FRACTIONATION OF PROTEIN HORMONES FROM HORSE PITUITARY GLANDS

ANNE STOCKELL HARTREE, JOHN B. MILLS,*
ROBERT A. S. WELCH† AND MARJORIE THOMAS

*Department of Biochemistry, University of Cambridge, and
Agricultural Research Council Unit of Reproductive Physiology and Biochemistry, Cambridge*

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Summary. A method developed for separation and partial purification of human pituitary hormones has been applied to acetone-dried horse pituitary glands. Growth hormone, follicle-stimulating hormone and luteinizing hormone activities were each obtained in separate fractions. Further purification of each hormone was achieved by the same or slightly modified procedures effective for the corresponding human hormones and the yields and potencies of the preparations were comparable to those obtained by other investigators. Hormones prepared from acetone-dried horse pituitaries stored for 25 years were of similar yield and potency to those prepared from recently collected glands. Horse and human luteinizing hormones cross-reacted immunologically and the horse hormone was assayed by an immunological method. Strong cross-reactions were observed between horse, pig and beef growth hormones and a very weak cross-reaction between horse and human growth hormones.

INTRODUCTION

A fractionation procedure developed for separation and purification of human pituitary hormones (Hartree, 1966) has been applied to acetone-dried horse pituitary glands. Although these studies have not been so extensive as those with human material, the methods used appear to be applicable, in general, to horse pituitaries. In addition we have had the opportunity to compare hormone activities extracted from acetone-dried horse glands stored for 25 years with glands stored for only a few months. These procedures have resulted in purification of horse pituitary FSH, LH and GH. Methods for purification of these hormones have also been described by Saxena & Henneman (1965, 1966), and Reichert & Wilhelmi (1965) have prepared horse LH. The hormone activities of our purified fractions appear to be comparable to their preparations on which potency data are available. The successful fractionation of human and horse pituitary hormones by this procedure suggests that it may be of wide applicability.

* Present address: Department of Biochemistry, Emory University, Atlanta, Georgia.

† Present address: Ruakura Agricultural Research Centre, Hamilton, New Zealand.

MATERIALS AND METHODS

Source of glands and preparation of pituitary powder

Horse pituitary glands were collected by Pink Bros., horse slaughterers, in Cambridge. The glands were removed within 10 min of slaughter, placed in 15 vol. of cold acetone and stored at 4° C. The first batch of 159 glands (Batch A) was minced in an electric meat grinder (model AC 22; Crypto Ltd, London). The mince was filtered with suction, washed with 5 litres of acetone and spread out in a large tray to dry. The yield of powder was 85 g. A second batch of 160 pituitaries (Batch B) yielded 86.4 g of powder. An additional batch (No. 22) of horse pituitary glands which were acetone-dried in 1940 as described by Chance, Rowlands & Young (1939) and stored in an air-tight glass jar at room temperature was kindly provided by Professor A. S. Parkes.

Column chromatography

The cellulosic ion-exchangers, Whatman CM-70 and Whatman DEAE-50 were treated and equilibrated as described previously (Hartree, 1966). Amberlite IRC-50 (Amberlite CG-50, 200-mesh) was equilibrated as described by Heideman, Bakke & Lawrence (1959). Whatman DE-32 was precycled and equilibrated by the method recommended by the makers. All column fractionations were carried out at 4° C.

Extraction of glycoproteins

The procedure was as described previously (Hartree, 1966). Acetone-dried pituitary powder was extracted twice at 4° C with 6% (w/v) ammonium acetate, pH 5.1, in 40% (v/v) ethanol. The soluble extracts were combined and glycoprotein precipitated at 4° C by addition of 2 vol. of cold 96% (v/v) ethanol slowly with stirring. The mixture was left at 4° C for 2 days and the precipitate recovered by centrifugation. After washing once with cold 96% (v/v) ethanol and twice with cold ether, it was dried *in vacuo*. The pituitary residue was washed with cold acetone and air-dried for subsequent extraction of growth hormone (GH).

