SUMMARY

1. Endopolygalacturonase has been separated from the culture fluid of *Aspergillus niger* with a purification factor of 400-fold and a recovery of 34 %.

2. The preparation obtained was homogeneous on paper electrophoresis at pH 7.2 and pH 8.0, and of moderate stability with an optimum for stability of about pH 6.

3. The enzyme rapidly lowered the viscosity of a solution of pectic acid and released reducing groups from it according to a random pattern of hydrolysis, but the digestion was not complete. Pectin underwent only a very slight degradation, sufficient to show a fall in viscosity but with little release of reducing groups.

4. Tetragalacturonic acid was rapidly split to tri- and mono-galacturonic acid, and trigalacturonic acid was slowly split to di- and mono-galacturonic acid, but the digalacturonic acid was not attacked.

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REFERENCES

- Ashby, J., Brooks, J. & Reid, W. W. (1955). *Chem. & Ind.* p. 360.
 Brooks, J. & Reid, W. W. (1955). *Chem. & Ind.* p. 325.
 Demain, A. L. & Phaff, H. J. (1954). *J. biol. Chem.* **210**, 381.

- Demain, A. L. & Phaff, H. J. (1957). *Wallerstein Labs Commun.* **20**, 119.
 Dixon, M. & Webb, E. C. (1958). In *Enzymes*, p. 39. London: Longmans, Green and Co. Ltd.
 Gomori, G. (1955). In *Methods in Enzymology*, vol. 1, p. 138. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
 Kertesz, Z. I. (1951). In *The Pectic Substances*, p. 128. New York: Interscience Publishers Inc.
 Kunkel, H. G. & Tiselius, A. (1951). *J. gen. Physiol.* **35**, 89.
 Lineweaver, H., Jang, R. & Jansen, E. F. (1949). *Arch. Biochem.* **20**, 137.
 Luh, B. S. & Phaff, H. J. (1954). *Arch. Biochem. Biophys.* **51**, 102.
 McCready, R. M. & Seegmiller, C. G. (1954). *Arch. Biochem. Biophys.* **50**, 440.
 McCready, R. M., Swenson, H. A. & Maclay, W. D. (1946). *Industr. Engng Chem. (Anal.)*, **18**, 290.
 Myers, P. B. & Baker, G. L. (1934). *Bull. Del. agric. Exp. Sta.* no. 187.
 Owens, H. S., McCready, R. M., Shepheard, A. D., Schultz, T. H., Puppen, E. L., Swenson, H. A., Miers, J. C., Erlandsen, R. F. & Maclay, W. D. (1952). In *Methods Used at the Western Regional Research Laboratory for Extraction and Analysis of Pectic Material*, Circ. U.S. Dep. Agric., AIC 340.
 Patel, D. S. & Phaff, H. J. (1959). *J. biol. Chem.* **234**, 237.
 Peterson, E. A. & Sober, H. A. (1956). *J. Amer. chem. Soc.* **78**, 751.
 Phaff, H. J. & Demain, A. L. (1956). *J. biol. Chem.* **218**, 875.
 Reid, W. W. (1952). *Biochem. J.* **50**, 289.
 Roboz, E., Barrett, R. W. & Tatum, E. L. (1952). *J. biol. Chem.* **195**, 459.
 Saito, H. (1955). *J. gen. appl. Microbiol.* **1**, 38.
 Trevelyan, W. E., Procter, D. P. & Harrison, J. S. (1950). *Nature, Lond.*, **166**, 444.
 Tuttobello, R. & Mill, P. J. (1961). *Biochem. J.* **79**, 51.

Biochem. J. (1961) **79**, 64

Chemical Reactions which Affect the Biological Activity of Human Gonadotrophins

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The biological activity of the gonadotrophic hormones is diminished by the action of various reagents. The effects of oxidation, reduction, acetylation and enzymic hydrolysis have been extensively investigated and certain differences in the relative stabilities of follicle-stimulating hormone (FSH) and interstitial-cell-stimulating hormone (ICSH) have been reported.

The gonadotrophins are glycoproteins and the carbohydrate groups appear to be important for

biological activity. Thus Whitten (1950) demonstrated that the activity of ICSH in serum gonadotrophin was retained after treatment with 2 mM-periodate but the activity of FSH was diminished. Geschwind & Li (1958), however, claimed that both gonadotrophins prepared from the anterior-pituitary glands of sheep were destroyed by treatment with 0.2 M-potassium periodate.

