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Factors Affecting Extraction and Recovery of Follicle Stimulating Hormone From Sheep Pituitary Glands.* (22532)

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A method was reported previously for extracting gonadotrophin from dry sheep pituitary tissue with saturated solutions of NaCl and KCl at 70°C(1). Further work resulted in a short simple procedure for obtaining follicle stimulating hormone by extracting pituitary tissue with saturated NaCl solution, removal of a protein fraction from the extract at pH 2.5, dialysis to remove the salt from the soluble fraction, and recovery of the activity quantitatively by use of an anion exchange resin(2). This procedure provides a method for obtaining relatively large amounts of follicle stimulating hormone (FSH) essentially free of luteinizing hormone (LH). The basic steps in this procedure were studied to determine the optimum conditions for increasing the yield and/or biological purity of the FSH preparation. The factors studied were concentration of salt and pH during extraction, and temperature and pH during fractionation. These factors were varied as indicated in the procedure, and the results obtained are reported in this paper.

Materials. Acetone dried whole sheep pituitary tissue was the starting material for these experiments. The NaCl solutions used for extraction were 80, 90 and 100% saturated. The saturated solution was prepared by shaking for one hour a given volume of distilled water with an excess of NaCl. The 80 and 90% saturated solutions were prepared by diluting the saturated solution with the proper volume of distilled water. The anion exchange resin XE-59 and the cation exchange resin XE-97 were obtained from the Rohm and Haas Co., Philadelphia, Pa.

Method of preparation. The amounts of pituitary powder extracted varied from 10 to 100 g but in most cases either 50 or 100 g were used. The dry pituitary tissue was mixed with the salt solutions in the ratio of 1 g of powder to 10 ml of the proper concentration of NaCl solution. The mixture was adjusted either to pH 4, 5, 5.5 or 6 with concentrated HCl or to pH 7, 8, 9 or 10 with NaOH. The mixture was allowed to stand for

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at least 20 minutes to reach equilibrium and a stable pH value. The mixture was maintained at 70°C for 1 hour by heating in a water bath during continuous stirring. The extract was recovered by centrifuging and the residue was extracted a second time in the same way. It was necessary to readjust the pH on the alkaline side for the second extraction. The 2 extracts were combined and allowed to cool to 30°C before adjusting to pH 2.5 by adding concentrated HCl dropwise while stirring. A few extracts were adjusted either to pH 3.0 or to pH 2.0. A heavy precipitate which formed at these pH values contained considerable follicle stimulating and luteinizing activities. This precipitate was utilized for other purposes. In some experiments the fractionations were also done at 50°, 60° and 70°C. The extraction was done as described above. After the first extract was recovered by centrifuging it was heated again to the desired temperature, adjusted immediately to pH 2.5 with HCl and centrifuged. The residue obtained after the first centrifugation was extracted again and the second extract was fractionated in the same way. The two fractions soluble at pH 2.5 were combined. The pH 2.5 soluble fractions

TABLE I. Effect of Follicle Stimulating Hormone Preparations Prepared at Different pH and Salt Concentrations on Ovaries of Normal Female Rats. (Fractionations were done at 30°C.)

				Concentration of NaCl solution used for extraction					
				% saturation					
				100		90		80	
				Assay of fractions—wt of glands (mg)					
Prep. No.	Extraction pH	Fractionation pH	Dose, g eq.	Protein, mg/g eq.	Ovaries*	Protein, mg/g eq.	Ovaries	Protein, mg/g eq.	Ovaries
A1	4	2.5	.5	8.6	14 ± .9†(9)‡				
			1.0		17 ± .2 (6)				
			2.0		14 (3)				
A2	5	2.5	.5	4.9	26.5± 1.3 (21)	4.6	34±4.1 (6)	5.7	40± 2.8(6)
			1.0		45.7± 1.8 (18)		53±6.0 (6)		117± 3.3(6)
			2.0		86 ±14.4 (5)				
A3	5.5	2.5	.5	4.8	40 ± 3.5 (6)				
			1.0		84 ±28.7 (6)				
A4	6	2.0	.5			3.6	74±8.0(13)		
		2.5	.5	5.5	38 ± 2.6 (20)	3.8	116±6 (15)§	4.6	102± 6.9(6)
			1.0		69 ± 5.6 (20)		147(3)		145±19 (6)§
			2.0		103 ± 9.0(11)§				
		3.0	.5			20.4	112±8 (24)§		
			1.0				116±9 (9) §		
A5	7	2.5	.5	4.3	60 ± 3.8 (32)	4.2	96(3)		
			.5			1.0	116(3)‖		
A6	8	2.5	.5	3.9	46 ± 3.2 (6)				
			1.0		120 ±10 (6)§				
A7	9	2.5	.5	7.0	65 ±14.3 (6)§				
			1.0		132 ±16.3 (6)§				
A8	10	2.5	.5		14 ± .9 (6)				
			1.0		15 ± 1.1 (6)				
A8 + FSH			.5		109 (3)				
			.5						
Idem			1.0		106 (3)				
			.5						
FSH			.5		118 (3)				
Control (uninjected)					17 ± 1.5 (8)				

* Ovaries contained only follicles unless otherwise designated.

