & Andersen (1959). The acetone-dried powder was extracted in 10% ammonium acetate-ethanol (60:40, v/v) and then precipitated by addition of ethanol until the concentration was 80% (v/v). From this stage the subsequent steps were the same for all three preparations (Fig. 1). Amounts of 100–200 mg. were applied to columns (20 cm. $\times$ 1 cm.) of carboxymethylcellulose in 0.01M-ammonium acetate, pH 6.1. Fraction CM1 was not adsorbed under these conditions and fraction CM2, which was adsorbed, was eluted in 1M-ammonium acetate, pH 6.1. CM1 was further fractionated on a column of diethylaminoethylcellulose (10 cm. $\times$ 1 cm.) by the method described for urine by Butt *et al.* (1959). The fraction eluted in 0.2M-ammonium acetate was then put directly through a column of calcium phosphate (10 cm. $\times$ 1 cm.) and fractions eluted in 1 mM- and 0.02M-disodium phosphate were collected, dialysed and reprecipitated by adding 5 vol. of ethanol.

CM2 was refractionated on carboxymethylcellulose (20 cm. $\times$ 1 cm.) by stepwise elution in ammonium acetate

(0.01–1.0M). Gonadotrophin was detected only in the fraction eluted in 0.1M-ammonium acetate.

In all chromatographic work the elution of protein was detected by observing the ultraviolet absorption of the eluate at 280 m μ in a Unicam spectrophotometer SP. 500.

Ultracentrifugal studies

These were performed by Dr D. R. Stanworth and Miss Ratcliffe of the University of Birmingham, Department of Experimental Pathology. A Spinco analytical model E was used. The proteins were dissolved in barbitone buffer (pH 8.6, *I* 0.05; 0.2M-sodium chloride) and centrifuged at 60 000 rev./min. for 160 min.

Biological assay

Two assays with mice have been fully described previously (Butt *et al.* 1961). They are (a) the uterine-weight method, which is believed to measure the combined effect of follicle-stimulating hormone and interstitial-cell-stimulating hormone, and (b) the augmentation method, which is believed to be relatively specific for follicle-stimulating hormone.

Human chorionic gonadotrophin was measured by the method of Loraine (1950). This depends upon the increase in weight of the prostate gland in intact immature rats.

The results were calculated by the methods of Gaddum (1953) and Borth (1960). The standard used was the international reference preparation, HMG24. Relative potencies were calculated in terms of this standard with laboratory substandards calibrated against it. The ratio of the potencies of any preparation in terms of standard by two methods of assay has been termed the 'index of discrimination' (Gaddum, 1955). If this ratio is less than 2.0, or the 95% fiducial limits of error overlap, the preparation is not considered to differ qualitatively from the standard (Cunningham & Brown, 1958).

Immunological techniques

Production of antisera. Bentonite (British Drug Houses Ltd.) (approx. 1 g.) was suspended in 100 ml. of 0.9% sodium chloride solution. Coarse particles were allowed to

Fig. 1. Scheme for the chromatographic separation of pituitary-gland powders with carboxymethyl (CM)-cellulose and diethylaminoethyl (DEAE)-cellulose and calcium phosphate (CP).

settle and were then separated by decantation and discarded. Gonadotrophin was mixed with an equal weight of the suspended bentonite and was administered intravenously to rabbits (sandylops).

Amounts of 0.5 mg. of gonadotrophin in 2 ml. of suspension were given twice a week for 3 weeks or more. Rabbits were bled at intervals after the last injection and it was found that the highest activity in the antiserum occurred between 6 and 12 days afterwards. Subsequently therefore antiserum was withdrawn 7 days after the final injection as a routine. Usually satisfactory results were obtained after treatment for only 3 weeks.

Examination of the antisera. (1) Biological activity. Antiserum (0.025–0.4 ml.) was injected intraperitoneally into mice in two equal doses on the first and second days. Gonadotrophin (equivalent to 1–3 mg. of the standard) was injected subcutaneously in four equal doses, each in 0.5 ml., on the second and third days. On the fourth day the mice were killed and the uteri dissected and weighed. Control mice were given sodium chloride solution or serum which had been withdrawn from rabbits before immunization, instead of antiserum. This was followed by the same dose of gonadotrophin as was given to the experimental mice. The effect of antiserum on the activity of human chorionic gonadotrophin was examined by the rat prostate-gland method. The antiserum (0.025–0.4 ml.) was injected intraperitoneally on the first and second day and the human chorionic gonadotrophin (1.5 and 4.5 i.u.) subcutaneously on the second, third and fourth days, the total dose being in 1 ml. On the fifth day the prostate gland and surrounding structures were dissected and fixed in Bouin's fluid for 24 hr. Then the prostate gland was dissected and weighed.