Preparation of FSH (fraction CP-1) and LH (fraction CM-2)

All steps were at 4° C. The glycoprotein precipitate was extracted overnight with 4 mM-ammonium acetate, pH 5.5, and centrifuged. The insoluble portion was washed twice with the same buffer and the washings were combined with the original extract. This solution was applied to a column of carboxymethyl-cellulose (CM-cellulose), previously equilibrated with the same buffer and the column was washed with more buffer until the E_{280} of the effluent decreased to the base line value. The adsorbed fraction was eluted with M-ammonium acetate. The unadsorbed fraction, designated CM-1, was freeze-dried and the adsorbed fraction (CM-2) was adjusted to pH 5.5 with glacial acetic acid and protein precipitated by slow addition of 5 vol. of 96% (v/v) ethanol with stirring. After 2 days at 4° C the mixture was centrifuged and the precipitate washed and dried as described above. The insoluble portion of the glycoprotein

precipitate was washed and dried similarly. Fraction CM-1 was further purified by methods described by Butt, Crooke & Cunningham (1961a) except that calcium phosphate chromatography preceded DEAE-cellulose chromatography as recommended by Dr W. R. Butt (personal communication). Fraction CM-1 (100 mg) was dissolved in 10 ml of distilled water and mixed with 10 ml of calcium phosphate suspension in water (prepared according to Main, Wilkins & Cole, 1959, and containing 10 m-moles calcium). After mixing for 10 min the suspension was centrifuged and the supernatant fluid added to a suspension of DEAE-cellulose (1.5 ml equilibrated DEAE-cellulose plus 8.5 ml buffer) previously equilibrated with 0.01 M-ammonium acetate. After mixing for a further 10 min, and centrifugation, the supernatant fluid was treated a second time with the same amount of fresh ion-exchanger. The DEAE-cellulose from both treatments was combined and eluted on a column with 0.2 M-ammonium acetate. The effluent (fraction CP-1) was adjusted to pH 5.5 with glacial acetic acid and the protein precipitated with 5 vol. of cold 96% (v/v) ethanol, washed and dried as described above.

Preparation of horse growth hormone

Three methods for preparation of GH were used. Two were modifications of the method of Raben (1959) in which the hormone is extracted in hot glacial acetic acid, and in the third method extraction was with dilute alkali.

Method 1. The procedure of Raben (1959) was followed (starting with 20 g acetone-dried pituitary residue after glycoprotein extraction) except for the last step (ethanol precipitation of growth hormone). For further purification a method devised by Chen, Howard & Wilhelmi (unpublished) and Chen (1964) for purification of pig GH was employed. The GH-containing solution was adjusted to pH 4.0 with N-HCl and solid ammonium sulphate added with stirring to a concentration of 1.25 M. The mixture was kept at 4° C overnight and centrifuged. The precipitate was taken up in 75 ml H₂O and 10 N-KOH was added to the stirred mixture until the pH rose to 10.5 and a clear solution was obtained. Then 13.0 g of solid ammonium sulphate was added, to bring the concentration to 1.2 M, and the pH was adjusted to 7.0. After 18 hr at 4° C the mixture was centrifuged, the precipitate taken up in water, dialysed and freeze-dried to yield 296 mg (preparation E 192 A). Of this fraction 286 mg was further purified by dissolving it in H₂O, removing precipitates at pH 4.9 (in water) and at pH 7.0 (in 0.25 M-ammonium sulphate), and reprecipitating the GH with 1.2 M-ammonium sulphate. The precipitate was taken up in water, dialysed and freeze-dried to yield 68 mg (preparation E 198 C).

Method 2. The procedure of Raben (1959) was followed omitting the oxy-cellulose treatments for removal of ACTH.