† Stand. error of mean in this and subsequent tables.

‡ Numbers in parentheses in this and subsequent tables indicate No. of animals used for assaying preparations.

§ Ovaries contained 1-7 corpora lutea/ovary in ⅓ or less of animals in each group.

‖ This fraction was purified with anion exchange resin XE-59 and as indicated 96 mg ovaries were obtained before and 116 mg after purification.

TABLE II. Effect of Follicle Stimulating Hormone Preparations Prepared at Different pH and Salt Concentrations on Ovaries, Testes and Accessories of 29-Day-Old Hypophysectomized Female and Male Rats.

			Concentration of NaCl solution used for extraction (% saturation)											
			100				90				80			
			Assay of fractions—wt of glands (mg)											
Prep. No.*	Ext'n pH	Dose, g eq.	Ovaries†	Testes	Ventral prostate	Sem. ves.	Ovaries†	Testes	Ventral prostate	Sem. ves.	Ovaries†	Testes	Ventral prostate	Sem. ves.
A2	5	5	113 (3)	728 (3)	7.7	4.1	206±41(5)	923±30(8)	12.9±1.4	6.3±.9	198 (2)	942 (3)	13.0	4.9
		10	118 (3)	811 (3)	8.7	4.6								
A3	5.5	5	118 (3)	875 (3)	11.5	4.1								
		10	159 (4)	867±63(6)	15.2±.8	6.7±.2								
A4	6.0	5	113 (3)	781 (3)	8.5	5.3	123 (4)	907 (3)	16.4	7.9	166 (2)	882 (2)	38.8	18.5
		10	210 (3)	988 (3)	13.2	5.2								
A5	7.0	5	145 (3)	746 (3)	13.5	6.3	204 (1)	1137 (3)	24	15.2				
		5					269‡(1)	1214 (3)	25.6	20.2				
Hypophysectomized rats—uninj. controls			6.4±.4(6)	191±10(11)	6.3±.4	5.2±.2								

* These preparations from Table I were fractionated at 30°C and pH 2.5. † These ovaries contained only follicles. ‡ This fraction was purified further with anion exchange resin XE-59.

TABLE III. Biological Effect in Normal and Hypophysectomized Rats of FSH Preparations Extracted at pH 6 and Fractionated at High Temperatures.

Method of preparation					Assay of fractions—wt of glands (mg)						
					Normal rats	Hypophysectomized rats					
Prep. No.	Extraction Salt conc., % sat.	Fractionation Temp., °C	pH	Protein, mg/g eq.	Dose, g eq.	Ovaries*	Dose, g eq.	Ovaries*	Testes	Ventral prostate	Sem. ves.
1	100	70	2.5	4.3	.5	20 (3)					
					1.0	29 (3)	10		844 (4)	13.4	5.1
2	100	60	2.5	4.2	.5	39 (3)	5	110 (2)	769 (3)	7.2	4.5
					1.0	58 (3)	10		985 (3)	12.1	6.1
3	90	60	2.0	4.9	.5	35 (3)					
					1.0	94 (3)	10		1086 (4)	16	6.6
4	90	60	2.5	4.5	.5	44 ± 1.8 (15)	5	209 ± 20 (7)	960 ± 40 (15)	14.3 ± .7	6.3 ± .3
					1.0	110 ± 9 (15)	10	205 (3)	948 ± 49 (8)	14.5 ± .7	6.8 ± .5
5	90	60	3.0	11.5	.5	59 (3)					
					1.0	118 (3)					
6	90	50	2.5	3.5	.5	42 (3)					
					1.0	82 (3)					
7	90	50	3.0	12.7	.5	72 (3)					
					1.0	72 (3)					
Uninjected control rats						17 ± 1.5 (8)		6.4 ± 4 (6)	191 ± 10 (11)	6.3 ± .4	5.2 ± .2

* These ovaries contained only follicles.

were dialyzed against distilled water and finally against 0.01% KCl solution to remove the NaCl. A light inactive precipitate formed during dialysis was removed by centrifugation. The fractions were dried by lyophilization. *Assay.* The fractions prepared by the above variations in the general procedure were assayed for gonadotrophic activity by using 21-day-old normal female rats of the Holtzman strain. The volumes of the extracts 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. These fractions were also assayed in male and female rats which were hypophysectomized when 27 days of age. Injections were begun 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 after the last injection. The ovaries, testes, ventral prostate, seminal vesicles, thyroids and adrenals were removed, dissected free of other tissue, and weighed. The ovaries were examined macroscopically for the presence of follicles and corpora lutea.

