Journal of Chromatography, 398 (1987) 255–264
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 19 538

SULFATE AND PHOSPHATE ANALYSIS IN GLYCOPROTEINS AND OTHER BIOLOGIC COMPOUNDS USING ION CHROMATOGRAPHY

APPLICATION TO GLYCOPROTEIN HORMONES AND SUGAR ESTERS

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(Received March 6th, 1987)

SUMMARY

An ion chromatography procedure was devised for the simultaneous determination of phosphate and sulfate in the same sample. In order to eliminate interference from zwitterionic compounds (particularly amino acids and peptides) generated during hydrolysis of the phosphate- or sulfate-containing compounds a pretreatment step with a cation-exchange column was required. The detection of sulfate is approximately twice as sensitive as phosphate on a molar basis. The useful working range for sulfate was 200 pmole to 35 nmole with the ion chromatography employed; the range for phosphate was 400 pmole to 65 nmole. Linearity in this range was very satisfactory. Representative analyses are presented for hydrolyzates of several glycoprotein hormones and sugar sulfates and phosphate esters. Replicate analyses were ±3.0% or better. The glycoprotein hormone analyses for sulfate did not indicate whole integers per mole, suggesting mixtures of isohormones as has been found by others using chromatofocusing or isoelectric focussing and immunoassay.

INTRODUCTION

The demonstration that some glycoprotein hormones contain sugar sulfate esters[1] prompted us to examine other glycoprotein hormone preparations available in our laboratory. Our initial attempts employed the colorimetric procedure used by Parson and Pierce[1] in their original studies. The microbarium chloranilate method, originally described by Spencer[2], proved capricious in our hands. The signal-to-noise ratios were unacceptable given the limited amount of sample available for some of the preparations we hoped to examine; systematic addition of potassium sulfate did not produce a linear curve; and some simple proteins gave spurious positive sulfate tests. These observations prompted us to seek a more reliable and sensitive analysis for sulfate.

The ion chromatography procedure of Buchholz *et al.*[3], originally developed to measure chloride, nitrate, and sulfate in air pollution and atmospheric precipita-

0021-9673/87/$03.50

tion studies, appeared adaptable to our application. The following report describes our adaptation of this method to the analysis of glycoproteins and related biologic materials. The conditions applied also allow simultaneous determination of phosphate in the same hydrolyzates. The conditions chosen for hydrolysis and sample handling are such that the method should be readily applicable to other biologic materials not specifically tested in the present study.

MATERIALS AND METHODS

The ion chromatography was done on a Waters ILC-2 ion/liquid chromatograph with a Model 430 conductivity detector (Waters Assoc., Milford, MA, U.S.A.). The design of this detector with a reference (working) electrode separate from the detection (sensing) electrode was particularly advantageous to these studies for the maintenance of stable baselines reproducible over several days without the need for a suppression device to reduce buffer conductivity. The method of Buchholz *et al.*[3] also used a nonsuppressed detection system. The buffer employed for our chromatography was the borate–gluconate buffer recommended by the manufacturer for anion analysis. Final concentrations were: 1.5 m*M* gluconate, and 11.1 m*M* borate in an aqueous solution of 12% acetonitrile and 0.25% glycerol, pH 9.2. All water used in the analyses was deionized, distilled, and filtered through a 0.22-μm filter unit (Sterivex-GS, Millipore, Bedford, MA, U.S.A.). The final buffer contains acetonitrile, thus an organic-compatible filter (Millipore Type FH, 0.5 μm) was used with an all-glass support system. The buffer was filtered and degassed immediately before use. The analytical column was a Waters IC-Pak-A anion-exchange column with an appropriate guard column. Baseline conductivity of the buffer was 306 μS.

