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Received December 16, 1952. P.S.E.B.M., 1953, v82.

Effect of Chlorides and Heat on Extraction and Activity of Sheep Pituitary Gonadotrophin.* (20098)

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Aqueous solutions of organic and inorganic compounds, and also water have been used for the extraction of pituitary gonadotrophin(s)(1-5). The concentration and, particularly, the separation in high yields of two biologically pure gonadotrophins from these extracts have been difficult to accomplish. Presumably this is due in part to the association of the gonadotrophins (FSH and LH) with inert material and with each other. On the basis of this assumption attempts were made to dissociate these hormones by extracting them with saturated solutions of inorganic chlorides, concentrated solutions of sucrose and urea, and water at a temperature just below that which would cause inactivation. Although separation of the gonadotrophins was not accomplished, they were concentrated by simple procedures and information was obtained relative to their stability to heat and their augmentation by concentrated salt solutions.

Methods and materials. The solvents used for extracting the dry sheep pituitary tissue were solutions of inorganic chlorides saturated at room temperature, 0.88 M sucrose, 6.6 M urea and water. The amounts of pituitary tissue extracted ranged from 2 to 100 g. Extractions with sodium chloride were made at 25, 60, 70, 80 and 90°C. Extractions with the other solutions were done at 70°C. The pituitary powder was mixed in the ratio of 1 g of dry tissue to 10 ml of solution, placed in a water bath regulated to the desired temperature, stirred for one hour, centrifuged and the residue reextracted in the same way. The two extracts were combined and designated as extract E. A third treatment with NaCl solution extracted little activity. On the basis of this, the residues that remained after two extractions with the other solvents were not studied. *The E extracts* were dialyzed to remove the chlorides and other substances used for extraction. This caused an inactive precipitate to form which was removed by centrifugation to give the dialyzed soluble fraction A which was adjusted to pH 4. The precipitate was removed by centrifugation and designated as fraction C, and the pH 4 soluble fraction (B) was concentrated by lyophilizing. This concentrated extract was dialyzed, then centrifuged to remove any insoluble material present and lyophilized to dryness to give fraction D. Fraction B was also recovered by precipitation with alcohol or acetone and dried by lyophilization. The *extracts and fractions* were assayed for gonadotrophic hormone activity by the use of 21-day-old female rats of the Holtzman-Rolfsmeyer strain. The volumes of the fractions were adjusted with

* This investigation was supported in part by research grant RG-2154(C2) from the National Institutes of Health and by a grant from funds supplied by the Wisconsin Alumni Research Foundation.

TABLE I. Gonadotrophic Activity of Fractions from Saturated Sodium Chloride Extracts of Sheep Pituitary Glands.

Fractions*	Dose, mg eq.†	Temp. of extraction, °C: 25	60	70	80	90	Protein (N × 6.25), mg/g eq. at 60°C
		Avg wt of ovaries, mg					
E	100	269±18.3 (7)‡	205±16.0 (5)	194(1)			
A	100	88±11.2(13)	93± 7.2(12)	101±11.8(6)	48(3)	17(6)	24.0(2)
B	100	107± 8.8(15)	97±12.7(12)	116(3)	24(3)	17(4)	12.8(2)
C	500	16± 0.9 (9)	15(3)				

* Extracts and fractions given in this and following tables are designated as follows: Undialyzed saturated extract, E; dialyzed soluble, A; dialyzed pH 4 soluble, B; and pH 4 insoluble, C. The pH 4 soluble B fractions were concentrated and are designated as D fractions in subsequent tables. The insoluble fraction that separated on dialysis was inactive. A total dose of 100 mg produced ovaries that averaged 14 mg, and 500 mg produced ovaries averaging 17 mg as compared with 13 mg for ovaries from uninjected rats of same age.

† Material equivalent to the mg of dry pituitary tissue indicated in this and subsequent tables.

‡ Figures given in parentheses following the stand. errors of the mean ovarian weights in this and other tables indicate the No. of rats used for assaying the various fractions.

normal saline so that each rat received the proper equivalent of dry pituitary tissue in nine 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 four days. Autopsy was performed on the morning following the last injection. The ovaries were removed, dissected free of other tissues, and weighed. The presence of follicles and corpora lutea was recorded.