Results. The gonadotrophic fractions obtained by extracting dry sheep pituitary tissue under different conditions of pH and salt concentration were assayed and the results from normal rats are summarized in Table I whereas those from hypophysectomized rats are given in Table II. These extracts were fractionated at 30°C and at pH 2.5 except for several extracts made at pH 6 with 90% saturated NaCl solution and fractionated at pH 2.0, 2.5 and 3.0. The data for saturated NaCl solution demonstrate that the amount of gonadotrophin extracted is dependent upon the pH over the range from pH 4 to 10. Inactivation occurred when the extracts were made at pH 4 (A1, Table I). The amount of FSH activity increased with increase in pH from 5 to 7. The ovaries produced by 0.5 and 1.0 g eq. of hormone contained only follicles (A2 to A5, Table I). The fractions from extracts made at pH 8 and 9 contained significant amounts of LH activity as shown by the fact that these ovaries contained corpora lutea (A6 and A7, Table I). Inactivation of FSH and LH occurred on extraction at pH 10 as shown by the lack of ovarian stimulation when the fraction was given alone and the lack of corpora lutea in the ovaries when it was given with FSH (A8, Table I).

Extractions were made with 90 and 80% saturated NaCl solutions at pH 5.0 and pH 6.0. Results obtained from the assay of these fractions and also those obtained with saturated salt solution show that when the pH was held constant the amount of hormone activity recovered increased as the salt concentration decreased (A2 and A4, Table I). The activity recovered at pH 6 was significantly greater than that recovered at pH 5 when fractions extracted with a given salt concentration are compared. When 90% NaCl extracts made at pH 6 were fractionated at pH 2.0, 2.5 and 3.0 there was an increase in activity with an increase in pH from 2.0 to 2.5 (A4, Table I). The ovarian response for the pH 2.0 fraction was only follicles whereas the response to the pH 2.5 and 3.0 fractions included a few corpora lutea in less than one-third of the ovaries. The protein contents of the pH 2.0 and 2.5 fractions were essentially the same (3.6 and 3.8 mg per g eq.) but the fraction obtained at pH 3.0 contained 20.4 mg per g eq.

The fractions (Table I) which appeared more promising in terms of being essentially free of LH were assayed in hypophysectomized male and female rats and the results are summarized in Table II. Doses 10 to 20 times greater than those given to normal female rats produced ovaries in the hypophysectomized rats that ranged in weight from 113 to 269 mg. These ovaries did not contain corpora lutea as determined by macroscopic examination. Since the ovary of the female rat is less sensitive to the action of LH than is the prostate to androgen pro-

duced by LH acting on the interstitial cells of the testes(4,5), the fractions of Table I were assayed in hypophysectomized males. When compared to those of uninjected control animals, the preparations made at pH 5 with saturated NaCl solution caused the least increase in ventral prostate weight of any of the fractions (Table II). When the pH was increased to 5.5, 6.0 and 7.0, the average prostate weights were about double the weight of those of the control animals. When the pH was held constant at 5.0 and the NaCl concentration was decreased from saturation to 90 and 80% saturation there was an increase in the weight of the prostate (A2, Table II). Similar results were obtained when the pH was held constant at 6.0 and the salt concentrations were decreased (A4, Table II). The fraction extracted with 80% saturated salt solution produced prostates that had an average weight of 38.8 mg which indicates that this fraction contained significant amounts of LH activity.

Another factor studied was the effect of fractionating at elevated temperatures. The rationale for these experiments is as follows. It was postulated that extracting with saturated salt solution at 70°C would dissociate weak protein linkages that might possibly result in releasing FSH from LH and other proteins(2). Furthermore, if this release of FSH by dissociation was reversible and was temperature sensitive, the dissociation might be greater at 70°C than at 30°C, the temperature at which the extracts were fractionated previously. On the basis of these assumptions it was thought that the yield of FSH might be increased by fractionating at 70° rather than 30°C and, also, that a cleaner separation from LH might be attained. The fractions were assayed in normal females and in hypophysectomized female and male rats. The results are given in Table III. The data from normal rats indicate that fractionation of the saturated NaCl extract at 70°C resulted in a great loss of FSH activity, whereas at 60°C considerable activity was recovered (1 and 2, Table III), but this was still less than that obtained at 30°C (A4, Table I). When 90% saturated NaCl extracts were fractionated at 60°C and at pH 2.0, 2.5 and 3.0 there was an increase in activity with an increase in the pH of fractionation (3 to 5, Table III). Fractions obtained at 50° and 60°C and at pH 2.5 contained essentially the same amount of activity (4 and 6, Table III). There was an increase in the activity when the fractionation was done at pH 3 and 50° and 60°C (5 and 7, Table III). Comparison of the ventral prostate response of the hypophysectomized male rats (Table III with results obtained from the same kind of rats (Table II) suggests that the fractions prepared at the high temperatures contained slightly less LH activity than those preparations made at 30°C. The ovaries from the hypophysectomized female rats contained only follicles.

