A Simplified Procedure for Obtaining Follicle-Stimulating Hormone from Sheep Pituitary Glands.* (20892)

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It was reported previously(1) that saturated solutions of NaCl and KCl could be used at 70°C for extracting gonadotrophins from dry sheep pituitary tissue, but that fractionation of the dialyzed extract did not result in the separation of the follicle-stimulating and luteinizing activities. On the basis of the hypothesis that pituitary gonadotrophins are associated with each other and with inert protein, a procedure was sought for dissociating follicle-stimulating hormone from these materials so that separation and purification could be effected readily. This was the rationale for heating dry pituitary tissue in saturated sodium chloride solution and fractionating the resulting extract. It was assumed that under these conditions weak linkages in the tissue constituents might be disrupted and liberate at least a part of the follicle-stimulating hormone. Results reported in this paper show that luteinizing hormone is removed from the saturated salt extract by isoelectric precipitation and that a part of the follicle-stimulating activity is subsequently recovered from the soluble fraction after removal of the salt by dialysis. This provides a short simple procedure for obtaining relatively large amounts of pituitary follicle-stimulating hormone.

Materials and methods. The sheep pituitary powder was prepared by dehydrating ground fresh glands with acetone. The saturated sodium chloride solution was prepared by shaking an excess of the salt with water at room temperature. The anion exchange resin XE-59 (Rohm and Haas, Philadelphia, Pa.) was used for concentrating the follicle-stimulating activity. Sixteen g of resin were vigorously shaken with 500 ml of distilled water, and the supernatant liquid was decanted after centrifugation. The washed resin was suspended in 1200 ml of 2% NaOH, mixed thoroughly by shaking, allowed to stand for 5 minutes and centrifuged. The recovered resin was washed free of NaOH with liberal amounts of water by repeated suspension, centrifugation and decantation. This procedure was repeated with 1200 ml of 2% HCl (4.55 ml concentrated HCl in 100 ml). After the resin was washed free of HCl with water it was treated again with 2% NaOH and washed free of the base. The activated resin was resuspended in 2 liters of water and recovered with suction on a Buchner funnel using No. 40 Whatman filter paper. The washed resin was suspended in 500 ml of distilled water and adjusted to pH 5.8 by careful addition of 2% HCl. The pH was checked again after 10 minutes to determine whether it was stabilized at 5.8. The amount of resin present was determined by drying two 5-ml samples for 24 hours at 75°C. This made it possible to measure a definite amount in terms of dry weight for an experiment. The activated resin was prepared one day before being used.

Preparation. The acetone dried pituitary powder was mixed in the ratio of one g of powder to 10 ml of saturated sodium chloride solution and extracted by stirring at 68°C or at 72°C for one hour. The pituitary mixture was maintained at these temperatures by placing it in a water bath which was 2 to 3 degrees above the temperature of the mixture. The extract was recovered by centrifuging and the residue was extracted a second time in the same way. The 2 extracts were combined. Usually 200 g of pituitary powder were used, but larger amounts could be processed with the proper facilities. The combined extract was allowed to cool to 30°C before adjusting it to pH 2.5 by addition of single drops of concentrated HCl during stirring. A heavy precipitate formed at pH 2.5 which contained considerable follicle-stimulating and luteiniz-

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TABLE I. Effect of Follicle-Stimulating Preparation on the Ovaries of Normal and Hypophysectomized Female Rats.