Results and discussion. Sodium chloride. The results of the gonadotrophic assays of the fractions obtained from the concentrated NaCl extracts are given in Table I. The A and B fractions obtained at 25, 60 and 70°C had essentially the same activity; fraction C contained little activity. The gonadotrophin was stable when heated at 70°C in saturated NaCl solution, whereas at 80°C the hormone was partially inactivated and almost completely inactivated at 90°C. On the basis of these results extractions with concentrated solutions of other compounds were done at 70°C.

Potassium chloride. Saturated KCl extracts prepared at 70°C were fractionated and the results of the assays are summarized in Table II. The D fractions recovered from the NaCl and KCl extracts contained both follicle stimulating and luteinizing activities indicating that a separation was not accomplished by the procedures of extraction and fractionation given above. Fraction D obtained from the KCl extracts was not inactivated when dissolved in saturated KCl and heated a second time at 70°C for one hour followed by dialysis against 0.5% KCl. A total dose of 100 mg of this fraction produced ovaries that had an average weight of 100 mg before, as compared with 102 mg after, the second heat treatment.

TABLE II. Gonadotrophic Activity of Fractions from Saturated Potassium Chloride Extracts of Sheep Pituitary Tissue.

Fractions*	Dose, mg eq.	Avg ovarian wt, mg	Protein (N × 6.25), mg/g eq.
E†	100	201± 8.1 (5)	
A	100	104± 5.7(41)	40.9±2.3(8)‡
B	100	102± 7.9(27)	16.4±1.2(9)
C	250	29±10.0 (4)	
C	500	59±11.0 (9)	28.9±2.6(7)
D	100	104± 9.4(13)	10.5±1.2(4)

* Extracts were made by heating at 70°C.

† Letters have same meaning as given in footnote 1 of Table I.

‡ No. of preparations on which avg protein values are based.

Barium, calcium, magnesium and lithium chlorides. The fractions obtained by dialysis of the extracts made with saturated solutions of these chlorides were less active than the corresponding ones obtained from NaCl and KCl extracts. The $BaCl_2$ fraction was the most active of the 4, the LiCl one was essentially inactive. The ovaries produced by the $BaCl_2$ fraction consisted mainly of follicles which may bear some relation to the fact that the pH of the $BaCl_2$ solution was 4.9

TABLE III. Gonadotrophic Activity of Aqueous, Sucrose and Urea Extracts of Sheep Pituitary Tissue.

		Kind of extract				
		Water		Sucrose (0.88 M)		
Fraction*	Dose, mg eq.	Ovarian wt, mg	Protein, mg/g eq.	Ovarian wt, mg	Protein, mg/g eq.	Urea (6.6 M), ovarian wt, mg
E†	100	112± 8.4(9)	93.7(2)			
A	100			142±13.4(6)	51.0(2)	21(3)
A	500					178(3)
B	100	127±10.7(9)	62.5(2)	132±10.5(6)	33.4(1)	
C	500	21(3)		25(3)		
D	100	113±10.3(6)	25.8(2)			

* Extractions made at 70°C.
† Letters have same meaning as in footnote 1, Table I.

TABLE IV. Augmentation of Sheep Pituitary Gonadotrophin by Concentrated Solutions of Sodium Chloride and Sucrose.

Fraction	Dose, mg eq.	Fraction dissolved in:	No. of rats	Avg ovarian wt, mg	Protein, mg/g eq.
KCl (S30A)	100	.9% NaCl	10	107	12.3
		.5 sat. NaCl	4	159	
Aqueous (P89)	100	.9% NaCl	9	106	40.2
		.5 sat. NaCl	6	235	
		.44 M sucrose	3	150	
		.88 M "	3	137	

as compared with 6.9 and 7.1, respectively, for the saturated NaCl and KCl solutions.

Water, sucrose and urea. The results given in Table III show that a major part of the sheep pituitary gonadotrophin was extracted with water at 70°C. The aqueous extract E and fraction B had essentially the same activity, and the latter fraction was concentrated without loss of activity to give fraction D. Fraction C contained little activity. Sheep pituitary gonadotrophin was also extracted at 70°C with 0.88 M sucrose. This solvent removed less protein from the dry tissue than did water. Fraction A obtained from the 6.6 M urea extract contained little gonadotrophin.