Fraction 21A (Table IV) was obtained by extracting at pH 6 with 90% saturated NaCl solution and fractionating at 60°C and pH 2.5. It was further purified with anion exchange resin XE-59 according to the procedure reported previously by McShan *et al.* (2). The results substantiate further the effectiveness of the anion resin in eliminating a large amount of inert protein (81%) from fraction 21A without loss of FSH activity to give 21A(A-4). The latter fraction was fur-

TABLE IV. Biological Effect of a Preparation Fractionated at High Temperature and Purified with Ion Exchange Resins.

FSH preparation	Protein, mg/g	Normal rats: Dose, g eq.	Normal rats: Ov. wt,* mg	Hypophysectomized rats: Dose, g eq.	Testes, mg	Prostate, mg	Sem. ves., mg	Adrenals, mg	Thyroids, mg
21A	3.63	.5	43 (3)	5	936 (2)	10.6	5.7	13	4.3
				10	1156 (2)	17.6	7.9	13	4.3
21A (A-4)	.72	.5	46 (3)						
49F8	.118			10	952 (3)	11.6	5.1	6.7	3.6

* These ovaries contained only follicles.

ther purified by use of a column of cation resin XE-97(3) to give fraction FSH49F8 which contained only 0.118 mg of protein per g eq. of dry pituitary tissue. The results of the assay of the crude 21A and the purified FSH49F8 fractions in hypophysectomized male rats indicate that a significant amount of LH activity was removed by the purification steps as indicated by the decrease in the ventral postate weight from 17.1 mg for the crude to 11.6 mg for FSH49F8.

The weights of the adrenals and thyroids from the hypophysectomized rats used for assay of the various FSH fractions were not significantly different from the weights of these glands obtained from control uninjected rats. This indicates that the FSH preparations were free of ACTH and TSH at the high dosage levels used for the assay of the FSH.

Summary. (1) The effect of salt concentration and pH during extraction of sheep pituitary tissue, and temperature and pH of fractionation of these extracts on the preparation of FSH were studied. The most highly purified FSH from the biological standpoint was obtained by extracting at pH 5 with saturated NaCl solution and fractionating at pH 2.5 and 30°C. (2) The FSH preparations made by extracting with 90% saturated and saturated NaCl at pH 5, 5.5 and 6 and fractionating at 30°C and pH 2.5 contained little LH. (3) These preparations were free of ACTH and TSH at the levels tested. (4) The FSH can be concentrated and purified by use of anion and cation exchange resins. (5) Preparations made from 80% saturated NaCl extracts and from extracts made at pH 8 and 9 with saturated NaCl contained significant amounts of LH.

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Antagonists to Neuromuscular Block Produced by Sarin. (22533)

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The action of cholinesterase inhibitors on the neuromuscular apparatus has been explained by Burns and Paton(1) as originating by increased spread from the end-plate of the membrane depolarization normally elicited by ACh release. After complete cholinesterase inactivation, the end-plate region is thought to become sufficiently depolarized through the action of the ACh released by a single nerve discharge to be responsive no longer to indirect stimulation. The present work was undertaken to determine whether or not the block of neuromuscular transmission induced by comparatively large doses of Sarin can be overcome by chemical means, and whether any light on the mechanism of action of Sarin can be derived from a knowledge of chemicals capable of overcoming the neuromuscular block.

Methods. Sciatic nerves of cats lightly anesthetized with sodium pentobarbital were stimulated electrically with square wave stimuli at a frequency of 1 every 2 seconds, a pulse duration of 0.1 millisecond, and a supramaximal voltage (0.2 to 0.7 volt). Contraction of the gastrocnemius-soleus muscle group was recorded by a partially isometric lever. After control muscle responses had been obtained, the cat was given 200 μg/kg of Sarin through one antebrachial vein. Artificial respiration (interrupted positive pressure) was administered when required to maintain life. Five minutes later the treatment agent, if any, was administered by the