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Factors Affecting the Initial Extraction of Ovine Gonadotropins[1] (38676)

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(Introduced by A. Clark Griffin)

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Several procedures for the extraction, separation and partial purification of the gonadotropins from sheep pituitary glands have been described (1–4). Since these reports, procedures for more extensive purification of oLH (5–14) and oFSH (15–18) have been devised. Notable variations in yield, relative potency and electrophoretic behavior of the oLH and oFSH purified by the cited procedures are evident. In this paper we demonstrate the effect different methods of tissue homogenization as well as solvents used for extraction have on the initial extraction of oLH and oFSH.

For this study we simultaneously processed, by four methods, 1 kg aliquots of ovine pituitary glands from a single source.

Materials and Methods. Fresh, frozen ovine pituitary glands (The Davidson Commission Co., Chicago, IL) were submitted to four methods of extraction for gonadotropin-rich fractions.

Method 1. The frozen glands were ground in a meat grinder with sufficient dry ice to prevent thawing, using a modified Koenig and King (3) procedure. The ground glands with admixed dry-ice were thawed in the pH 5.0 ethanol–acetate buffer (40% ethanol and acetate at 0.5 ionic strength). This mixture was verified to be pH 5.0 and the first extraction was carried out at 4° in the pH 5.0 ethanol–acetate solvent for 24 hr. The resulting residue was collected at the centrifuge and the supernatant rendered 80% with respect to ethanol. After 2 days at 4° the 80% ethanol precipitate was collected at the centrifuge. The residue from the 40% ethanol extract and the precipitate from the 80% ethanol fractionation were reconstituted with distilled water, dialyzed free of salt, centrifuged to remove inert material, and lyophilized.

Method 2. Fresh frozen pituitary glands were thawed, homogenized in pH 5.0 ethanol–acetate buffer at 4° with a Tekmar homogenizer, Super Dispax System, Model SD-45 (Tekmar Company, Cincinnati, OH) and allowed to extract at 4° for 24 hr. Except for the method of homogenization Method 2 was identical to Method 1. The resultant products were the 40% ethanol residue and 80% ethanol precipitate.

Method 3. Frozen pituitary glands were thawed then homogenized with the Tekmar homogenizer in water. The pituitary pulp was adjusted to pH 5.0, allowed to extract at 4° for 4 hr, then the residue was collected at the centrifuge. The supernatant was adjusted to pH 4.0 with 2 *N* HCl, brought to 40% saturated ammonium sulfate by slow addition of 100% saturated ammonium sulfate and allowed to stand at 4° overnight. The 40% ammonium sulfate precipitate was collected by centrifugation and the supernatant brought to 80% saturation ammonium sulfate by slow addition of solid ammonium sulfate. After 48 hr at 4° the 80% ammonium sulfate precipitate was collected at the centrifuge. The two gonadotropin-rich fractions, 40% and 80% ammonium sulfate precipitates, were reconstituted in water, dialyzed free of salt and lyophilized.

Method 4. Since preliminary studies indicated the aqueous extraction procedure (Method 3) left some LH in the residue it was reprocessed by the modified Koenig and King (3) procedure described in Method 1.

In order to recover any remaining gonadotropins the 80% ethanol–acetate supernatants and 80% ammonium sulfate

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TABLE I. COMPARISON OF THE PROTEIN YIELD, LH AND FSH POTENCY OF FRACTIONS FROM 1 Kg OVINE PITUITARY GLANDS SUBMITTED TO FOUR FRACTIONATION PROCEDURES.[a]