(2) Agar-gel method. Solutions of 1% (w/v) agar (Ionagar, Oxo Ltd.) were prepared in several buffers. These were 0.1 M-sodium acetate in 0.9% sodium chloride, adjusted with acetic acid to pH 5.5 and pH 6.5, and phosphate buffer (0.1 M in 0.9% NaCl) at pH 7.6. Antigen and antibody were placed in wells made in the gel, of 5 mm. diameter, with the centres 8 mm. apart. Diffusion into the gel was allowed to proceed overnight, or longer in some experiments, at room temperature. If a permanent record of the experiment was required the gels were washed in a solution of 1% formalin in phosphate buffer (pH 7.6) for 20 min. and then in running water for several hours. Next they were stained in a 0.2% solution of chlorazol black (Gurr and Co. Ltd.) for about 30 min. and washed in water until the background was pale. The gel was then transferred to a glass slide and dried.

(3) Red-cell-haemagglutination technique. Sheep cells preserved in Alsever solution were obtained from Wellcome Research Laboratories Ltd. In early experiments the cells were tanned and coated with antigen exactly as described for human growth hormone (Read & Bryan, 1960), 100–500 μ g. of antigen being used for 6 ml. of red-cell suspension. In later experiments the modification of Ling (1960) was used. Red cells were washed three times with sodium chloride solution and were then treated with pyruvic aldehyde (25% solution, L. Light and Co. Ltd.). Reagent (1.6 vol.) was added to sodium chloride solution (3.0 vol.) and the pH was brought to approximately 7.0 with 10% sodium carbonate solution. Phosphate buffer (0.15 M, pH 8.0, 0.7 vol.) and red-cell suspension (1 vol. of 50% suspension) were then added to the mixture and stored at 4° for 2 days with occasional mixing. The cells were

thoroughly washed with sodium chloride solution and stored as a 10% suspension containing 0.1% of azide as preservative.

Antigen was then attached to the cells. A portion (2 ml.) of the 10% suspension of cells was centrifuged and the supernatant discarded. The cells were washed once with dilute buffer (sodium chloride–water–phosphate, 0.15 M, pH 6.0; 10:10:1, by vol.) and then suspended in the same buffer (1.5 ml.) containing 100–500 μ g. of antigen. After heating at 50° for 1 hr. the cells were centrifuged and washed three times with sodium chloride solution containing rabbit serum (0.5%) and finally were suspended in 10 ml. of sodium chloride solution containing rabbit serum (1%, v/v) and 0.1% of azide.

Antiserum was heated at 56° for 30 min. and was then absorbed with sheep red cells as described by Read & Bryan (1960). A series of dilutions in serum–sodium chloride solution (1%, v/v) was made and 0.2 ml. was pipetted into Perspex agglutination trays (R. B. Turner and Co. Ltd.). A portion (0.2 ml.) of serum–sodium chloride solution was added to each well, followed by two drops (0.05 ml. approx.) of red-cell suspension. After mixing the cells were covered and allowed to settle overnight. If no haemagglutination occurred the cells sank to the bottom of the well, forming a discrete button; if haemagglutination occurred the cells spread out evenly over the bottom of the well.

RESULTS

Urinary gonadotrophin

Human menopausal gonadotrophin prepared by the benzoic acid–tungstic acid method followed by fractionation on diethylaminoethylcellulose was assayed by the mouse uterine-weight and augmentation methods. The doses of this preparation were 47.5 and 95.0 μ g. in the uterine-weight assay and 110.0 and 220.0 μ g. in the augmentation assay. The results (Table 1) showed that the two assays gave approximately the same result as we have found previously for this method of extraction (Butt *et al.* 1959). Ultracentrifugal analysis showed that the main component (82% of the total protein) had sedimentation coefficient ($S_{20,w}$) 2.6. There was one other recognizable component with a higher $S_{20,w}$ which could not be calculated accurately.