Fraenkel-Conrat, Simpson & Evans (1939, 1940) and McShan & Meyer (1940) showed that reduction

with cysteine diminished the activity of sheep FSH, and Li, Simpson & Evans (1940) and Fraenkel-Conrat *et al.* (1940) showed that it completely destroyed sheep ICSH. Similar results were obtained after acetylation with keten (Li, Simpson & Evans, 1939). The activity of ICSH may therefore be connected with the presence of -S-S- linkages reducible by cysteine or with amino groups accessible to keten.

Other differences between FSH and ICSH have been demonstrated by experiments with enzymes. Chow, Greep & van Dyke (1939) found that under certain conditions hog FSH is stable to tryptic digestion and is only partially destroyed by chymotrypsin, but that hog ICSH is completely destroyed by both.

Reactions with certain cations have also been studied. Antoniadou *et al.* (1957) used precipitation with zinc and ethanol for the extraction of gonadotrophin from human plasma, and Legault-Démare, Clauser & Jutisz (1958) claimed that a 20-fold increase in the purity of pregnant-mare serum gonadotrophin was obtained by the removal of inert material precipitable by barium.

These observations on gonadotrophins extracted from the anterior pituitary gland and from the serum of various animals led us to investigate similar reactions with the human gonadotrophins. The study was designed to show whether any reaction differentially affected the biological activity of FSH or ICSH. Such a reaction might be of practical value in the purification of the hormones from human urine or pituitary tissue.

METHODS

Biological assay

Immature female mice of an albino strain (supplied by Mr W. Clarke, Oldham), weighing 8–10 g. at the start of the assay, were used in each test. The gonadotrophin in 0.9% sodium chloride soln. was injected subcutaneously in five equal doses of 0.5 ml. over a 3-day period. Injections were made twice a day on the first 2 days and once on the third. The animals were killed 72 hr. after the first injections. The uteri or ovaries were dissected and weighed fresh.

Three to six animals were used for each dose and the interval between successive doses was either 1.5 or 2.0. Two methods were used, which have been described in detail by P. S. Brown (1955).

(a) *Uterine-weight method.* This is believed to measure the combined effect of follicle-stimulating hormone (FSH) and interstitial-cell-stimulating hormone (ICSH). For the construction of the dose-response curve the logarithm of the dose and the mean of the combined uterine weights were used. The response was linear with doses between 0.5 and 2.0 mg. of the International Reference Preparation (HMG 24). Whenever possible at least three doses were used so that the linearity of the response line could be checked.

(b) *Augmentation method.* This is believed to be relatively specific for FSH. It depends on the increase in weight of the ovary of the immature mouse treated simultaneously with the test material and with a great excess (40 i.u.) of human chorionic gonadotrophin (HCG). The HCG has an action similar to ICSH and causes only a small increase in ovarian weight. The effect of ICSH in the test material is rendered insignificant by the excess of HCG. Any increase in weight of the ovary above that caused by the HCG alone is considered to be due to FSH. The specificity of this method has been discussed elsewhere (Brown, 1959). The log dose-response curve was linear in the range 0.5–1.5 mg. of HMG 24.

The results were calculated by the standard methods given by Bliss (1952) or by the simplified methods of Gaddum (1953). The activity of the material recoverable after chemical treatment was calculated from the assay results and expressed as a percentage of the activity in the original material. The results of two assays are considered to be qualitatively similar if their 95% fiducial limits of error overlap and the ratio of calculated potencies (index of discrimination) by the two methods does not exceed two (Cunningham & Brown, 1958).

A laboratory substandard was prepared from urine of post-menopausal women by the benzoic acid-tungstic acid method of Butt (1958). Its potency was determined by both assays in terms of HMG 24: 1 mg. was equivalent to 0.73 (95% fiducial limits 0.62–0.84) mg. of HMG 24 by the uterine-weight method and 1.04 (0.37–2.10) mg. of HMG 24 by the augmentation method.