D-Gluconic acid, grade V; D-glucose-6-sulfate, potassium salt, crystalline; and D-galactose-6-phosphate, disodium salt were from Sigma (St. Louis, MO, U.S.A.). Cytosine triphosphate, sodium salt was from P. L. Biochemicals (Milwaukee, WI, U.S.A.). Sodium sulfate (anhydrous) and sodium phosphate, tribasic, were analytical reagent grade (MCB, Cincinnati, OH, U.S.A.). Acetonitrile was HPLC grade (Fisher, Pittsburgh, PA, U.S.A.). Sodium lauryl sulfate was specially pure, from BDH Chemicals (Poole, U.K.). Dowex AG 50W-X12 (200–400 mesh) was obtained from Bio-Rad (Richmond, CA, U.S.A.). All other chemicals were the best grade available commercially.

Sulfate and phosphate are commonly encountered in laboratory detergents, thus all glassware to be used in the analyses should be scrupulously cleaned. The cleaning routine adopted should be specifically tested to make sure residual phosphate or sulfate cannot be detected in a concentrated rinse with high quality distilled water. If this proves to be a problem it may be necessary to utilize an aqua regia rinse step.

Glycoproteins for analysis were routinely dried to constant weight in an Abderhalden drying pistol over phosphorus pentoxide, *in vacuo*. The apparatus (Ace Glass, Cat. No. 6692, Vineland, NJ, U.S.A.) was heated by water reflux. The sample was handled on a thin stainless-steel rectangle (4.5 × 1.2 cm) bent in a V-shape and placed inside a "Pregl pig" weighing vessel with a standard taper cap. With this device the sample could be transferred from the drying pistol closed to the atmosphere. The sample was then transferred quantitatively and conveniently by dumping

the stainless-steel holder directly onto the pan of a Cahn electrobalance and the weighing completed with no more than 2–3 min atmospheric exposure. Based on time studies, drying free of bound water was complete within 15 min (shortest time tested) but we routinely use a 2-h drying period. The drying step is important with glycoproteins. Moisture content varied from 5 to 17% with various preparations. The moisture content appears to reflect the history of the sample rather than the nature of the glycoprotein involved based on our experience of several years.

For removal of amino acids from protein hydrolyzates the samples after removal of HCl were redissolved in 500 μl of 0.1% acetic acid and (generally) a 400 μl aliquot passed over a Dowex AG 50W-X12 resin in the acid form. (For scarce samples a quantitative transfer may be necessary.) In practice, the resin was prepared in bulk by washing through several acid–base cycles as suggested by the supplier, and converted to the acid form with 2 *N* hydrochloric acid. The resin was then washed with 1 *N* acetic acid until a negative chloride test was obtained in the effluent. The resin was stored in the refrigerator until use. As required in a given analysis several small columns (35 × 4 mm) were packed to a height of 12 mm. The columns were washed with 0.1% acetic acid just prior to use. Individual samples were assigned individual columns, and the columns were discarded after use.

The column treatment provided an additional useful check for the analysis. Samples prepared by ammonium sulfate precipitation or from phosphate buffers could conceivably retain inorganic phosphate or sulfate that was not an integral part of the molecule. This proved readily testable by dissolving the sample in 0.1% acetic acid and applying it directly (without hydrolysis) to the AG 50W column as above. The effluent and wash was collected, dried, and analyzed as above. This unhydrolyzed "blank" for certain samples has proved necessary.

Hormone preparations analyzed were highly purified, highest potency preparations available, obtained from the following sources: human lutropin (hLH, preparation AFP-4179C) was obtained from the National Hormone and Pituitary Program, Baltimore, MD, U.S.A.). Bovine lutropin (bLH, preparation JGP-fraction 1a) from Dr. J. G. Pierce, University of California at Los Angeles. The preparations of equine lutropin (eLH), equine follitropin (eFSH), equine chorionic gonadotropin (eCG), human chorionic gonadotropin (hCG), and ovine lutropin (oLH) were prepared in this laboratory by previously described procedures[4–7].

Data analysis was done with the MINITAB statistical computing program obtained from the statistics department, Penn State University, run on a Cyber 174 CDC computer.