Kind of rats	Fraction	Dose, mgEq	No. of rats	Avg ovarian wt, mg	Ovarian response	Protein, mg/g
♀ Normal	SAC*	250	6	47	F†	
(21 days old, inj. 4.5 days)		500‡	31	63	F‖	3.56
		500§	6	74	F¶	4.40
Hypophysectomized	SAC	1000	6	106	F	
(29 days old, inj. 10 days)		2000	12	155	F	
		3000	4	151	F	
		5000	3	202	Cloudy F	

* Fraction soluble at pH 2.5 dialyzed and dried by lyophilization.
† F = Follicles.
‡ Made by extracting at 68°C.
§ *idem* at 72°C.
‖ Three pairs of ovaries contained a few corpora.
¶ One pair of ovaries contained 4 corpora.

ing activities. The fraction soluble at pH 2.5 contained follicle-stimulating activity. The salt was removed from this fraction by dialysis first with distilled water and finally against 0.01% KCl solution. After dialysis a light inactive precipitate was removed by centrifugation. The activity was recovered from the solution either by lyophilization or by using the anion exchange resin XE-59 in batch operation. Since recovery by lyophilization is time consuming and somewhat expensive, the exchanger was usually used for concentrating the hormone. Another factor favoring the use of the resin is that the final product contained about 80% less protein than when the recovery was done by lyophilization. When the exchanger was used the pH 2.5 dialyzed fraction, approximately 4500 ml from 200 g of pituitary powder, was adjusted to pH 5.8, and mixed with the activated anion exchanger. An equivalence of 40 mg of dry resin obtained by suction filtration of the pH 5.8 resin suspension was used per g of starting dry pituitary. This mixture was shaken for 90 minutes, the resin was recovered on a Buchner funnel, washed with one liter of water previously adjusted to pH 11.2 with NaOH, suspended in 500 ml of 3.72% sodium acetate and shaken for 45 minutes. The sodium acetate eluted the follicle-stimulating activity from the resin and was recovered in the acetate solution by filtering off the resin. The acetate was removed by dialysis against 0.01% KCl and the hormone was recovered in the dry form by lyophilization.

Assay. The preparations were assayed for gonadotrophic activity by using 21-day-old normal female rats of the Holtzman-Rolfsmeyer strain. The volumes of the fractions were adjusted with 0.9% saline solution so that each rat received the proper gram equivalent (g eq.) of dry pituitary tissue in 9 injections of 0.5 ml each. The first injection was made on the afternoon of the first day followed by injections on the morning and afternoon of each of the next 4 days. Autopsy was performed on the morning following the last injection. The ovaries were removed, dissected free of other tissues, weighed and examined by means of strong transmitted light for the presence of follicles and corpora lutea. Assays were also made with male and female rats which were hypophysectomized when 27 days of age. Injections were started when the rats were 29 days old, and were made on the morning and afternoon of each day for 10 days with autopsy on the morning of the 11th day. The ovaries and testes were removed, dissected free of other tissues, weighed and the ovaries examined for the presence of corpora lutea and follicles. The tissues were preserved in Bouin's solution and subsequently prepared for microscopic study.

Results. Follicle-stimulating preparation. Results given in Table I show that, with few

TABLE II. Effect of Follicle-Stimulating Preparation on Testes and Accessories of 29-Day-Old Hypophysectomized Male Rats.

Kind of rats	Fraction	Dose, mgEq	No. of rats	Wt of glands (mg) Sem. vesicle	Prostate	Testes	Thyroids
♂ Hypophys. (inj. 10 days)	SAC	1000	6	5.2	6.9	640	3.5 (2)
		2000	12	5.3	10.2	677	4.4 (4)
		3000	2	5.0	10.5	735	
		5000	2	6.3	13.5	796	5.7 (2)
Hypophys. (non-inj. controls)			8	5.1	6.3	127	4.5 (3)

exceptions, the preparation recovered from the pH 2.5 soluble fraction by dialysis and lyophilization produced ovaries that contained only follicles when 0.5 g eq. was given to normal female rats. This is true for preparations made by extracting either at 68°C or 72°C. The high temperature is necessary for extracting the hormone since little follicle-stimulating activity was recovered when the extractions were run for 4 to 8 hours at room temperature. On the basis of the above results it was decided to assay the preparations by administering them for 10 days to 29-day-old hypophysectomized male and female rats. The amounts given ranged from 1 to 5 g eq. and the average ovarian weights ranged from 106 mg to 202 mg, respectively. Macroscopically, all of the ovaries contained follicles only but those from rats which received 5 g eq. were cloudy (Table I).