HMG-J5 was assayed after fractionation on a column of calcium phosphate (10 cm. $\times$ 1 cm.). Active material was obtained in the fractions eluted in 1 mM-disodium phosphate (CP1) and in 0.02 M-disodium phosphate (CP2). In the uterine-weight assay doses of 10.0, 20.0 and 40.0 μ g. of CP1 and 20.0 and 40.0 μ g. of CP2 were used. In the augmentation assay doses of 2.5, 5.0 and 10.0 μ g. of CP1 and 10.0, 20.0 and 40.0 μ g. of CP2 were used. Both fractions were considerably more potent in the augmentation assay than in the uterine-weight assay and appeared therefore to be qualitatively different from the standard. Ultracentrifugal investigations indicated that CP1

contained a major component (90.9%) with $S_{20,w}$ 2.0, and CP2 contained 64% of a component with $S_{20,w}$ 1.64. There was one minor component with a higher $S_{20,w}$ recognized in each fraction.

Pituitary-gland gonadotrophin

Assay of initial materials. The potencies of the three starting materials (the acetone-dried powder, the potassium chloride extract and HGH.1/3) are given in Table 2. The potencies of the potassium chloride extract and HGH.1/3 were considerably greater than the acetone-dried powder; however, whereas there was no significant qualitative difference in the results by the two methods of assay when applied to the potassium chloride extract, there was a difference in HGH.1/3. This was considerably more potent in the augmentation assay than in the uterine-weight assay (index of discrimination 2.5) with no overlap of fiducial limits, which suggests that during its preparation some interstitial-cell-stimulating hormone had been removed from the follicle-stimulating hormone.

Fractionation procedures. The fractions CM1 and CM2 were first prepared from the potassium chloride extract. Details of the assays are given in Table 3.

The index of discrimination in the assay of CM1 was greater than 1 and in the assay of CM2 was considerably less than 1. This suggests that CM1 was relatively richer in follicle-stimulating hormone than the standard and conversely CM2 was richer in interstitial-cell-stimulating hormone. The total recovery was reasonably good and the procedure was therefore considered to be a useful initial step. Because of shortage of potassium chloride extract further batches of CM1 and CM2 were prepared from HGH.1/3. These were then used for subsequent fractionations.

Fractionation of CM1. Three separate batches of CM1 were fractionated on diethylaminoethyl-cellulose. DEAE1 was eluted in 0.2M-ammonium acetate and contained nearly all the detectable activity. Material left on the column was eluted in 0.5M-ammonium acetate (at pH 9.5) made up in

Table 1. *Potency of urinary gonadotrophin*

The first figure in the design of each experiment gives the number of doses of unknown, the second the number of doses of standard. In each assay here and in the subsequent Tables there was no significant (P 0.05) divergence from parallelism in the log dose-response lines, and where more than two points were used no significant departure from linearity. The index of precision (λ) was between 0.14 and 0.23 and the number of animals used in these assays was between 13 and 37. The index of discrimination is the result for the augmentation assay divided by the result for uterine-weight assay.

Preparation	Design	Uterine-wt. assay (mg. of standard/mg.)	Design	Augmentation assay (mg. of standard/mg.)	Index of discrimin- ation
DEAE 1	2:2	19 (13-28)	2:2	15 (10-27)	0.8
HMG-J5 {	CP1 3:2	107 (72-207)	3:2	345 (225-520)	3.2
	CP2 2:2	23 (12-33)	2:2	116 (85-158)	5.0

Table 2. *Potency of pituitary-gland gonadotrophin*

λ was between 0.11 and 0.28. The number of animals used in these assays was between 12 and 28.

Preparation	Design	Uterine-wt. assay (mg. of standard/mg.)	Design	Augmentation assay (mg. of standard/mg.)
Acetone-dried powder	3:2	15 (11-19)	3:2	14 (9.5-21)
Potassium chloride extract	3:2	59 (37-255)	2:2	63 (30-192)
HGH.1/3	2:2	74 (63-86)	3:2	183 (125-285)

Table 3. *Fractionation of pituitary-gland gonadotrophin on carboxymethylcellulose*

Each assay was at least 2:2-point design. λ was 0.23-0.28. The number of animals used in the assays was between 16 and 28.