Augmentation of pituitary gonadotrophin by concentrated salt solutions. The undialyzed extract E obtained with saturated NaCl at 25°C produced ovaries that had an average weight of 269 mg as compared with 88 mg for the soluble fraction A obtained from extract E by dialysis. This effect was also obtained with NaCl extracts made at 60 and 70°C (Table I), and with saturated KCl extracts made at 70°C (Table II). These saturated extracts were diluted about 1:1 with 0.9% NaCl to give approximately 0.5 saturated solutions for injection purposes. This concentration of NaCl and KCl was toxic. Results given in Table IV were obtained by the administration of two gonadotrophic preparations (P89 and S30A) in 0.9% NaCl and 0.5 saturated NaCl solutions. These preparations were equivalent in activity when administered in 0.9% NaCl, but P89 which contained over three times as much protein as S30A was more effectively augmented by 0.5 saturated NaCl than was S30A. These results show that the degree of augmentation produced by concentrated NaCl is influenced by the protein content of the preparation. The protein contents (N x 6.25) of the various fractions are given in Tables I, II, III and IV. Slight augmentation occurred when P89 was administered in 0.44 M and 0.88 M sucrose solutions. These data support the hypothesis that the augmentation of gonadotrophin by concentrated NaCl and sucrose solutions is due to the slow release of the hormone from the injection site. This explanation has been used to explain the augmentation of gonadotrophin by non-specific substances such as heme(6) and insoluble metallic hydroxides(7).

Summary. Concentrated fractions of dry

sheep pituitary tissue obtained from extracts made at 70°C with water, saturated solutions of NaCl and KCl, and with 0.88 M sucrose contained the major part of the gonadotrophic activity. Fractions obtained from extracts made with saturated solutions of barium, calcium and magnesium chlorides, and 6.6 M urea at 70°C were much less active. Dialyzed soluble fractions obtained from saturated NaCl extracts made at 80°C contained little activity and those from extracts made at 90°C were essentially inactive. Extraction at 70°C with the saturated solutions and fractionation of the dialyzed extracts did not separate FSH from LH. The activity of the gonadotrophin was augmented when administered in solutions which were 0.5 saturated with NaCl and KCl.

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Received December 1, 1952. P.S.E.B.M., 1953, v82.

Naturally Occurring Fibromas of Grey Squirrels Related to Shope's Rabbit Fibroma. (20099)

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Six grey squirrels (*Sciurus carolinensis*) collected within a year in Maryland counties adjacent to the District of Columbia were covered with multiple tumors which in gross appearance, histologic structure, and on immunological grounds show close relationship to the fibromas originally described by Shope (1) in cottontail rabbits. Afflicted squirrels, which weighed around 300 g or less, appeared to be juveniles. The squirrel fibromas measured from a few mm to 2.5 cm in diameter and varied in number from 5 on one squirrel to between 50 and 100 on others. They were located indiscriminately on all parts of the animals from eyelids to tail and toes. A squirrel with naturally occurring tumors is shown in Fig. 1. As discussed below, woodchucks (*Marmota monax*) were experimentally infected.

Materials and methods. Fibromas when harvested for passage were minced with scissors and ground with sand before being made into 10% emulsions with meat infusion broth. Due to the presence of skin and hair follicles, especially in woodchuck and squirrel biopsies, such emulsions were often contaminated as originally prepared. Addition of penicillin and streptomycin, followed by centrifugation for ½ hour, however, appeared to eliminate contaminants as a factor of importance. In addition animals inoculated with woodchuck or squirrel preparations were given 150,000 units of penicillin in oil. Some of the woodchuck fibroma emulsions were free from contaminants when tested in whole meat broth and on blood agar slants. In *neutralization* tests, the OA strain of Shope rabbit fibroma* was diluted with meat infusion broth, so that after addition to equal volumes of undiluted sera, the final virus dilutions were 10^{-2}, 10^{-3}, and 10^{-4}. Such mixtures in amounts of 0.5

* Kindly supplied by Dr. David R. Ginder, Emory University, Atlanta, Ga.