Preparation of gonadotrophin for chemical experiments

Urine. Urine of post-menopausal women was extracted by the benzoic acid-tungstic acid method of Butt (1958) or by the kaolin-adsorption method (Brown, 1959). The powders obtained were then purified by use of ion-exchange materials (Butt & Round, 1958). The mixed resin, Bio-Deminrolit (The Permutit Co.), was used in the first stage of this procedure instead of De-Acidite FF in the borate form, as previously described, since less preparation of the resin was required and equally good results were obtained. The active material was then adsorbed on diethylaminoethyl (DEAE)-cellulose prepared from Whatman cellulose powder (Peterson & Sober, 1956) and was eluted by 0.5M-ammonium acetate (at pH 9.5) made up in 0.2M-sodium chloride as previously described. The potency of different batches measured by the two methods of assay varied slightly but was usually similar to that of the International Reference Preparation (HMG 24). These products therefore contained both FSH and ICSH in about the same proportions as the standard.

Pituitary gland. A powder was supplied by Dr A. Korner (University of Cambridge, Department of Biochemistry) which had been prepared from acetone-dried human pituitaries as a by-product in the extraction of growth hormone. This preparation involved extraction of the acetone-dried powder in potassium chloride (0.34M) at pH 5.5, after which the pH was adjusted to 8.6. Ethanol was added until the concentration was 30% (v/v) and any precipitate was removed by centrifuging. Solid ammonium sulphate was added to 50% saturation (based on the water content) and the precipitate was discarded. The super-

nant was dialysed and freeze-dried and the dry powder was then fractionated in this laboratory.

It was first applied to a column of carboxymethyl (CM)-cellulose as described by Steelman, Segaloff & Andersen (1959) but ammonium acetate was used instead of sodium acetate for elution. The fraction eluted in 0.01 M-ammonium acetate was precipitated by adding 3 vol. of ethanol and then was dissolved in water and fractionated on DEAE-cellulose as described by Butt, Croke & Cunningham (1959) for urinary gonadotrophin. The material eluted from DEAE-cellulose in 0.2 M-ammonium acetate was precipitated by adding 3 vol. of ethanol, washed with ethanol and ether and dried. Biological assay by the uterine-weight method showed that 1 mg. was equivalent to 86 (65–144) mg., and by the augmentation method to 240 (180–320) mg., of the standard HMG 24. Histological examination of the ovaries of hypophysectomized rats given this material showed that it was a potent follicle-stimulating hormone and it will be referred to in this paper as pituitary 'FSH'. Ultracentrifugal analysis by Dr D. R. Stanworth (University of Birmingham, Department of Experimental Pathology) indicated that it contained one major component with a sedimentation coefficient, $S_{20,w}$, 3.95. Centrifuging was performed in barbitone buffer (pH 8.6; 1.0.5; 0.2 M-sodium chloride) at 60 000 rev./min. at 20°. After sedimentation for 144 min. there was a suggestion of some heterogeneity in the form of a slight shoulder on the trailing edge.

Material eluted in M-ammonium acetate from the CM-cellulose column was reprecipitated by adding 3 vol. of ethanol and then refractionated on CM-cellulose. The eluate in 0.1 M-ammonium acetate was precipitated by adding 3 vol. of ethanol, washed with ethanol and ether and dried. There was insufficient material for ultracentrifugal analysis. The product from approx. 20 mg. of material was dissolved in 1 ml. of 0.9% sodium chloride soln. It contained the equivalent of 780 (480–1460) mg. of HMG 24 by the uterine-weight method and 850 (255–1720) mg. of HMG 24 by the augmentation method. This shows that it is qualitatively similar to the standard by these assays but it differs from the standard in its effect on the ovaries of immature mice in that corpora haemorrhagica are produced by a dosage of only twice that which is required to double the uterine weight. It corresponds with the luteinizing or interstitial-cell-stimulating hormone fractions of Steelman *et al.* (1959) and for this reason it will be referred to as pituitary 'ICSH'.

EXPERIMENTAL AND RESULTS

In all experiments, unless otherwise stated, the products were finally precipitated by adding 5 vol. of ethanol and were washed with ethanol and ether.

Oxidation

Periodate. Gonadotrophin (20 mg. of urinary, or 200 µg. of pituitary FSH) in 2 ml. of 0.2 M-ammonium acetate at pH 7.0 was mixed with 2 ml. of 4 mM-potassium periodate. The reaction proceeded for 30 min. at room temperature and was then stopped by the addition of excess of glucose.