Method 3. Crude GH was prepared by suspending 20 g acetone-dried pituitary residue (after glycoprotein extraction) in 400 ml 0.1 N-NaOH at 4° C. The mixture was stirred for 15 min, the pH was adjusted to 10.5 with 4 N-HCl and stirring was continued overnight at 4° C. The pH was adjusted to 7.0, the mixture centrifuged and the residue re-extracted at pH 10.5 for 1 hr. After adjustment to pH 7.0 and centrifugation, the extract was combined with the first extract and 10 N-KOH added, with stirring, until the solution was 0.3 M in potassium

ion. This solution was adjusted to pH 8.5 with glacial acetic acid and centrifuged. The supernatant fluid was treated with an equal volume of 96% (v/v) ethanol at 4° C with stirring. After centrifugation the precipitate was washed with cold ethanol and acetone and dried *in vacuo* to yield 6.4 g of crude GH.

Biological assays

FSH was assayed in mice by the augmentation assay (Brown, 1955; Butt, Crooke, Cunningham & Evans, 1961b) which depends on the increase in weight of the ovary of the immature mouse treated simultaneously with the test material and human chorionic gonadotrophin. The precision of the assays was poor initially, but was greatly improved by using litter mates as suggested by Brown & Wells (1966). Growth hormone was assayed by its effectiveness in increasing the weight of hypophysectomized rats (Hartree, Kovačić & Thomas, 1965).

Immunological studies

These were performed by the haemagglutination inhibition method which has been described previously (Hartree, 1966). Antiserum to human GH was prepared as described by Ehrlich & Randle (1961). Antiserum to human pituitary LH (of potency five times standard NIH-LH-s1) was raised in rabbits using Freund's complete adjuvant. (A horse antiserum to the same LH preparation was prepared by Miss Marion Wood, Wellcome Research Laboratories.) Antiserum to pig GH (Wilhelmi preparation P 511 A of potency 2.3 i.u./mg) was produced in guinea-pigs by Dr Neil Powers using Freund's complete adjuvant. Immunological estimates for horse LH were performed using sheep red blood cells coated with human chorionic gonadotrophin (of potency 2790 i.u./mg kindly provided by Dr W. J. Tindall of Organon Laboratories) and antiserum to human pituitary LH. The cross-reaction between horse pituitary fractions and antiserum resulted in neutralization of the antiserum and inhibition of haemagglutination. The concentration of horse LH was estimated by the same procedure used for human LH (Hartree, 1966) and the results are expressed on a weight for weight basis as percentage of a human LH standard (of biological potency five times standard NIH-LH-s1). The immunoassay values are not a measure of biological potency since they are based on a relatively weak cross-reaction, but they provide a basis of comparison of LH content of various horse pituitary fractions.

RESULTS

Extraction of glycoproteins and separation of FSH from LH

The procedures, which were developed for fractionation of human glycoprotein hormones (Hartree, 1966), are outlined in Scheme 1. The weight yields for each fraction together with biological assays for FSH and immunological estimates for LH are given in Table 1. Although the yield of glycoprotein fraction from acetone-dried powder stored for 25 years (Batch 22) was somewhat

less than the yield from recently collected pituitaries, the FSH and LH activities in the fractions are comparable. Chromatography on CM-cellulose resulted in concentration of FSH in fraction CM-1 and a second fraction (CM-2) containing LH and low in FSH.

SCHEME 1. Flow sheet for separation of horse pituitary GH, FSH and LH into individual fractions.

TABLE 1

WEIGHT YIELDS AND ASSAYS OF FSH AND LH IN HORSE PITUITARY FRACTIONS

Fraction	Yield (g/100 g of pituitary powder)	FSH assay with fiducial limits (P = 0.95) (i.u./mg) ^a	Index of precision (λ) [*]	Immunological estimate of LH ^b (% of human LH std)
Acetone-dried powder (Batch A)	100	Not assayed		0.009
Glycoprotein ppt. (from Batch A)	3.55	14 (5.7 to 33)	0.5	0.37; 0.25
Acetone-dried powder (Batch 22)	100	0.64 (0.44 to 1.5)	0.21	Not assayed
Glycoprotein ppt. (from Batch 22)	2.14	20 (14 to 37)	0.18	0.31; 0.10
CM-1 (28-7-65) ^c	0.73	35	0.45	Not assayed
CM-2 (28-7-65) ^c	0.45	< 10		Not assayed
CM-1 (8-11-65) ^d	1.54	40 (14 to 110)	0.31	0.31; 0.09
CM-2 (8-11-65) ^d	0.93	< 15		0.33
CM-1 (10-1-66) ^e	1.4	23 (11 to 70)	0.22	0.12

* λ = The standard deviation/The slope of dose-response curve.

