pattern during the period of cyst development.

Summary. Ovarian cysts were experimentally induced by injecting hypothyroid rats with human chorionic gonadotropin. Cystic ovarian tissue synthesized *in vitro* a large quantity of 20α-hydroxypregn-4-en-3-one but estrogen synthesis was low.

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Purification of Ovine Luteinizing Hormone-Releasing Factor by Gel Filtration and Ion Exchange Chromatography.* (30028)

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Release of hypophyseal luteinizing hormone (LH) appears to be controlled by a hypothalamic LH-releasing factor (LH-RF). Crude or partially purified rat, beef, or sheep hypothalamic extracts prepared from the stalk-median eminence (SME) region evoke the release of LH in a variety of test animals (1-4). Since the extracts are active after destruction of the median eminence region(2), an operation which eliminates neural control of LH, it is believed that they act directly on the pituitary to release LH. This belief is strengthened by the finding that microinjection of these extracts into the adenohypophysis induces ovulation in both rats(5) and rabbits(6). More recently, it has been possible to demonstrate a decline in stored LH-releasing activity in rats at proestrus(7, 8), and to observe LH-releasing activity in plasma of hypophysectomized rats(9). These observations suggest that variations in secretion rate of the LH-RF may bring about the altered rates of LH release which occur under various physiological conditions, such as the stages of the estrous cycle and alterations in gonadal hormone titers.

We have reported the purification of bovine LH-RF on a small scale by gel filtration

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on Sephadex G-25(10). We have modified our procedures to obtain a better purification on a larger scale for subsequent studies of amino acid composition and structure of this releasing factor. This paper describes the preparation of highly purified ovine LH-RF by gel filtration, using larger columns of Sephadex and different methods of extraction and elution, which was followed by chromatography on columns of carboxymethylcellulose (CMC).

Methods. Extractions. SME tissue from recently slaughtered sheep§ was collected on dry ice and converted into acetone powder prior to extraction with 4 M acetic acid as previously described(11). The acetic acid extract was lyophilized and reextracted with glacial acetic acid, diluted 3-4 times with glass distilled water and relyophilized. This step was found useful to remove the bulk of inert proteins and some insoluble salts.

Chromatography. Gel filtration on a column (150 × 4.5 cm) of Sephadex G-25 equilibrated in 0.1 M ammonium acetate (pH 5) was performed first to separate LH-RF from follicle stimulating hormone releasing factor (FSH-RF), and the bulk of vasopressin and other peptides as reported earlier (11). The zones containing the LH-RF were combined and lyophilized. The lyophilized material was dissolved in 30-40 ml of glacial acetic acid to remove salt, diluted 3-4 times with glass distilled water, and relyophilized. This lyophilized material was dissolved in the starting buffer and applied to a CMC column (2 × 100 cm) which had been equilibrated with 0.002 M ammonium acetate buffer at pH 4.5. Gradients to pH 6.5, 0.04 M buffer at tube no. 10, and to 0.2 M buffer at tube no. 30, were applied through a 500 ml mixing flask. The volume of liquid in each tube collected was 20 ml. When necessary, further desalting was accomplished by passage through the CMC column and by glacial acetic acid extraction.

CMC columns were packed under 8 lb of pressure and flow rates were maintained at 30-50 ml/hour. Columns were regenerated with 1 M acetic acid followed by water and re-equilibration with the starting buffer.

Polypeptide concentration in the effluent was followed by the Folin-Lowry reaction (12). Conductivity measurements were made by a conductivity bridge, and concentration of sodium (Na) and potassium (K) was measured with the Baird flame photometer.

Bioassays. LH-releasing activity was estimated by determining the percentage ovarian ascorbic acid depletion (OAAD) which followed intravenous (iv) administration of the various fractions into immature female rats which had been pretreated with gonadotrophins‖(1,13). Three to 7 test rats were used for every fraction evaluated.

Pressor activity was estimated by the method of Dekanski(14) with Pitressin as the standard.

Results. Crude extracts. Preliminary experiments revealed that the acetone powder obtained from sheep SME fragments was active in depleting ovarian ascorbic acid. A dose of 4 mg of the powder gave an ascorbic acid depletion of 22%. The acetic acid extract made from the acetone powder was likewise active; 1 mg of extract made from this lyophilized powder gave depletions which averaged 17%. This powder was extracted with glacial acetic acid, relyophilized, dissolved in ammonium acetate, and subjected to gel filtration.