Hydrogen peroxide. Urinary gonadotrophin (20 mg.) was dissolved in water (4 ml.) and 0.25 ml. of 6% (w/v) hydrogen peroxide was added. Two further portions of 0.25 ml. of hydrogen peroxide were added at 15 min. intervals. Oxidation proceeded for a further 45 min. and the gonadotrophin was then reprecipitated.

The potency of FSH and ICSH was partially diminished by the effect of periodate and was considerably diminished by hydrogen peroxide but there was no significant difference between the results by the two methods of assay (Table 1).

Reduction

Urinary gonadotrophin (20 mg.) was dissolved in 1 ml. of 0.1 M-ammonium acetate (pH 7.0) and was reduced at room temperature by: (a) ascorbic acid (20 mg.) for 6 hr.; (b) thioglycollic acid (0.2 ml.) for 6 hr.; (c) methionine (20 mg.) for 6 hr.; (d) cysteine (20 mg.) for 6 hr.; (e) sodium borohydride (8–20 mg.) for 1 hr.

There was slight loss of active material after treatment with ascorbic acid, thioglycollic acid or methionine and rather more loss with cysteine (Table 2). Reduction with sodium borohydride (0.4 mg./mg. of protein) caused no loss of activity but with 1.0 mg./mg. of protein the activity was modified. Results by the uterine-weight method showed that less than 33% of the original activity remained whereas in the augmentation assay for

Table 1. *Oxidation of human urinary gonadotrophins*

The 95% fiducial limits are given in parentheses. All assays were of at least 2:2 point design except in the experiment with H_2O_2 , which was of 1:3 point design. Divergence from parallelism of the dose-response lines is not significant ($P > 0.05$) in any assay. The index of precision (λ) is calculated by dividing the standard deviation of the responses by the slope of the line connecting the response with the logarithm of the dose. It varied in these assays between 0.05 and 0.23.

Method of oxidation	Type of gonadotrophin	Relative potency (% of control)	
		Augmentation assay for FSH	Uterine-weight assay for FSH + ICSH
2 mM-Periodate	Urine	55 (38–68)	38 (31–47)
	Pituitary 'FSH'	35 (22–47)	46 (36–57)
	Pituitary 'ICSH'	30 (19–62)	36 (4–65)
H_2O_2	Urine	≤ 40	≤ 30

Table 2. *Reduction of human urinary gonadotrophin*

The designs of the assays are shown by the number of doses of reduced (first number) and initial materials (second number) and the significance of the divergence in slopes of the dose-response lines [$P(G)$] is also given. The index of precision (λ) in these assays varied between 0.06 and 0.17.

Method of reduction	Design	Augmentation assay for FSH (relative potency as % of control with 95% fiducial limits)	$P(G)$	Design	Uterine-weight assay for FSH + ICSH (relative potency as % of control with 95% fiducial limits)	$P(G)$
Ascorbic acid	2:2	71 (19-76)	0.6-0.7	2:3	51 (36-84)	0.9
Thioglycollic acid	2:3	73 (59-77)	0.6-0.7	2:3	53 (30-77)	0.9
Methionine	1:3	78 (55-110)	—	1:3	140 (80-1900)	—
Cysteine	1:2	51 (20-68)	—	1:3	43 (5-74)	—
Sodium borohydride						
0.4 mg./mg.; 1 hr.	2:3	150 (85-447)	0.6-0.7	3:3	81 (36-168)	0.1-0.2
1.0 mg./mg.; 1 hr.	3:3	Invalid due to divergence in slopes of dose-response lines	0.001	1:3	<33	—

Table 3. *Treatment of urinary and pituitary gonadotrophin with borohydride*

All the assays were of at least 2:2 point design except in the experiments with pituitary 'ICSH', where the design was 1:2. There was no significant deviation from parallelism in any assay and the index of precision (λ) varied between 0.07 and 0.20.

Preparation	Potency (% of control with 95% fiducial limits)
	Augmentation assay for FSH Uterine-weight assay for FSH + ICSH
Urinary { 1	72 (68-78) 34 (30-43)
2	93 (56-215) 36 (16-59)
3	87 (51-132) 49 (40-70)
Pituitary 'FSH'	67 (50-104) 66 (28-142)
Pituitary 'ICSH'	<12 <20

FSH the slope of the log dose-response line was significantly less than the original.