^a The Second International Standard of Human Menopausal Gonadotrophin was used as standard.

^b Rabbit antiserum to human LH was used for all immunoassays.

^c Prepared from 135 mg glycoprotein ppt. from Batch A; CM-cellulose column dimensions: 1 × 30 cm.

^d Prepared from 500 mg glycoprotein ppt. from Batch A; CM-cellulose column dimensions: 2 × 30 cm.

^e Prepared from 4.0 g glycoprotein ppt. from Batch A and from Batch 22; CM-cellulose dimensions: 5 × 30 cm.

Further purification of FSH

Fraction CM-1 was further purified by calcium phosphate adsorption and DEAE-cellulose chromatography as described in the Materials and Methods section. The purified material (CP-1) was partitioned between ethyl cellosolve, salt and urea as described by Butt, Crooke & Wolf (1965). The yields and

TABLE 2
WEIGHT YIELDS AND ASSAYS OF PURIFIED HORSE FSH PREPARATIONS

<i>Preparation</i>	<i>Yield (g/ 100 g of pituitary powder)</i>	<i>FSH assay with fiducial limits (P = 0.95) (i.u./mg)^a</i>	<i>Index of precision (λ)</i>
CP-1 (17-1-66)	0.13	215 (160 to 320)	0.14
CP-1 (14-4-66)	0.18	252 (125 to 590)	0.36
CP-1 (5-5-66)	0.12	162 (124 to 257)	0.06
CP-1 (10-6-66)	0.14	109 (63 to 237)	0.22
Urea/saline/cellosolve (3-5-66)	0.063	< 100	
Urea/saline/cellosolve (31-5-66)	0.056	38 (12 to 150)	0.24

^a The Second International Standard of Human Menopausal Gonadotrophin was used as standard.

TABLE 3
WEIGHT YIELDS AND IMMUNOASSAYS OF HORSE LH PREPARATIONS PURIFIED BY CHROMATOGRAPHY ON DEAE-CELLULOSE

<i>Fraction</i>	<i>Conditions for elution</i>	<i>Yield (g/100 g of pituitary powder)</i>	<i>Immunological estimates of LH (% of human LH std)</i>
DEAE-1 (16-11-65)	0.1 M-glycine, pH 9.5	0.051	1.8*
DEAE-2 (16-11-65)	1 M-ammonium acetate	0.39	0.22*
DEAE-I (25-11-65)	0.25 M-glycine, pH 9.5	0.26	1.2*
DEAE-II (25-11-65)	1 M-ammonium acetate	0.32	0.18*
DEAE-I (19-1-66)	0.25 M-glycine, pH 9.5	0.21	3.0*; 3.0† 2.5†; 2.4†
DEAE-II (19-1-66)	1 M-ammonium acetate	0.20	0.14*; 0.33†
DEAE-I (13-9-66)	0.25 M-glycine, pH 9.5	0.22	1.1†; 1.6†
DEAE-II (13-9-66)	1 M-ammonium acetate	0.16	1.3†; 1.2†

* Assayed using horse antiserum to human LH.

† Assayed using rabbit antiserum to human LH.

potencies of the preparations are given in Table 2. Calcium phosphate adsorption followed by DEAE-cellulose chromatography resulted in a more than five-fold increase in potency over CM-1 and approximately 50% recovery of activity. Partitioning of CP-1 between cellosolve, salt and urea resulted in low recovery of activity and the product was less potent than the starting material.