Preparation	Potency (mg. of standard/mg.)			Total (mg. of standard)		Recovery (%)	
	Uterine-wt. assay	Augmentation assay	Index of discrimin- ation	Uterine- wt. assay	Augmen- tation assay	Uterine- wt. assay	Augmen- tation assay
Potassium chloride extract	59 (37-255)	63 (30-192)	1.1	2600	2750	—	—
CM1	113 (46-299)	183 (84-355)	1.6	1140	1820	60	72
CM2	78 (27-220)	28 (4-57)	0.36	410	146		

0.2M-sodium chloride. This fraction accounted for less than 0.5 % of the original activity of the CM1 put on the column. Details of the assay on DEAE1 are given in Table 4. The potencies of the three batches were in reasonable agreement but in each the index of discrimination was high, suggesting that DEAE1 was rich in follicle-stimulating hormone compared with the standard.

Ultracentrifugal investigations indicated that the first batch contained one recognizable component ($S_{20,w}$ 3.95) (Fig. 2*a*). After 144 min. sedimentation

there was a suggestion of some heterogeneity in the form of a slight shoulder on the trailing edge. The other two batches each contained a component with similar $S_{20,w}$ (71 % in the one and 67 % in the other) and in addition a second component with lower $S_{20,w}$ (2.6–3.0).

Fractionation of DEAE1 on calcium phosphate. Details of the assays of CP1 and CP2 fractions are given in Table 5. In the uterine-weight assay doses of 0.5 and 1.0 μ g. of CP1 and 2.0, 4.0 and 8.0 μ g. of CP2 were used. In the augmentation assay doses

Table 4. Potency of DEAE1 fraction

λ was between 0.10 and 0.20. The number of animals used in each assay was between 13 and 22.

Batch no.	Design	Uterine-wt. assay (mg. of standard/mg.)	Design	Augmentation assay (mg. of standard/mg.)	Index of discrimination
1	3:2	86 (65–144)	3:3	240 (180–320)	2.8
2	2:2	81 (72–129)	2:2	220 (144–525)	2.7
3	3:2	83 (47–290)	2:2	195 (132–478)	2.4

Fig. 2. Ultracentrifugal patterns obtained with gonadotrophins prepared from human pituitary glands. Protein concentration in each was approx. 1 %. Temp. 20°. Barbitone buffer, pH 8.6, I 0.05, in 0.2M-NaCl. (a) Pituitary-gland DEAE 1; (b) pituitary-gland CP2. Numbers refer to time (min.).

Table 5. Potency of CP1 and CP2 fractions

λ was between 0.11 and 0.28. The number of animals used in each assay was between 29 and 40.

Preparation	Design	Uterine-wt. assay (mg. of standard/mg.)	Design	Augmentation assay (mg. of standard/mg.)	Index of discrimination
CP1	2:2	1318 (999–1722)	2:2	3002 (1938–6956)	2.2
CP2	3:2	271 (194–391)	3:2	1014 (753–1555)	3.7

Table 6. *Potency of CM 2 and CM 2/2 fractions*

λ was between 0.12 and 0.21. The number of animals used was between 18 and 47.

Preparation	Design	Uterine-wt. assay (mg. of standard/mg.)	Design	Augmentation assay (mg. of standard/mg.)	Index of discrimination
CM 2	2:2	49 (41-68)	2:2	30 (12-35)	0.62
CM 2/2	2:2*	79 (22-134)	2:2	20 (11-28)	0.25

* In the weighed uterine response the log dose-response line for fraction CM 2/2 had a lower slope than the standard line and it was difficult to assemble a satisfactory quantitative assay. It was possible to obtain a valid assay by a probit method (Brown, 1955), a uterine weight of 10 mg. being classified as a positive response, and these results have been calculated by such a method. λ was 0.26 and the number of animals used was 42.

Table 7. *Relative potencies of pituitary-gland fractions*

λ was between 0.15 and 0.27. The number of animals used in each assay was between 29 and 60.