Method	Fraction	Protein yield[b] (gm/Kg)	LH potency[c] (relative to NIH-LH-S18)		λ	LH total units	FSH potency[e] (relative to NIH-FSH-S9)		FSH total units
1.	40% Ethanol residue	1.030	0.008	(0.005–0.013)	0.16	8	<0.01		
2.	40% Ethanol residue	0.252	0.026	(0.018–0.037)	0.16	6	<0.01		
3.	40% Ammonium sulfate ppt	6.170	0.079	(0.055–0.115)	0.16	487	<0.01		
4.	40% Ethanol residue	0.562	0.059	(0.041–0.083)	0.16	32	0.012	(0.0042–0.017)	6
1.	80% Ethanol ppt	2.904	0.16	(0.082–0.32)	0.22	474	0.42	(0.31–0.68)	1226
2.	80% Ethanol ppt	0.747	3.20	(1.88–5.44)	0.23	2390[d]	0.69	(0.48–1.59)	514
		1.374	2.23	(1.13–4.40)	0.19	3057			
3.	80% Ammonium sulfate ppt	3.256	0.081	(0.046–0.14)	0.22	262	0.32	(0.25–0.40)	1035
4.	80% Ethanol ppt	0.617	0.085	(0.048–0.15)	0.22	52	1.07	(0.62–1.16)	658
1.	25% Acetone ppt	3.350	0.004	(0.002–0.008)	0.28	12	<0.01		
2.	25% Acetone ppt	1.305	0.013	(0.007–0.026)	0.28	17	<0.01		
3.	25% Acetone ppt	0.301	0.005	(0.003–0.011)	0.28	1	0.0099	(0.0027–0.016)	3
4.	25% Acetone ppt	0.504	0.63	(0.21–1.91)	0.28	318	0.0161	(0.108–0.021)	8

[a] Details of the four fraction procedures are described in the Materials and Methods section of the text.

[b] Determined from Biuret reaction.

[c] Values in parentheses are 95% confidence limits.

[d] Values in parentheses are for two separate experiments.

[e] Values in parentheses are 95% confidence limits. Values denoted less than 1 (<1) indicate no significant response at 200 times the dose of the standard.

supernatants obtained after the last centrifugation described in Methods 1–4 were submitted to 25% acetone (v/v) precipitation. The 4 supernatants were allowed to stand at 4° for 2 days then centrifuged to yield an acetone precipitate. The precipitates were reconstituted in water, dialyzed and lyophilized.

oLH Bioassay. The potency of the LH-containing fractions was estimated by the Parlow ovarian ascorbic acid depletion bioassay (19). Bioassays were analyzed for relative potency according to the log-dose response statistical analysis described by Bliss (20); NIH-LH-S18 was used as standard.

oFSH Bioassay. Bioassays for oFSH activity in the residues and precipitates obtained from ethanol, ammonium sulfate and acetone fractionations were carried out according to the procedure described by Jacobs and Ward (21), a modification of the method of Steelman and Pohley (22). Relative potency was evaluated by the slope-ratio analysis described by Steelman and Pohley (22), using 90 and 180 μg NIH-FSH-S9 as standard and each unknown in three dose concentrations at least two of which were appropriate for optimal responses with respect to the standard.

Disc gel electrophoresis was carried out in 7.5% acrylamide at pH 8.4 in Tris–HCl buffer for 2 hr at 1.5 ma per gel according to the method of Davis (23). The gels were stained with 1% Amido Schwartz and destained in 7% glacial acetic acid containing 5% methanol. Protein determinations were made using the Biuret reagent (24).

Results. The 40% ethanol residue was essentially devoid of both oLH and oFSH biological activity (Table I). The 2.9 gm of protein recovered in the 80% ethanol fraction contained 474 total units of oLH and 1226 total units of oFSH. Further precipitation with acetone (25%) yielded 3.35 g of protein containing negligible oLH and oFSH biological activity.

Comparing Method 1 with Method 2 (Table I), the most striking difference the means of homogenizing the tissue produced

was a substantial increase in the yield and potency of the oLH. Since this result was so impressively different from our previous experience (e.g. Ref. 11), the experiment was repeated for confirmation. The Tekmar homogenizer (which utilizes a very high shear effect with counter-rotating blades for homogenization) released oLH into the ethanol-acetate buffers with greater efficiency. Moreover, the specific activity was higher, probably due to denaturation and insolubilization of higher molecular weight proteins to produce less contamination with other soluble proteins (Col. 3 Table I). On the other hand, this procedure was not as effective for FSH (500 units, Method 2 vs. 1200–1600 for Method 1 or Methods 3 and 4; Table I). The increase in LH with Method 2 was visually apparent in the PAGE profile (Fig. 1, gel 2-B) where LH was the predominant protein and the total protein content was less heterogeneous than the corresponding fraction from Method 1 (gel 1-B).