Further purification of LH

When fraction CM-2 was chromatographed on DEAE-cellulose in 0.005 M-glycine buffer at pH 9.5 virtually all of the 280 mμ-absorbing material was adsorbed by the ion-exchanger, and was eluted only at glycine concentration

of 0.1 M and above. The remaining protein was eluted with 1 M-ammonium acetate. The fractions obtained, buffer concentration of eluant, yields and immunoassay data for LH are given in Table 3. The best conditions for purification of the immunological LH activity in good yield are chromatography in 0.25 M-glycine buffer at pH 9.5. However, for one preparation (13-9-66) the DEAE-II fraction still contained as much LH as DEAE-I. Bio-assay of DEAE-I (19-1-66) by the OAAD method (Hartree, Butt & Kirkham, 1964) was performed by Dr W. R. Butt who obtained a potency of 1380 i.u. HCG/mg with 95% fiducial limits 670 to 3000.

TABLE 4

WEIGHT YIELDS AND IMMUNOASSAYS OF HORSE LH PREPARATIONS PURIFIED BY CHROMATOGRAPHY ON IRC-50

<i>Fraction</i>	<i>Conditions for elution</i>	<i>Yield (g/100 g of pituitary powder)</i>	<i>Immunological estimate of LH (% of human LH std)</i>
IRC-1 (21-3-66)	pH 8.0 tris-phosphate, 0.005 M in Na ⁺	0.042	1.0
IRC-2 (21-3-66)	1 M-ammonium acetate	0.118	3.2
IRC-1 (28-6-66)	pH 8.2 tris-phosphate, 0.005 M in Na ⁺	0.040	0.53
IRC-2 (28-6-66)	1 M-ammonium acetate	0.108	4.3

Rabbit antiserum to human LH was used in all immunoassays.

TABLE 5

WEIGHT YIELDS AND BIO-ASSAYS FOR HORSE GROWTH HORMONE PREPARATIONS

<i>Preparation</i>	<i>Method used</i>	<i>Yield (g/100 g of pituitary powder)</i>	<i>Growth hormone bio-assay with fiducial limits (P = 0.95) (i.u./mg)</i>	<i>Index of precision (λ)</i>
Horse GH E 198 C from Batch A	1	0.34	0.88* (0.57 to 1.34)	0.19
Horse GH (7-2-66) from Batch 22	2	0.68	0.44 (0.20 to 0.91)	0.32
Horse GH (22-3-66) from Batch A	2	0.75	0.57 (0.26 to 1.16)	0.32
Crude horse GH (8-11-66) from Batch 22	3	28.6	< 0.04	

* Assayed at Emory University. The other preparations were assayed in Cambridge.

The methods used for preparation of horse GH are described in detail in the Materials and Methods section.

Further purification of fraction DEAE-I was attempted by chromatography on IRC-50. Columns of ion-exchanger (1 × 15 cm) were equilibrated with tris-phosphate buffer at pH 8 containing 0.005 M-sodium ion, and 50 mg of fraction DEAE-I (19-1-66) dissolved in the same buffer was applied and the column washed with additional buffer. After elution of the unadsorbed fraction, the protein remaining on the column was eluted with 1 M-ammonium acetate. The yields and immunoassay data from these experiments are given in Table 4. The fraction adsorbed by IRC-50 (IRC-2) appears to be somewhat more potent than the starting material.

Assay of GH preparations and further purification

Weight yields and bio-assay data on GH prepared by the three methods described in the Materials and Methods section are given in Table 5. The yield and potency of GH prepared by Raben's method (1959) is only slightly higher for recently collected glands than for acetone-dried glands stored for 25 years. Because of the imprecision of the bio-assays, this difference cannot be considered significant. The yield of GH prepared by Method 1 is only half as much as that prepared by Method 2, but the potency is appreciably higher. Method 3 yields a large amount of very low potency material.