Reduction with borohydride was therefore re-investigated. Three different samples of urinary gonadotrophin and samples of pituitary 'FSH' and 'ICSH' were reduced with 1.0 mg. of sodium borohydride/mg. of gonadotrophin for 1 hr. The potencies of the products were then compared by bioassay with the respective controls (Table 3). In the experiments with urinary gonadotrophin the results of the uterine-weight assays were always lower than those of the augmentation assays. This suggests that ICSH is destroyed by borohydride more readily than is FSH. The experiments with pituitary 'FSH' and 'ICSH' support this conclusion since the potency of FSH is only slightly diminished whereas that of ICSH is greatly diminished. In contrast with the previous experiment in Table 2, there was no evidence of lack of parallelism in the log dose-response lines in these assays.

Table 4. *Effect of sodium hydroxide on activity of gonadotrophins*

Gonadotrophins were prepared by the benzoic acid-tungstic acid method. They were dissolved in water and brought to pH 11.5 with *N*-NaOH. After 20 min. the pH was readjusted to approx. 7.0 and the gonadotrophin was reprecipitated by adding 5 vol. of ethanol. Assays were of 2:2 point design and the index of precision (λ) varied between 0.05 and 0.10.

	Potency (% of control with 95% fiducial limits)
	Augmentation assay for FSH Uterine-weight assay for FSH + ICSH
Post-menopausal gonadotrophin	123 (101-150) 96 (80-137)
Male gonadotrophin	— 95 (76-115)

Effect of alkali

Gonadotrophin prepared by the benzoic acid-tungstic acid method was dissolved in water and brought to pH 11.5 with *N*-sodium hydroxide (glass electrode). After 20 min. at 20° the pH was adjusted to approx. 7.0 and the gonadotrophin was reprecipitated.

This treatment had no effect on the potency of gonadotrophin from the urine of post-menopausal women or from normal men (Table 4).

Other reactions

Methylation. About 150 mg. of urinary gonadotrophin was dissolved in 22 ml. of water, which was then divided equally in experimental and control

tubes. To each tube was added 0.18 g. of boric acid and 0.8 ml. of 20% sodium hydroxide. After shaking to dissolve the boric acid, 0.2 ml. of dimethyl sulphate was added to the experimental tube and 0.2 ml. of water to the control. The tubes were shaken and left at room temperature for 15 min., when a further 0.2 ml. of dimethyl sulphate and 0.4 ml. of 5*N*-sodium hydroxide were added to the experimental tube and 0.6 ml. of water to the control, and left for 15 min. About 2 g. of Bio-Deminrolit was then added to each tube and after filtration the gonadotrophins were precipitated by adding 4 vol. of ethanol and were washed with ethanol and ether.

The potency of the methylated gonadotrophin was less than 19% of the initial potency by both methods of assay. Methoxyl groups were measured by the Zeisel method. The control preparation contained no detectable methoxyl groups whereas the methylated preparation contained 1.8%.

Hydroxylamine. Urinary gonadotrophin (about 80 mg.) was dissolved in 10 ml. of 0.2*M*-ammonium acetate (pH 7.4) and divided equally between experimental and control tubes. Hydroxylamine hydrochloride (2 ml.) was brought to pH 7.4 with sodium hydroxide and added to the experimental tube and 2 ml. of water added to the control. After 1 hr. at 15° no precipitate had formed in the experimental tube and the gonadotrophins were therefore precipitated with 3 vol. of ethanol. The precipitates were washed five times with ethanol and finally with ether. There was no loss of FSH or ICSH by this treatment (Table 5).

Treatment with salivary amylase. Urinary gonadotrophin (about 10 mg.) was dissolved in 6.0 ml. of 0.5*M*-ammonium acetate (pH 6.6) and divided equally between experimental and control tubes. Filtered saliva (6 ml.) was added to the experimental tube and 6 ml. of water to the control. After 4 hr. at 25° each was treated with 4 g. of Bio-Deminrolit for 10 min. This amount was sufficient to remove the amylase present. After filtration the gonadotrophins were precipitated by adding 3 vol. of ethanol in the cold, and washed with ethanol and ether. This treatment did not decrease the potency of FSH or ICSH (Table 5).