Preparation	Design	Uterine-wt. assay (mg. of CM 2/mg.)	Augmentation assay (mg. of CM 2/mg.)	Index of discrimination
CP 1	3:2	13.4 (10.6-17.8)	81.3 (56.3-159.4)	6.1
CP 2	2:2	4.0 (3.0-5.4)	33 (19-130)	8.25

of 0.5 and 1.0 μ g. of CP 1 and 1.0, 2.0 and 4.0 μ g. of CP 2 were used. The potency of each was considerably greater than DEAE 1 and in CP 2 the index of discrimination had increased further. When examined in the ultracentrifuge CP 2 appeared to be homogeneous with $S_{20,w}$ 3.07 (Fig. 2b), whereas CP 1 contained a major component (64%) with $S_{20,w}$ 1.64 and a minor component (36%) with a higher $S_{20,w}$.

Fractionation of CM 2. CM 2 was fractionated by chromatography on carboxymethylcellulose. The material eluted in 0.1 M-ammonium acetate (CM 2/2) contained all the detectable gonadotrophin. The doses of CM 2 were 16.0 and 32.0 μ g. in the uterine-weight assay and 32.0 and 64.0 μ g. in the augmentation assay, and the doses of CM 2/2 were 3.0, 6.0 and 12.0 μ g. in the uterine-weight assay and 40.0 and 80.0 μ g. in the augmentation assay. Details of the assays are given in Table 6.

In contrast with the results with the CM 1 fraction the index of discrimination was less than 1.0. The shape of the log dose-response curve in the uterine-weight method for CM 2/2 was different from standard, the slope of the line being significantly less than that of the standard (P 0.01). The result was therefore calculated by a probit method (Brown, 1955), by which it was possible to obtain a valid assay. Ultracentrifugal examination of CM 2/2 indicated a major component (95%) with $S_{20,w}$ 4.17.

Comparison of biological activities. The figures suggested that the fractions CP 1 and CP 2 were relatively rich in follicle-stimulating hormone and that CM 2 was rich in interstitial-cell-stimulating hormone when compared with the standard. They were therefore compared directly with one another

Table 8. *Recovery of gonadotrophin during fractionation of HGH.1/3*

The total amount of starting material (HGH.1/3) and the amounts recovered in CP 1, CP 2 and CM 2 were measured by the two methods of assay. The total percentage recovered in the three fractions is given in the last two columns.

	Total (mg. of standard)		Recovery (%)	
	Uterine-wt. assay	Augmen- tation assay	Uterine- wt. assay	Augmen- tation assay
HGH.1/3	42 300	105 000	—	—
CP 1	17 600	40 500	67.5	53
CP 2	2 950	11 000		
CM 2	8 000	4 270		

and the results are given in Table 7. The index of discrimination increased to 6.1 when CP 1 was assayed against CM 2 and to 8.25 when CP 2 was assayed against CM 2.

Recovery of biological activity. The recovery of biological activity in fractions CP 1, CP 2 and CM 2 was good in view of the number of steps involved. The overall yield in the three fractions combined was over 50% of the original activity in HGH.1/3. The results are shown in Table 8. The recovery of CM 2/2 from CM 2 was, however, only 22.5% by the uterine-weight assay and 10.7% by the augmentation assay. These results are given in Table 9.

Immunological investigations

Rabbit antisera were raised against the DEAE 1 fraction from both a urinary and a pituitary-gland extract. A total dose of 19 mg. of the

urinary and 5 mg. of the pituitary-gland preparation was used.

Experiments in vivo. Both antisera were effective in inhibiting the activity of urinary and pituitary-gland gonadotrophins. Three different urinary DEAE1 fractions and two different pituitary-gland DEAE1 fractions were investigated. They were given in doses equivalent to 1.5 and 3.0 mg. of the standard. In each experiment the potency was reduced by more than 50 % by 0.1 ml. of antiserum. The pituitary-gland antiserum was also examined for effectiveness against pituitary-gland CP1, CP2 and CM2 used at doses equivalent to 1 and 2 mg. of the standard. Again 0.1 ml. of antiserum reduced the potency by more than 50 %.

In contrast with these results both antisera were ineffective against human chorionic gonadotrophin in rats. Doses of 0.025–0.4 ml. of each antiserum caused no reduction in the weight of the prostate gland of rats given 1.5 and 4.5 i.u. of human chorionic gonadotrophin compared with control animals given human chorionic gonadotrophin alone ($P > 0.2$). Higher doses than 0.4 ml. caused toxic effects. These results are summarized in Table 10.