TEXT-FIG. 1. Chromatography of crude horse growth hormone on DEAE-cellulose. The column was equilibrated with 0.01 M-tris buffer (adjusted to pH 8.0 with formic acid) and the protein was applied in the same buffer. At tube No. 16 gradient elution to 0.3 M-tris-formate at pH 8.0 through a 500-ml mixing chamber was begun. At tube No. 105 M-ammonium acetate was applied to the column. The tubes of effluent, containing 8.5 ml each, were combined as fractions indicated by dotted lines.

Further purification of GH prepared by Method 3 was carried out using DEAE-cellulose chromatography. Gradient elution with tris-formate buffer at pH 8.0 was employed since it had been effective in purification of pig and human growth hormones (Ashworth & Wilhelmi, unpublished observations). Horse GH (352 mg; Method 3) was mixed with 35 ml water and 2 N-NaOH was added dropwise with stirring until it dissolved. It was then dialysed against 0.01 M-tris buffer (adjusted to pH 8.0 with formic acid) and a large amount of

insoluble material was removed by centrifugation. The soluble portion was applied to a column of DEAE-cellulose (Whatman DE-32, 2.1×43.5 cm) previously equilibrated with 0.01 M-tris-formate buffer at pH 8.0. Under these conditions all of the 280 m μ -absorbing material was adsorbed by the ion-exchanger. Gradient elution to 0.3 M-tris-formate at pH 8.0 through a 500-ml mixing chamber resulted in elution of a small peak of 280 m μ -absorbing material (Text-fig. 1). Additional protein was eluted with 1 M-ammonium acetate. The tubes of effluent were pooled into Fractions 1 and 2 as shown in Text-fig. 1. Each fraction was adjusted to pH 5.5 with glacial acetic acid and the protein precipitated at 4° C by dropwise addition of an equal volume of cold ethanol with stirring. After 48 hr at 4° C (to complete precipitation) the protein was centrifuged at 4° C, washed with ethanol and acetone and dried *in vacuo*.

TABLE 6

WEIGHT YIELDS AND BIO-ASSAYS OF HORSE GROWTH HORMONE PREPARATIONS AFTER DEAE-CELLULOSE CHROMATOGRAPHY

Preparation	Conditions for elution	Yield (g/100 g of pituitary powder)	Growth hormone bio-assay with fiducial limits (P = 0.95) (i.u./mg)	Index of precision (λ)
DE-1 (28-11-66) ^a	Gradient from 0.01 to 0.3 M-tris formate, pH 8.0	0.43	0.56 (0.24 to 1.09)	0.27
DE-2 (28-11-66)	1 M-ammonium acetate	3.2	Not assayed	
DE-1 (7-12-66) ^a	0.1 M-tris formate, pH 8.0	0.71	0.75 (0.34 to 1.48)	0.27
DE-2 (7-12-66)	1 M-ammonium acetate	2.8	0.03	
DE-1 (8-5-67) ^b	0.035 M-tris formate, pH 8.0	0.03	0.78 (0.47 to 1.26)	0.25
DE-2 (8-5-67)	1 M-ammonium acetate	0.08	0.40 (0.23 to 0.68)	0.25

^a The starting material was crude horse GH (8-11-66) prepared by Method 3.

^b The starting material was horse GH (7-2-66) prepared by Method 2.

As a result of this experiment, a simpler procedure was developed in which the ion-exchanger was equilibrated with 0.1 M-tris-formate buffer and GH (Method 3) was equilibrated with the same buffer and applied to the column. The column was washed with additional buffer and the unadsorbed material was pooled as fraction DE-1. Additional protein (fraction DE-2) was eluted with 1 M-ammonium acetate. Data on yields and potencies of these preparations are given in Table 6.

Further purification of horse GH (Method 2) was performed by DEAE-cellulose chromatography at pH 8.0 and stepwise changes in buffer concentration. The yields and potencies of these fractions are also given in Table 6.

Dr Isabel Forsyth has assayed two preparations for prolactin activity by local injection into the pigeon crop gland. Raben horse GH (7-2-66) contained 0.1 to 0.5 i.u. prolactin/mg and preparation DE-1 (7-12-66) contained less than 0.1 i.u. prolactin/mg.