The data obtained from hypophysectomized male rats are given in Table II. The seminal vesicles of all animals given the follicle-stimulating preparation were not significantly increased over those from non-injected animals. The average weight of the prostate of the animals given 5 g eq. was 13.5 mg as compared to 6.3 mg for the glands of the control animals. The testes, however, were increased from an average control value of 127 mg to 796 mg for those of the animals given 5 g eq. of the preparation. On the basis of relatively few data it appears that this preparation is free of thyrotrophic activity as indicated by little or no increase in the weight of the thyroids from hypophysectomized rats as compared to those of the control animals (Table II). The preparation contains no lactogenic activity as indicated by its failure to stimulate the crop glands of pigeons. The preparation is free of adrenotrophic hormone as 5 g eq. did not decrease the ascorbic acid in the adrenals of young adult hypophysectomized rats.

TABLE III. Effect of Follicle-Stimulating Preparation Recovered by Anion Exchange Resin on Ovaries of Normal 21-Day-Old Female Rats.

Fraction of saturated NaCl extract*	No. of preparations	Dose, mgEq	No. of rats	Avg ovarian wt, mg	Ovarian response†	Protein, mg/g
1. SAC	7	500	22	*57*	F	4.58 (8)
2. SA(A-4)ND	8	500	24	74	" ‡	1.36 (1)
3. SA(A-4)DC	7	500	21	65	" §	1.11 (1)
4. SA(A-4)D	6	500	22	*58*	"	1.06 (8)
	3	1000	12	93	"	
5. SA(A-1)	7	1000	23	17.6	Nonstim.	1.40 (7)
6. SA(A-5)D	7	1000	21	16.6	"	0.17 (7)

* Fractions in this and other tables are designated as follows: SAC—fraction soluble at pH 2.5 dialyzed and concentrated by lyophilization; SA(A-4)ND—activity from SAC eluted from anion resin XE-59 with NaAc-nondialyzed; SA(A-4)DC—NaAc removed by dialysis and concentrated; SA(A-4)D—No. 3 dried by lyophilization; SA(A-1)—supernatant from SAC after treatment with resin; SA(A-5)D—second NaAc eluate from resin.

† F = Follicles.

‡ Corpora lutea in 3 pairs of ovaries.

§ " " " 1 " " "

TABLE IV. Effect of Follicle-Stimulating Preparation Recovered with Anion Exchange Resin on Gonads of Male and Female Rats.

Kind of rats	Age	Fraction	Dose, mgEq	No. of rats	Ovaries	
					Wt, mg	Response
Normal female	21	SA(A-4)D	500	10	41	F*
			1000	6	79	F
Hypophys. females	29	"	5000	6	145	F & cloudy F
					Wt of glands (mg)		
					Sem. vesicle	Prostate	Testes
Hypophys. males	29	"	5000	6	4.4	9.5	745
Controls (non-inj.)				8	5.1	6.3	127

* F = Follicles.

The data given in Table III show that essentially all the follicle-stimulating activity was recovered from the pH 2.5 soluble dialyzed fraction by adsorption on the anion exchanger XE-59 and elution by one treatment with sodium acetate solution. This is shown by the fact that the average ovarian weight was 57 mg (line 1) when a dose of 500 mg was assayed in normal female rats before treatment as compared to an average ovarian weight of 58 mg (line 4) for the preparation after recovery of the activity from the exchanger. Seven preparations were used for these assays. Further support for this conclusion is found in the data of line 5 which show that the supernatant remaining after treatment of the solution with the exchanger did not contain significant activity. Furthermore, the fraction obtained by treating the exchanger a second time with sodium acetate did not contain significant gonadotrophic activity as shown by the data of line 6.