Combination with cations

Zinc. The method of Antoniadou *et al.* (1957) was used in this experiment. A portion (0.02 ml.) of a mixture of 0.25*M*-zinc acetate and 0.25*M*-glycine was added to urinary gonadotrophin (20 mg.) in 1 ml. of water. The mixture was refrigerated (5°) for 30 min. and the precipitate separated by centrifuging. It was washed with a mixture of 1 ml. of water and 0.02 ml. of zinc reagent and the wash was combined with the first supernatant. A further 0.5 ml. of the zinc reagent and ethanol

(0.6 ml.) was then added to the combined washes and the precipitate which again formed was separated by centrifuging. A further 7.5 ml. of ethanol was added to the supernatant and a third precipitate was separated by centrifuging. The three precipitates were each taken up separately in small volumes of 0.15*M*-sodium chloride and 0.1*M*-sodium citrate to dissolve the zinc. The protein in each was then precipitated by the addition of 5 vol. of ethanol and the precipitates were finally washed with ethanol and ether. All the detectable biological activity was recovered in the second precipitate (Table 6) and there was no significant difference between results by the two assays. The recovery of active material was good.

Copper. A portion (0.2 ml.) of 0.2*M*-cupric sulphate was added to urinary gonadotrophin (20 mg.) in 1 ml. of 0.2*M*-ammonium acetate. The precipitate which formed was separated by centrifuging and dissolved in 0.2*M*-ethylenediamine-tetra-acetic acid (sodium salt). Protein was reprecipitated by adding 5 vol. of ethanol and was washed with ethanol and ether. The recovery of biologically active material in this fraction is given

Table 5. *Treatment of urinary gonadotrophin with salivary amylase and with hydroxylamine*

Gonadotrophins were prepared by the kaolin method. The 95% fiducial limits are given in parentheses. The design in each assay was 2:2. The divergence from parallelism in the dose-response lines was not significant and the index of precision (λ) varied between 0.08 and 0.14.

	Potency (% of control with 95% fiducial limits)	
	Augmentation assay for FSH	Uterine-weight assay for FSH + ICSH
Hydroxylamine	100 (78-158)	125 (101-157)
Amylase	135 (68-168)	159 (112-250)

Table 6. *Precipitation of gonadotrophins with zinc, copper and barium*

In experiments with zinc and copper the gonadotrophin was precipitated by the metal: in the experiment with barium the gonadotrophin remained in solution. There was no evidence of any divergence from parallelism in the dose-response lines in these assays and the index of precision (λ) was between 0.05 and 0.26.

	Potency (% of control with 95% fiducial limits)	
	Augmentation assay for FSH	Uterine-weight assay for FSH + ICSH
Zinc	62 (47-234)	99 (80-230)
Copper	91 (74-111)	86 (59-130)
Barium	66 (41-150)	60 (42-101)

in Table 6, which shows that there was no significant difference between the results by the two assays.

Barium. A portion (2.0 ml.) of 2M-barium acetate was added to urinary gonadotrophin (about 100 mg.) in 10 ml. of 0.2M-sodium carbonate in an ice bath. It was added drop by drop with shaking and when the pH had fallen to 7.0 the barium carbonate which had precipitated was removed by centrifuging and the supernatant was treated with about 2 g. of Bio-Deminrolit and filtered. Protein was precipitated by adding 3 vol. of ethanol and then washed with ethanol and ether. About 60% of the original weight of material was recovered in this fraction and the relative potency compared with the original was also about 60% (Table 6). The potency per milligram had not therefore been increased by treatment with barium as in the experiment of Legault-Démare *et al.* (1958).

Reactions with triazine dyes

The dyes Procion Brilliant Red 2BS, Yellow RS and Blue 3G5 were gifts from Imperial Chemical Industries Ltd. (Dyestuffs Division). They all contain the dichlorotriazinyl grouping and sulphonic acid groups. Under the conditions of the following experiments they are believed to be attached to proteins through the amino groups (Hess & Pearce, 1959).

Urinary gonadotrophin (20 mg.) in 2.0 ml. of 0.9% sodium chloride soln. was mixed with 0.2 mg. of triazine dye in 0.5 ml. of 0.9% sodium chloride soln. Protein was precipitated after 30 min. by adding 5 vol. of ethanol. The product was washed with ethanol and ether. Gonadotrophin was recovered after treatment with the blue and yellow dyes with no appreciable loss of biological activity (Table 7) but the recovery with the red dye was poor.