The method which gives the best yield of high potency GH (DE-1, 7-12-66)

C*

with negligible contamination with prolactin is thus alkaline extraction (Method 3) followed by chromatography on DEAE-cellulose in 0.1 M-tris-formate at pH 8.0.

Immunological studies of growth hormones

Antiserum to pig GH was effective in agglutinating sheep red blood cells coated with horse GH or with pig GH. In addition to this demonstration of immunological cross-reaction, pig, horse, beef and human growth hormones were tested for inhibition of haemagglutination in both systems and also with human GH antiserum and erythrocytes coated with the same hormone. The

TABLE 7

IMMUNOLOGICAL COMPARISON OF GROWTH HORMONES FROM DIFFERENT SPECIES

Antiserum:	Antiserum to pig GH (P 511 A) raised in guinea-pigs diluted 1:9600		Antiserum to human GH (MY-61) raised in rabbits diluted 1:2400
	Pig GH (P 511 A)	Horse GH (DEAE-1, 25-7-66)	Human GH (MY-61)
Antigen attached to cells:	Dilution of antigen (mg/ml) at which haemagglutination was inhibited		
Antigen			
Pig GH (P 511 A)	1:5540	1:695	<2
Horse GH (E 198 C)	1:980	1:2770	1:1.5
Beef GH (B 809 A)	1: > 2560	1:2770	<2
Human GH (MY-61)	Not tested	1: < 5	1:9600

results are given in Table 7. High dilutions of pig, beef and horse growth hormones were effective in neutralizing antiserum to pig GH, thus inhibiting agglutination of cells coated with pig or horse GH, but human GH was ineffective. Conversely, pig and beef hormones did not prevent haemagglutination of cells coated with the human hormone in the presence of antiserum to human GH. Horse GH showed a slight cross-reactivity with human GH. At low dilution it neutralized antiserum to human GH and prevented haemagglutination.

DISCUSSION

Saxena, McShan & Meyer (1962) have described purification of horse FSH and Saxena & Henneman (1965, 1966) have given methods for separation and purification of horse FSH, LH and GH. A comparison of the potency of the purified FSH of Saxena *et al.* (1962) with our CP-1 can be made if we use the values determined by Butt, Crooke & Wolf (1965) for converting standard NIH-FSH into i.u. 2nd IRP-HGM (8 i.u. 2nd IRP-HGM = 0.31 mg NIH-FSH-s1). Their preparation would thus be 181 i.u. 2nd IRP-HGM/mg, which is in the potency range of our CP-1 preparations. Assuming that horse pituitary glands contain 80% water, our average yield of CP-1 (0.14 g/100 g pituitary powder = 280 µg/g fresh glands) is about four times higher than theirs (59 µg/g fresh glands). Standard hormone preparations were not included in the assays of Saxena & Henneman (1965) for FSH and LH activities and so the yields and potencies of

our gonadotrophin fractions cannot be compared with theirs. Reichert & Wilhelmi (1965) have purified horse LH by ethanol and trichloroacetic acid fractionation followed by chromatography on DEAE-cellulose and IRC-50. This method yielded 367 mg LH/kg of whole glands of potency $0.9 \times$ standard NIH-LH-S1. Our DEAE-I (19-1-66), which was bio-assayed in terms of an HCG standard, is of approximately the same potency if we use the conversion factor reported by Butt *et al.* (1965), i.e. that 0.0053 mg NIH-LH-S1 is equivalent to 10 i.u. HCG. Thus the assay value of 1380 i.u. HCG/mg is equivalent to $0.73 \times$ standard NIH-LH-S1. Again assuming an 80% water content of the glands, our yield of LH (0.21 g/100 g pituitary powder = 420 mg/kg whole gland) is also comparable to theirs. Although we used different conditions of pH and ionic strength for DEAE-cellulose chromatography, our IRC-50 purification was performed with a similar buffer system and our finding that LH was adsorbed to the ion-exchanger under these conditions is in complete agreement with theirs.