Before treatment with the anion exchanger these preparations contained nitrogen equivalent, on the average, to 4.58 mg of protein per g eq. but after treatment the active fraction represented by SA(A-4)D contained nitrogen equivalent to 1.06 mg of protein per g eq. of original pituitary powder. Thus, a 77% decrease in nitrogen or protein content was effected by treatment with the anion exchanger and essentially all the hormone activity was recovered. The final preparation dried by lyophilization was slightly pink in color. This was presumably due to the concentration of pigment from the soluble extract by the resin.

Two preparations obtained by use of the exchanger were assayed in normal female and hypophysectomized female and male rats. The results given in Table IV show that 0.5 g and 1.0 g eq. produced ovaries in normal rats that had average weights of 41 mg and 79 mg, respectively. Five g eq. given for 10 days to hypophysectomized female rats produced ovaries which weighed 145 mg and which contained only follicles as shown by microscopic examination. The uteri of these animals were distended with fluid. When the same amounts of these preparations were given to hypophysectomized male rats for 10 days, the weight of the seminal vesicles was not greater than those of non-injected controls, whereas the weight of the prostate was increased only 3.2 mg over the control value. The weight of the testes, however, was increased 618 mg over the control weight.

Microscopic study of the ovaries from hypophysectomized animals which received 5 g eq. of preparation SA(A-4)D (Table IV) showed no distinct lutein tissue although the theca interna and interstitial cells of some ovaries showed evidence of slight secretory activity. The tubules in the testes from hypophysectomized rats were highly stimulated and numerous spermatids were present, but there was little stimulation of the interstitial tissue. These results indicate that the preparation obtained by use of the anion exchanger is predominantly follicle-stimulating in action being essentially free of luteinizing activity. The activity of this preparation is about the same as the preparation obtained by tryptic digestion(2) when they are compared on the basis of activity recovered from a gram of dry pituitary glands. This is

not the case, however, when they are compared on the basis of protein content since the new preparation contains 10 to 15 times less protein per gram of dry pituitary than does the preparation obtained by tryptic digestion.

A preparation recovered by use of the anion exchanger was subjected to electrophoretic analysis. The material was prepared for this experiment by dissolving it in 0.1 ionic phosphate buffer of pH 7.4. A fast moving component which spread with time was withdrawn from the electrophoretic cell and found to contain the major part of the gonadotrophic activity. There were two other components which migrated slowly and on assay they were found to contain little gonadotrophin. Attempts to separate the active component by column chromatography using the anion exchanger XE-59 were not successful.

A light precipitate which formed on removal of the salt from the saturated extract by dialysis was discarded since it did not show gonadotrophic activity when one and 2 g eq. were given to normal female rats.

The results of preliminary experiments indicate that essentially all the lactogenic and adrenotrophic hormones are present in the residue that remains after the extraction with saturated sodium chloride solution. These hormones probably can be recovered from this residue by known procedures.

The precipitate obtained by adjusting the saturated salt extract to pH 2.5 contained luteinizing hormone and considerable follicle-stimulating activity (Table V). This precipitate may form at a pH as high as 3 but the major precipitation occurs at a pH lower than 4 where considerable gonadotrophin precipitates from an aqueous extract.

Treatment of the pituitary tissue with saturated sodium chloride solution at a high temperature may result in aggregation, denaturation and dissociation of proteins. Each of these processes may play a part in making the above separation possible. The fact that a good part of the follicle-stimulating hormone remains in solution on isoelectric precipitation of the luteinizing activity from the salt extract suggests that dissociation of linkages in the tissue protein may account in part for this sharp separation. Further support for this concept is found in results reported previously(1) which showed that the two gonadotrophic activities are not separated by isoelectric fractionation of the salt extract after dialysis, or by fractionation in the same way of an aqueous extract made by heating.

TABLE V. Gonadotrophic Activity of Saturated Sodium Chloride Insoluble Fraction.

Fraction*	Dose, mgEq	Avg ovarian wt, mg
P95 (dried)	100	30 (3)†
" "	200	100 (3)
" "	300	121 (3)
" "	500	134 (3)

* pH 2.5 insoluble.
† No. of rats used for assay.