Other investigations of antisera. By the double-diffusion technique in agar gel at least two precipitin lines were observed between the antisera to urinary DEAE1 and urinary DEAE1 antigen (Fig. 3). The pituitary-gland DEAE1 antigen gave only one line against this antiserum. The fractions CP1 and CP2, however, gave no reaction and the antigen responsible for the precipitin line

was found to be in CP3, which was eluted from the calcium phosphate column by a pH gradient with 0.02M-disodium phosphate and N-sodium hydroxide. Fraction CP3 was biologically inert.

The antiserum to pituitary DEAE1 gave no precipitin lines under any conditions tried. Buffer of pH 5.5–7.6 and widely differing concentrations of antigen and antibody were used in these experiments.

Reactions were obtained with the red-cell-haemagglutination technique. Cells treated with pyruvic aldehyde were coated with pituitary-gland DEAE1, 100 μ g./2 ml. of the 10 % suspension of cells being used. Haemagglutination occurred in those mixtures in which the final concentration (the titre) of the antiserum to pituitary-gland DEAE1 fraction was not less than 1 in 1500. Titres as high as 1 in 10 000 were obtained with the antiserum to the urinary DEAE1 fraction. The agglutination was inhibited by human menopausal gonadotrophin and by pituitary-gland gonadotrophin. For these experiments it was found convenient to use a dilution of antiserum of one-half of the titre (i.e. 1 in 750 for a titre of 1 in 1500). The gonadotrophin was dissolved in sodium chloride solution (usually 1 mg./ml.) and doubling dilutions were made. A portion (0.2 ml.) of each dilution was added to 0.2 ml. of the diluted antiserum, followed by 0.05 ml. of the red-cell suspension. Inhibition of the agglutination occurred in those mixtures in which sufficient gonadotrophin had been added to neutralize the antibody. An example of this reaction is shown in Table 11. In the preliminary titration of the antisera the last agglutination occurred in the mixture containing a dilution of 1 in 6400 of the antiserum to urinary and 1 in 1152 of the antiserum to pituitary-gland gonadotrophin. A dilution of 1 in 576 of antiserum to pituitary-gland gonadotrophin was chosen in the inhibition experiment and it was found that a varying degree of inhibition occurred with different preparations of human menopausal gonadotrophin. The inhibition was not specific for human menopausal gonadotrophin, however, since human chorionic gonadotrophin also inhibited the reaction.

Agglutination and inhibition reactions have also been demonstrated with red cells coated with other pituitary-gland fractions including CP1 and CM2.

Table 9. *Recovery of gonadotrophin during fractionation of CM2*

The total amount of starting material (CM2) and the amount recovered in CM2/2 were measured by the two methods of assay. The percentage recovered in CM2/2 is given in the last two columns.

Preparation	Total (mg. of standard)		Recovery (%)	
	Uterine- wt. assay	Augmen- tation assay	Uterine- wt. assay	Augmen- tation assay
CM2	2600	1380	—	—
CM2/2	590	145	22.5	10.7

Table 10. *Inhibition of gonadotrophin by antisera*

+, Antiserum was effective in reducing the activity of the gonadotrophin; —, antiserum was ineffective at the highest dose tested. Dose of antiserum used was between 0.025 and 0.4 ml.; more than 0.4 ml. was usually toxic.

Antiserum	Human menopausal gonadotrophin	Pituitary gonadotrophin	Human chorionic gonadotrophin
Urinary DEAE 1	+	+	—
Pituitary-gland DEAE 1	+	+	—

Fig. 3. Agar-gel precipitation reactions between extracts of gonadotrophin and antiserum to urinary DEAE 1. A-F are doubling dilutions of the antigens; the central hole contains the antiserum. (a) 0.1% of urinary DEAE 1; (b) 0.1% of pituitary-gland DEAE 1; (c) 0.1% of pituitary-gland CP 1; (d) 0.1% of pituitary-gland CP 3.

Table 11. Titration of antisera and inhibition reaction

+, Haemagglutination; -, no agglutination. In the titration 0.2 ml. of antiserum was mixed with 0.2 ml. of serum-sodium chloride solution and 0.05 ml. of red cells. With urinary DEAE 1 the end point was $\frac{1}{3200}$ and with pituitary-gland DEAE 1 the end point was $\frac{1}{1158}$. In the inhibition experiments a dilution of $\frac{1}{3200}$ of the pituitary-gland antiserum was used and the agglutination was inhibited by dilution as far as $\frac{1}{1158}$ % of a urinary human menopausal gonadotrophin DEAE 1, and to $\frac{1}{2}$ % of a benzoic acid-tungstic acid extract of human menopausal gonadotrophin. By biological assay the DEAE 1 fraction was 250 times as potent as the crude human menopausal gonadotrophin.