The dyed gonadotrophins were used for electrophoretic experiments on cellulose strips (Oxo Ltd., Medical Department). Horizontal electrophoresis

in 0.1M-sodium acetate containing 0.9% sodium chloride at pH 5.5 was continued for 16 hr. with 0.5 mA/cm. width. Usually a single dyed area was obtained, about 1 cm. on the positive side of the origin, and this was biologically active. Staining with Nigrosine WS (Hopkin and Williams Ltd.) or Lissamine green (Imperial Chemical Industries Ltd.), however, revealed a slower-moving fraction which did not carry the triazine dye and this was also biologically active. These two fractions appeared to be approximately equal in biological activity and no significant differences were found between the results by the two methods of assay. In a typical experiment with the yellow dye the dyed area contained the equivalent of 11.3 (9.0–12.2) mg. of HMG 24 by the uterine-weight assay and 7.8 (3.0–10.8) mg. of HMG 24 by the augmentation method, whereas the second, slower-moving fraction contained 9.5 (8.0–11.3) mg. of HMG 24 and 11.2 (2.0–24.0) mg. of HMG 24 by the same two assays.

On columns of DEAE-cellulose the dyed gonadotrophins were eluted in 0.2M-ammonium acetate in the same way as untreated gonadotrophins. An appreciable quantity of the dye was retained at the top of the column and this contained no detectable biological activity.

DISCUSSION

The two biological assays used here have been developed in order to recognize qualitative differences in human urinary gonadotrophins (Brown, 1958, 1959). Borth, Linder & Lunenfeld (1959) suggested that such differences may be due not to changes in the ratio of ICSH to FSH but to other factors such as rates of absorption, toxicity or the presence of synergists or antagonists to gonadotrophin in the extracts. Thus different methods of extraction might give products which appear to contain different ratios of ICSH to FSH but this might be explained by different quantities of impurities contained in each. In the present experiments this possibility does not arise since the treated material was always assayed against gonadotrophin from the same batch. The possibility remains that certain reagents may modify the impurities rather than the gonadotrophin and so alter the relative potency. However, the results obtained with highly purified pituitary fractions are essentially the same as with relatively crude urinary extracts, suggesting that the main effect is on the gonadotrophin rather than on the impurities.

The biological activity of gonadotrophin was altered by reactions which may affect the carbohydrate groups. Oxidation with periodate and methylation with dimethyl sulphate diminished both ICSH and FSH activity. Reduction with

Table 7. *Combination of urinary gonadotrophin with triazine dyes*

Assays for the blue and yellow derivatives were of 2:2 point design and for the red derivatives were 1:2 point design. There was no evidence of lack of parallelism in the dose-response lines and the index of precision (λ) varied between 0.05 and 0.15.

	Potency (% of control with 95% fiducial limits)	
	Augmentation assay for FSH	Uterine-weight assay for FSH + ICSH
Procion blue	106 (63–200)	93 (41–130)
Procion yellow	87 (66–108)	71 (50–100)
Procion red	<50	<50

borohydride diminished ICSH more than FSH, as shown by the experiments with crude extracts of urine containing both ICSH and FSH and with purer preparations of human pituitary 'ICSH' and 'FSH'. With urinary extracts the results by the uterine-weight method were consistently lower than those by the augmentation method. With pituitary 'ICSH' the biological activity became undetectable whereas with pituitary 'FSH' the activity was only slightly lowered.

None of the other reactions which were investigated affected the activity of ICSH or FSH differentially. Some of these reactions may be useful, however, as steps in the purification of gonadotrophins. Copper and zinc salts were found to precipitate urinary gonadotrophins with only slight loss of activity. Barium was investigated because it was used successfully for the purification of pregnant-mare serum gonadotrophin by Legault-Démare *et al.* (1958). They obtained a 20-fold increase in potency per milligram but the present experiment showed that no such increase was obtained with human urinary gonadotrophin.

Methylation under the mild conditions used by J. B. Brown (1955) greatly diminished the activity of urinary gonadotrophin. Chemical measurement of the methoxyl groups showed that the treated material contained 1.8%, but it was not possible to show with the available material whether carbohydrate or amino acids had been methylated.