Gel filtration. Minimal OAAD was obtained in the non-retained, protein-rich fraction which was eluted from the column just after the void volume, when tested in one experiment. The activity in this large molecular zone was probably caused by minimal contamination of the extract with LH. The major ovarian ascorbic acid depleting activity was found in a narrow zone from tubes 112 to 126, with a peak at tube 118 (Fig. 1). Pressor activity began to emerge from the column just after the LH-releasing activity, and reached a maximum 4 tubes after that for LH-release. Fractions were obtained which had LH-releasing activity and very little pressor activity (75 to 160 mU) at

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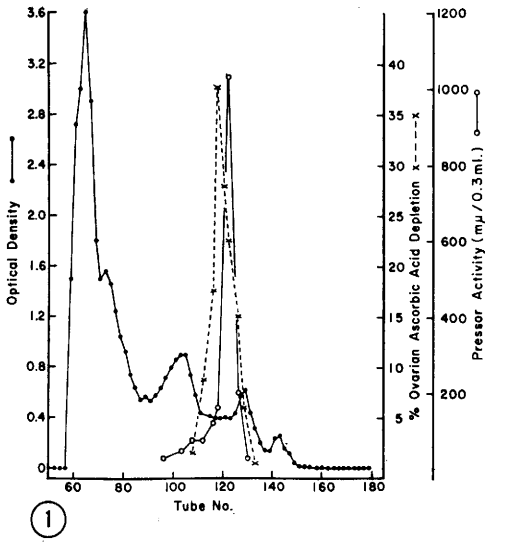

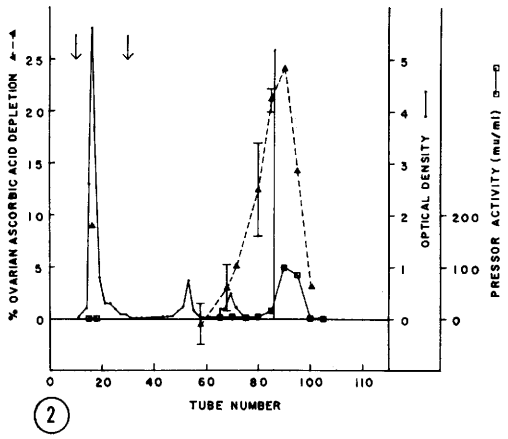

FIG. 1. LH-releasing and pressor activity of ovine hypothalamic extracts obtained by gel filtration on Sephadex G-25. Optical density (at 750 mμ) represents peptide concentration as determined by the Folin-Lowry reaction. LH-releasing activity is estimated by ovarian ascorbic acid depletion.

FIG. 2. LH-releasing (% ovarian ascorbic acid depletion) and pressor activity of fractions obtained from the CMC column. Arrows represent application of gradient to 0.04 M (pH 6.5) ammonium acetate buffer at tube 10 and gradient to 2.0 M (pH 6.5) buffer at tube 30. All tubes to the left of vertical line at tube 87 contain little or no vasopressin. Vertical bars give standard error where 5 or more test rats were used.

the 0.3 ml dose used for evaluation of ascorbic acid depletion. Although the OAAD observed on the ascending limb of the curve for LH-release could hardly have been caused by vasopressin, in view of the small degree of pressor contamination, that on the descending limb may have been in part caused by vasopressin, since doses of 200 mU of this polypeptide can give an OAAD of 10% (15); however, the displacement of the pressor peak from that for LH release indicates that a separation of these two activities had been obtained.

Three such fractionations have been carried out on Sephadex. In one case, the pressor peak was separated from that for LH release by 6 tubes, instead of the 4 tubes illustrated in Fig. 1; whereas only in one fractionation did the peak of pressor and LH-releasing activity coincide. Even in this case, tubes on the ascending limb of the LH-releasing curve had activity which could not be accounted for by vasopressin contamination. The peptide content in the 0.3 ml of effluent injected into the test rats averaged approximately 70 μg in the tubes which gave maximal OAAD.

Chromatography on CMC. The LH-releasing zones from the 3 Sephadex fractionations were pooled, lyophilized, and extracted with glacial acetic acid to reduce the concentration of salts. Since the conductivity and concentration of Na and K were found to be still quite high, the extract was relyophilized and reextracted 2 times in a further effort to reduce contamination with these cations which interfere with chromatography on the CMC ion exchanger. Approximately 500-600 mg of peptide (estimated by Folin-Lowry determination) were applied to the column. Despite these precautions nearly all the LH-releasing activity, and roughly 60-70% of the pressor activity (which still contaminated the extract) were not retained on the exchanger in our first run. After determining its Na and K content, the non-retained material was relyophilized, reextracted as before, and again chromatographed. In all, 4 chromatograms were run. In none of the cases were the columns overloaded with peptide since the size of the column was quite large (2 × 100 cm). Progressively larger fractions of the LH-releasing and pressor activity were retained on the exchanger as the Na and K concentrations were progressively reduced by passage through CMC and extraction with glacial acetic acid. The Na and K contami-

TABLE I. LH-Releasing Activity of Fractions Purified by Gel Filtration and Chromatography on CMC.