(i) Titration of antisera

		Dilution of antiserum								
		$\frac{1}{20}$	$\frac{1}{100}$	$\frac{1}{200}$	$\frac{1}{400}$	$\frac{1}{800}$	$\frac{1}{1600}$	$\frac{1}{3200}$	$\frac{1}{6400}$	$\frac{1}{12800}$
Antiserum	Urinary DEAE 1	+	+	+	+	+	+	+	+	-
	Pituitary-gland DEAE 1	$\frac{1}{144}$	$\frac{1}{288}$	$\frac{1}{576}$	$\frac{1}{1152}$	$\frac{1}{2304}$	$\frac{1}{4608}$	-	-	-

(ii) Inhibition experiment with pituitary-gland DEAE 1 antiserum at a concn. of $\frac{1}{3200}$

		Concn. of human menopausal gonadotrophin added (% w/v)							
Gonadotrophin	HMG DEAE 1	$\frac{1}{2}$	$\frac{1}{4}$	$\frac{1}{8}$	$\frac{1}{16}$	$\frac{1}{32}$	$\frac{1}{64}$	$\frac{1}{128}$	$\frac{1}{256}$
	HMG crude	4	2	1	$\frac{1}{2}$	$\frac{1}{4}$	$\frac{1}{8}$	$\frac{1}{16}$	$\frac{1}{32}$

DISCUSSION

Several methods have been described for the preparation of human gonadotrophin in highly purified form. Li, Squire & Gröschel (1960) employed zone electrophoresis on columns of ethanolized cellulose and column chromatography on diethylaminoethyl cellulose and Amberlite IRC50 to purify follicle-stimulating and interstitial-cell-stimulating hormone from human pituitary glands. Ultracentrifugal analysis of the follicle-stimulating-hormone fraction showed remarkable similarity to our CP1 fraction. The major component (70 %) had sedimentation coefficient 1.67 and CP1 contained a major component (64 %) with sedimentation coefficient 1.64. The interstitial-cell-stimulating-hormone fraction of Li *et al.*, however, contained a major component (82 %) with sedimentation coefficient 2.4 differing considerably from our CM2/2, which contained 95 % of a component with sedimentation coefficient 4.17. It is difficult to compare the biological activities because no comparison with the International Reference Preparation HMG 24 was reported by Li *et al.*

The initial steps of our method were adopted from the work of Steelman *et al.* (1959) and our observations are largely in agreement. Their follicle-stimulating-hormone fraction corresponds with our DEAE1 fraction and their interstitial-cell-stimulating hormone with our CM2/2. Comparisons of biological activity of products are again difficult owing to the lack of a common standard. Roos & Gemzell (1960) have prepared a pituitary-gland fraction rich in follicle-stimulating hormone which has been assayed against an international reference substance. They used chromatography on carboxymethyl- and diethylaminoethyl-cellulose followed by zone electrophoresis on a copolymerizate of polyvinyl chloride and polyvinyl acetate. The resulting follicle-stimulating-hormone fraction was tested by the augmentation method with rats instead of mice (Steelman & Pohley, 1953). It was assayed against HMG 20A, a reference preparation similar in biological activity to HMG 24. The relative potency was 2040 times that of the standard, which compares well with 3002 of our CP1.

We found a second fraction which was rich in follicle-stimulating hormone: this was CP2. It was essentially homogeneous in the ultracentrifuge but had a higher sedimentation coefficient (3.07) than CP1. It may be that the active principle in CP1 was attached to a larger inert protein in CP2 but its relationship to CP1 is not yet clear.

The active material recovered in the three fractions CP1, CP2 and CM2 accounted for over 50 % of the activity in the starting material HGH. 1/3. The only fraction containing detectable gonadotrophin, which was discarded, was left on

the column of diethylaminoethylcellulose during the fractionation of CM1. Its activity (per mg.) was very low compared with that of the other active fractions and in quantity it did not account for more than 0.5 % of the original material. The fractionation of CM2 on carboxymethylcellulose was not very efficient. No active material could be detected on the column although only about 20 % could be found in CM2/2. Other methods are being investigated for the fractionation of CM2.