The stability in alkali of the urinary gonadotrophins was investigated to determine whether any changes, particularly qualitative changes, occur during elution in the kaolin-extraction procedure. Gonadotrophin was prepared by the benzoic acid-tungstic acid method since a high pH is not used. This material was quite stable for 20 min. at room temperature at pH 11.5, which is the maximum pH normally used for elution from kaolin. It indicates that no qualitative changes are likely to occur at this stage.

Coloured derivatives of the urinary gonadotrophin with yellow and blue triazine dyes were obtained with satisfactory biological activity. They were prepared so that the behaviour of gonadotrophins in chromatographic and electrophoretic separations could be observed visually. Electrophoresis showed, however, that only a portion of the gonadotrophin carried the dye and there is no biological evidence that the electrophoretic fractions are qualitatively different.

A study of reactions such as those described may eventually lead to a greater knowledge of the chemical nature of those groups in the protein that are necessary for gonadotrophic activity. Quantitative data on the chemical changes would be of small value until pure preparations of gonadotrophins are available.

SUMMARY

1. The effects of various reagents on the activity of human urinary and pituitary gonadotrophins has been studied by two assay methods, the first measuring the combined effects of follicle-stimulating hormone and interstitial-cell-stimulating hormone and the second being more specific for follicle-stimulating hormone.

2. The only reagent to affect one type of activity more than the other was sodium borohydride. This appeared to destroy interstitial-cell-stimulating hormone more readily than follicle-stimulating hormone.

3. Methods are described for the combination of urinary gonadotrophin with zinc, copper and triazine dyes without loss of biological activity.

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REFERENCES

- Antoniades, H. N., Pennell, R. B., McArthur, J. W., Ingersoll, F. M., Ulfelder, M. & Oncley, J. L. (1957). *J. biol. Chem.* **228**, 863.
- Bliss, C. L. (1952). *The Statistics of Bioassay*. New York: Academic Press Inc.
- Borth, R., Linder, A. & Lunenfeld, B. (1959). *Acta endocr., Copenhagen*, **31**, 192.
- Brown, J. B. (1955). *Biochem. J.* **60**, 185.
- Brown, P. S. (1955). *J. Endocrin.* **13**, 59.
- Brown, P. S. (1958). *J. Endocrin.* **17**, 329.
- Brown, P. S. (1959). *J. Endocrin.* **18**, 46.
- Butt, W. R. (1958). *J. Endocrin.* **17**, 143.
- Butt, W. R., Crooke, A. C. & Cunningham, F. J. (1959). *Acta endocr., Copenhagen*, **32**, 509.
- Butt, W. R. & Round, B. P. (1958). *J. Endocrin.* **17**, 75.
- Chow, B. F., Greep, R. O. & van Dyke, H. B. (1939). *J. Endocrin.* **1**, 440.
- Cunningham, F. J. & Brown, P. S. (1958). *Nature, Lond.*, **182**, 1238.
- Fraenkel-Conrat, H. L., Simpson, M. E. & Evans, H. M. (1939). *J. biol. Chem.* **130**, 246.
- Fraenkel-Conrat, H. L., Simpson, M. E. & Evans, H. M. (1940). *Proc. Soc. exp. Biol., N.Y.*, **45**, 627.
- Gaddum, J. H. (1953). *J. Pharm., Lond.*, **6**, 345.
- Geschwind, I. I. & Li, C. H. (1958). *Endocrinology*, **63**, 449.
- Hess, R. & Pearce, A. G. E. (1959). *Nature, Lond.*, **183**, 260.
- Legault-Démare, J., Clauser, H. & Jutisz, M. (1958). *Biochim. biophys. Acta*, **30**, 169.
- Li, C. H., Simpson, M. E. & Evans, H. M. (1939). *J. biol. Chem.* **131**, 259.
- Li, C. H., Simpson, M. E. & Evans, H. M. (1940). *Endocrinology*, **27**, 803.
- McShan, W. M. & Meyer, R. K. (1940). *J. biol. Chem.* **135**, 473.
- Peterson, E. A. & Sober, A. A. (1956). *J. Amer. chem. Soc.* **78**, 751.
- Steelman, S. L., Segaloff, A. & Andersen, R. N. (1959). *Proc. Soc. exp. Biol., N.Y.*, **101**, 452.
- Whitten, W. K. (1950). *Aust. J. sci. Res.* **3**, 346.