The yield of GH obtained by Saxena & Henneman (1966) from the pituitary residue after extraction of gonadotrophin was about twice as high as ours although we followed the same method (Raben, 1959). After further purification by gel-filtration, their material was as potent as our most active preparation (0.9 i.u./mg). Although their purified GH still contained significant prolactin, our preparation which had been chromatographed on DEAE-cellulose was devoid of this activity.

Our procedure is the first elaboration of a method for obtaining horse pituitary FSH, LH and GH from the same starting material with known yields of activity. It is of interest that the yields of FSH, LH and GH from acetone-dried pituitaries stored for 25 years are similar to those obtained from glands stored for only a few months.

The initial extraction of horse FSH and LH followed by their separation on a CM-cellulose column has been achieved by the methods used for the corresponding human hormones. Further purification of horse FSH by calcium phosphate adsorption and DEAE-cellulose chromatography, as applied to human FSH (Butt *et al.*, 1961a), was also effective. The average recovery of activity in horse CP-1 is 26,300 i.u./100 g of pituitary powder which represents 41% of the FSH originally present (64,000 i.u./100 g of pituitary powder). Further purification of horse CP-1 by partitioning between cellosolve, salt and urea was unsuccessful and resulted in 90% loss of activity. Thus horse FSH is not as stable to this treatment as human FSH.

Chromatography of horse CM-2 (LH) on DEAE-cellulose at pH 9.5 in glycine buffer required higher ionic strength (0.25 M-glycine) than was necessary for human LH (0.1 M-glycine) in order to recover most of the activity in the un-adsorbed fraction. This suggests that horse LH is slightly more acidic than human LH.

The Raben method (1959) is effective for purification of both horse and human growth hormones. Horse GH is obtained in lower yield (0.7 g/100 g of pituitary powder) than is human GH (4.3 g/100 g) (Hartree, 1966), but Saxena & Henneman (1966) report higher yields of horse GH (1.5 g/100 g) by this method.

The biological potencies of horse CP-1 (FSH), DEAE-1 (LH) and Raben GH are all somewhat lower than the corresponding human preparations. Although it is possible that they are less highly purified, the horse hormones may be less active in the assay animals (rats and mice) than are the human hormones because of differences in response among species.

The immunological estimates of horse LH activities in various fractions cannot be directly compared with the corresponding human preparations because only a relatively weak cross-reaction between horse LH and antiserum to human LH is measured. The horse preparations have immunological potencies 7 to 10% of the corresponding human fractions in our assay system.

Although we have not shown correlation between biological assay and our immunoassay for horse LH, it is known that this immunoassay for human LH correlates well with bio-assay by the OAAD method (Hartree *et al.*, 1964). Moreover, comparison of bio-assay of sheep pituitary fractions with immunoassay (Hartree & Greenhalgh, unpublished results) suggests that in this species the two methods agree within their limits of precision. In the present study, horse LH activity was followed solely by immunoassay. Bio-assay of a purified fraction (DEAE-1, 19-1-66) demonstrated that its yield and potency were comparable to horse LH prepared by a very different method (Reichert & Wilhelmi, 1965). The use of an immunological cross-reaction between HCG (attached to sheep erythrocytes) and antiserum to human LH decreases the possibility that pituitary protein contaminants of human or horse LH will be measured in the immunoassay. Since chorionic gonadotrophin is obtained from pregnancy urine it is unlikely to possess the same contaminants present in pituitary LH fractions.

In our immunological studies of horse GH by the method of haemagglutination inhibition, we observed strong cross-reactions between horse, pig and beef growth hormones and a very weak cross-reaction between horse and human hormones. Saxena & Henneman (1966) reported no cross-reaction between horse and human GH in a system similar to ours, and Laron (quoted by Saxena & Henneman, 1966) was unable to demonstrate cross-reaction between horse GH and antiserum to beef GH in an Ouchterlony double diffusion system. These differences in immunological results may be explained by differences in the antisera or in the immunological methods employed.

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