Fractions rich in follicle-stimulating hormone have been obtained from postmenopausal urine by the method of Johnsen (1955), followed by chromatography on calcium phosphate. It has not been possible to reach such high potencies as with the pituitary-gland gonadotrophins, the best pituitary-gland fractions having a potency about 10 times that of the best urinary preparation with the augmentation assay. Bourillon, Got & Marcy (1960) have obtained a highly active gonadotrophin from postmenopausal urine by precipitation with ethanol, adsorption on kaolin, chromatography on ion-exchange materials and zone electrophoresis on starch. The product was active in the mouse uterine-weight assay at a dose of 1 μ g. It was not assayed directly against the standard, but other information given by the authors suggests that this represents a relative potency of about 1000 in terms of the standard, which implies that its potency lies between those of our best urinary and pituitary-gland preparations.

The gonadotrophins from both urine and from pituitary glands have shown remarkable stability. They have been stored in 0.9 % sodium chloride solution at -15° for over 12 months without detectable loss of activity. During this time they have been removed and thawed at frequent intervals for examination.

The production of antibodies to these gonadotrophins has been carried out by a new technique. They were adsorbed strongly by bentonite, which was given to rabbits as a suspension. A powerful antibody was detected after the administration of 3 mg. of pituitary-gland DEAE1 and it has been found that on immunizing the rabbit again after a rest of 9 months only 0.9 mg. of the same antigen was required to restore the titre to its original value. A statistical comparison of the new technique with other established techniques has not been made because of the limited supplies available, but the method of alum suspension has been ineffective in our hands. This is probably because the gonadotrophin remains in solution. Antibodies have also been produced with Freund's adjuvant (Butt, Crooke, Cunningham & Gell, 1960), but large quantities of antigen were required.

Antibodies to both urinary and pituitary-gland antigens were effective *in vivo* in inhibiting the

activity of gonadotrophins from urine or pituitary glands in the mouse but they were ineffective against human chorionic gonadotrophin in the rat. A continuation of our previous studies (Butt, Crooke & Cunningham, 1960) has confirmed that the antibody to human chorionic gonadotrophin is effective not only against human chorionic gonadotrophin but also against human menopausal gonadotrophin and pituitary-gland gonadotrophin.

It has not been possible to use immuno-electrophoresis in this study since no precipitation can be observed in agar gel. The red-cell-haemagglutination technique, however, gives a satisfactory end point. We have used the method of Ling (1960) for preparing the red cells since it was found that the sensitized cells were much more stable than those prepared by the tannic acid method. Some have been used satisfactorily 2 months after they were coated. Since inhibition of the haemagglutination reaction was obtained with microgram quantities of pituitary-gland or chorionic gonadotrophin the technique may be useful for the quantitative estimation of gonadotrophins in urine or serum. In view of the cross-reactions of pituitary-gland and chorionic gonadotrophins, however, the results will have to be interpreted with care.

SUMMARY

1. Gonadotrophins were prepared from human urine and pituitary glands and purified by chromatography on carboxymethyl- and diethylaminoethyl-cellulose and calcium phosphate.

2. Two fractions, one with mainly follicle-stimulating-hormone activity and one with mainly interstitial-cell-stimulating-hormone activity, were obtained.

3. The first follicle-stimulating-hormone fraction was 3002 times as potent as the International Standard Preparation HMG 24 by the ovarian-augmentation assay. It showed two components in the ultracentrifuge, the major one (64%) having sedimentation coefficient 1.64. The second follicle-stimulating-hormone fraction was 1014 times as potent as the standard and was homogeneous in the ultracentrifuge with sedimentation coefficient 3.07.

4. The best urinary fractions had about one-tenth of the activity of the best pituitary-gland fractions.

5. Antibodies were raised to some of the fractions from urine and pituitary glands and when

tested *in vivo* they were found to inhibit both urinary human menopausal gonadotrophin and pituitary-gland gonadotrophin but were ineffective against human chorionic gonadotrophin. They failed to produce precipitin lines by the agar-gel-diffusion technique but could be used in the red-cell-haemagglutination technique.

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