In the light of the above results and discussion it appears that the follicle-stimulating hormone may be present in dry pituitary tissue in different forms such as: (a) the "free" hormone, (b) in combination with luteinizing hormone, (c) in combination with inert protein, and (d) a form dissociated by heating in saturated sodium chloride solution. This may also be true for the follicle-stimulating hormone of fresh pituitary tissue and even for that of intact glands. If this hypothesis is correct, it would obviously account for a part of the difficulty workers have encountered in purification and separation of pituitary follicle-stimulating hormone. It would also seem probable on the basis of this hypothesis that there is some "free" or "complete" follicle-stimulating hormone present at any given time in the gland so that it is available for secretion into the blood as the endocrine and physiological balances demand it.

Summary. 1. A short simple method is given for obtaining from sheep pituitary glands a follicle-stimulating hormone preparation of high biological purity and low protein content. This method consists of extracting the glands with saturated sodium chloride solution at 72°C, adjusting the extract to pH 2.5, dialysis of the soluble fraction and recovery of the hormone from the dialyzed fraction by lyophilization or by the use of an anion exchanger. 2. When 5 g eq. were given to male and female hypophysectomized rats for 10 days, only a very small amount of luteinizing

activity was found. The fraction insoluble at pH 2.5 contained both follicle-stimulating and luteinizing activities. 3. The results are discussed from the viewpoint that follicle-stimulating hormone may exist in pituitary tissue in different forms.

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2. ———, *J. Biol. Chem.*, 1940, v132, 473.

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Failure to Demonstrate Skin Groups Analogous to Blood Groups Using Human Skin Cultures.* (20893)

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The question as to whether human skin partakes of the group characteristics of blood is still unsettled. The lack of definitive work on this problem is due in part to the fact that skin cells being fixed cells do not lend themselves to the typing procedures applicable to erythrocytes. In the present work the tissue culture method was utilized in a study of this problem.

Materials and methods. Specimens of human skin from donors of known blood types were obtained at the operating table, cut in varying thicknesses with the electric dermatome or in full thickness with the ordinary scalpel. The specimens were immediately placed in a mixture of 90% Tyrode's solution and 10% pooled human serum and refrigerated until explants were made in from one to two hours. The Maximow double coverslip method in which the explant was embedded in a plasma clot after the method of Burrows(1), was used. Under these conditions explants showed growth in from 3-4 days and reached maximum growth after 6-12 days, and after 15-17 days began to regress and were discarded. The following groups of experiments were performed: A. 115 cultures (from 29 donors) of skin grown with pooled human cord serum as nutrient and at their maximum growth, were first washed with Tyrode's solution and then treated as follows: 1. 82 cultures from blood Group A donors with one drop of Anti A serum. 2. 33 cultures from blood Group B donors with one drop of Anti B Serum. The sera were commercial "high potency antisera from donors whose antiserum titer had been stimulated by either A or B specific substances for a suitable period previous to their blood donation,"(2) and whose agglutinative power against the corresponding erythrocytes had been checked in this laboratory before they were used. B. 98 skin explants from donors whose blood groups had been previously determined (62 A and 36 B) were made according to the method described above, but with one drop of the corresponding antisera as nutrient from the start. C. A third group of experiments was suggested by the work of Bassett, Earle and Campbell(3) which in turn were suggested by an observation made by Farr and his co-investigators(4). Farr and his co-workers found that in the plasma and serum of rabbits there is "normally present a protective factor against the leukopenic effect of total body irradiation but that this factor is largely destroyed by total body or *in vitro* irradiation with 800r. They have also found that there is a naturally occurring protective factor against pyrogens in the blood of rabbits but the relationship between the irradiation factor and the pyrogen factor has not been studied as yet." Bassett, Earle and Campbell observing that the serum of rabbits previously inoculated with human skin material had no effect on cultures of human skin, suspected that a protective factor might likewise be

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