The results of Method 3 indicated aqueous extraction of pituitary glands was about as effective as ethanolic acetate buffers for removal of oFSH (Table I, Methods 1 and 3). However, the extraction of oLH was complicated in this procedure since activity was found in the residue (starting material for Method 4), and both the 40% and 80% ammonium sulfate precipitates. Nevertheless, the efficiency of LH extraction by

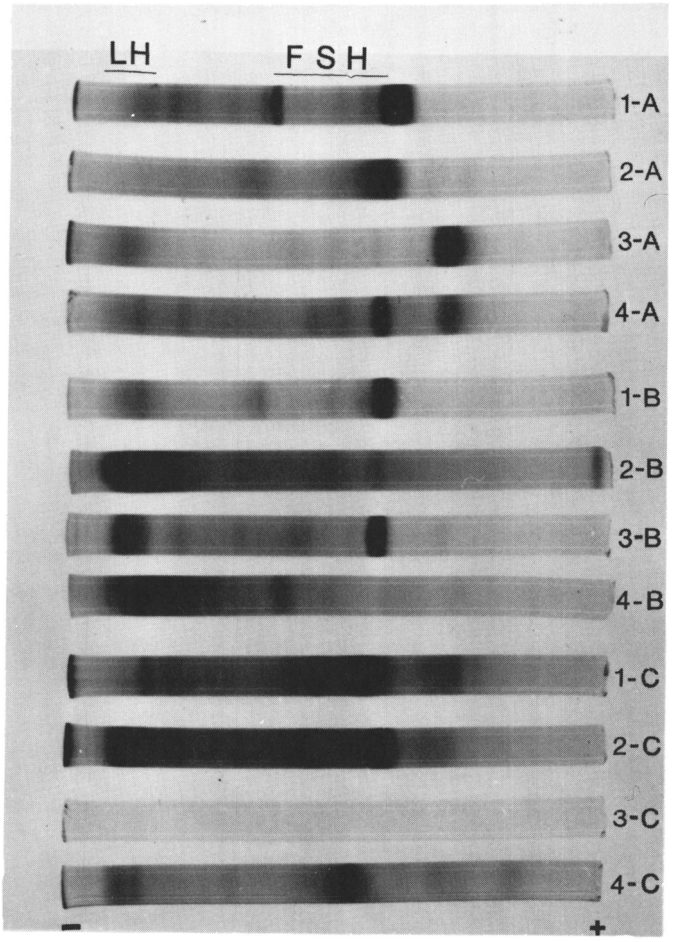

FIG. 1. Patterns in PAGE of 40% ethanol or ammonium sulfate fractions (series A), 80% ethanol or ammonium sulfate fractions (series B) and acetone fractions (series C). The first number designates the method of origin (Methods 1–4) as described in the text. Regions of oLH and oFSH activity are indicated.

Method 1 or Method 3 + 4, considering the total units obtained, was only 0.25–0.33 that of Method 2. In contrast, Method 3 + 4 gave threefold greater yield of FSH than Method 2 and 1.4-fold better than Method 1. The yields of oFSH obtained are not substantially different from those recorded in other laboratories using other procedures (15–18), but the yield of oLH obtained by Method 2 is approximately two to three times greater than those recorded by other laboratories (5–14).

The disc electrophoresis patterns in Fig. 1 indicate the number of components in each fraction described above and in Table I. The areas of the gel in which purified oLH and oFSH are found is indicated. To evaluate the chemical differences (if any) in the products obtained by the different procedures described will obviously require further purification. Such studies are in progress.

Summary. The method of tissue homogenization as well as the solvents used for extraction influenced the protein yield, the specific activity of the oLH and oFSH, and the total units of each hormone recovered. Use of the Tekmar homogenizer (Method 2) produced a fivefold increase in the total yield of oLH per Kg glands (2390 units, Method 2; 474 units, Method 1) and the relative potency of this partially purified LH from the 80% ethanol precipitate was increased (0.16 × NIH-LH-S18, Method 1; 2.2–3.2 × NIH-LH-S18, Method 2). By combining the aqueous extraction—ammonium sulfate precipitation with a subsequent ethanolic acetate extraction (Methods 3 and 4) the combined yields provided a 2.4-fold increase in oLH and 1.4-fold increase in oFSH. Thus the total units recovered of oFSH and oLH can be increased simply by changing the methods of homogenization and extraction.

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