Fractionation No.	Tube No.	Dose (μg peptide)	Pressor activity (mU)	% ovarian ascorbic acid depletion		P vs control
				Individual responses	Mean ± SEM	
APSD 2-14	80	3.5	0	8.7, 28.9, 11.4, 16.4, 26.9	17.3 ± 4.4	<.01
" " "	85	3.5	16	22.1, 18.2, 18.6, 22.2, 23.4	20.9 ± 1.1	<.001
APSD 2-11	50	7.2	0	18.8, 11.2, 14.5, 9.4	13.4 ± 1.7	<.01
" " "	57	2.4	7	12.8, 8.2, 10.0, 18.6, 13.0	12.5 ± 1.7	<.01
" " "	64	4.8	50	22.4, 16.4, 19.6, 16.5, 17.6	18.5 ± 1.2	<.001

nation of the extract applied to the column in the last experiment had been reduced to 1 and 0.2 meq respectively. Under these conditions, essentially all the LH-releasing and pressor activity was retained on the column (Fig. 2) and was eluted after application of the gradient to 0.2 M, pH 6.5 ammonium acetate buffer. The LH-releasing activity emerged just prior to the pressor peak, such that a number of active fractions (10) were obtained which contained little or no vasopressin (Fig. 2, Table I). Some LH-releasing activity was still obtained in the pressor zone; however, the contamination with vasopressin (120 mU) in the 1.2 ml dose was too little to account for the LH-releasing activity found. Considerable quantities of pressor free LH-RF were recovered from the last 2 fractionations on CMC (APSD 2-14 and 2-11); smaller quantities were obtained from the first 2 fractionations (APSD 2-1 and 2-8) where most of the LH-RF was not retained on the exchanger. Since the presence of Na and K in the extract inhibited retention of LH-RF more than that of vasopressin, the starting material for later fractionations had a higher LH-RF to vasopressin ratio, which aided in the separation achieved in the later columns. The pressor-free, LH-releasing fractions were active at a minimum dose of less than 3 μg of peptide (Table I) and represent highly purified LH-RF, at least as pure as any so far reported.

Discussion. The present method for purification of the LH-RF provides a highly purified material which is essentially free of vasopressin after 2 chromatographic steps. On the basis of its order of elution from Sephadex, the LH-RF appears to have a molecular weight slightly greater than that of vasopressin. That it probably is a polypeptide is suggested by the fact that it is inactivated by the proteolytic enzymes, pepsin and trypsin(2,3). Its retention on the cation exchanger CMC indicates that it is a basic polypeptide. Other experiments have revealed that gel filtration on Sephadex can give a separation of the LH-RF from other hypothalamic releasing factors, such as the corticotrophin-releasing factor(16), FSH-RF (11), and growth hormone-releasing factor (unpublished data).

Guillemin *et al*(17) have reported briefly on the purification of ovine SME extract by chromatography on CMC. Since they did not mention any attempts to desalt their extract prior to application to the column, it is rather surprising that appreciable LH-RF was retained. We would like to point out that this step is unavoidable in order to get retention of LH-RF on CMC.

Schally and Bowers(4) have just reported the purification of bovine LH-RF by means of gel filtration and ion-exchange chromatography on CMC. Their results, obtained with different solvent systems and conditions of extraction, are in agreement with ours. They also noted the importance of desalting the extract prior to loading on the CMC column, but attempted to remove anions too by passing the extract through Amberlite CG-45. This step may not be necessary, as shown by these experiments. The only disadvantage to the present method is the tedious removal of Na and K, and we are currently searching for easier ways to accomplish this step. Both Schally and Bowers, and Guillemin *et al* only tested zones for LH-releasing activity. A more complete picture of the behavior of the releasing factor is presented here where the entire curve of releasing activity has been followed.

Two puzzling points cannot yet be adequately explained. First, why was the LH-releasing zone obtained from the fractiona-

tion on Sephadex so high in salt content? Since molecular size is the principal factor which determines separations on Sephadex, one would have expected the polypeptide-containing zone to be relatively free of small ions. Second, why did the CMC fail to remove as much Na and K as would be expected from its rated capacity for these cations? Desalting with glacial acetic acid was also relatively less effective. We do not know the explanation for this peculiar behavior of the material, but it may be related to its high viscosity which might interfere with contact of the extract with the particles of the exchanger. Another possibility is that other material in the LH-releasing zone may have a high positive charge which saturates the exchange capacity of CMC, and consequently interferes with retention of salts and the releasing factor.

Summary. Acetone powders prepared from sheep SME fragments were extracted with acetic acid and subjected to gel filtration on Sephadex G-25. An LH-releasing zone was eluted from the column just before the appearance of the pressor zone. The LH-releasing zones from 3 such columns were pooled, applied to a CMC column, and eluted by application of gradients of ammonium acetate of increasing ionic strength and pH. A pressor free LH-releasing zone was eluted from the CMC just prior to emergence of vasopressin. This highly purified LH-RF was active at a dose of 3 μg of peptide. Preliminary desalting was required in order for the LH-RF to be retained on the CMC column. Various steps and stages of desalting have been discussed.

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Purification and Partial Characterization of Mouse Hemopexin (Beta 2-III Globulin).* (30029)

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A protein migrating in the beta-2 globulin region has been shown to be increased in the sera of tumor bearing mice(1,2,3,4), and to be present in soluble extracts of a mouse mammary carcinoma of the RIII mice(4), and in soluble extracts of a rhabdomyosarcoma, and mammary carcinoma (both spon-

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