RESULTS

Sensitivity and performance of the detector and chromatographic system

Initial tests were performed on standard solutions of sulfate and phosphate prepared with sodium sulfate and sodium phosphate. Samples ranging from 100 pmole to 80 nmole were used to establish the standard curves for the analyses. The useful working range for sulfate was 200 pmole to 35 nmole, and 400 pmole to 65 nmole for phosphate (actually HPO_4^{2-} at the pH of the analysis). At the lower end of the stated range the signal-to-noise ratio on our instrument was greater than 3 and useful quantitation was possible. The majority of the analyses have been done in the

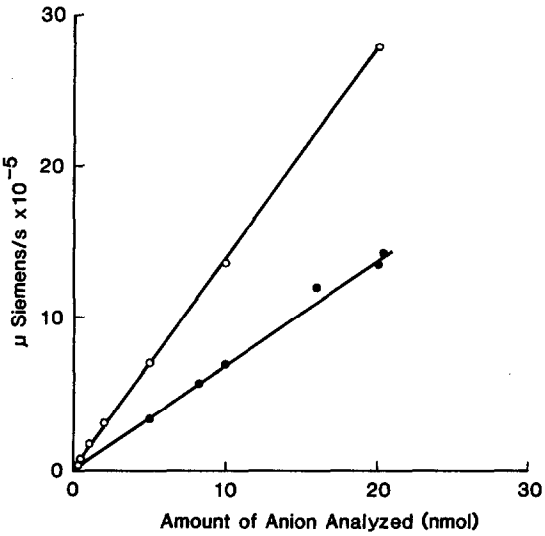

Fig. 1. Analysis of sulfate and phosphate by ion chromatography. The *Y*-axis represents the area (μS as a function of time) generated by the nmoles of sulfate or phosphate injected (*X*-axis). Details of the instrument and operating conditions are described in the text. (○) Sulfate; (●) phosphate.

range 1–20 nmole and this is the most convenient working range. Beyond the upper end of the stated range the peaks were too broad and/or off-scale and thus departed from linearity. In the range stated linearity was excellent (Fig. 1).

The calculated regression lines for the standard curves are: (1) phosphate analysis: $y = -0.031 + 0.681x$; (2) sulfate analysis: $y = 0.090 + 1.39x$, where y = conductivity/unit time (μS/s) $\times 10^{-5}$ and x is the nmoles of sulfate or phosphate analyzed. The absolute values are to an extent arbitrary in that they are influenced by the integrator settings and will vary slightly with the integrator used, but in any event will be related to the area under the curve as indicated by the above equations. From the slope ratio of these curves it is apparent that the sulfate response per nmole is about 2.0 times greater than the phosphate in this system. Based on 67 analyses with two to five replicates per point, in the analysis of variance, r^2 (coefficient of determination) adjusted for degrees of freedom was over 99.9%, indicating excellent precision on the slope with little variance about the individual points[8].

Hydrolysis conditions

For this part of the study we concentrated on conditions suitable for sulfate analysis and simply showed that the conditions selected were also suitable for phosphate analysis.

Studies were first done with lauryl sulfate* to evaluate efficiency of 4 *N* or 6 *N* hydrochloric acid hydrolysis at 110°C as a function of time. Hydrolysis for 2, 4, 5, 8 or 24 h was studied. The 4 *N* hydrochloric acid gave more variable results; values

* Aliquot sampling of lauryl sulfate solutions proved capricious until we established that the plastic pipet tips must be rinsed with the solution several times, and the sample drawn without frothing.

ranged from 85–96.5% of theory over this time period (data not shown). The 6 *N* hydrochloric acid was thus adopted for the protein hydrolyses.

Ovine and bovine lutropin were used as representative glycoproteins containing sulfate esters[1]. Hydrolysis for 4, 8, or 24 h was studied. The samples were introduced into glass hydrolysis tubes as aqueous solutions with 40–500 μg of hormone. The samples were dried in a desiccator *in vacuo*. The hydrochloric acid was next added. The sample was frozen and the tube was sealed *in vacuo* and placed in the hydrolysis oven for the designated time. The sample was opened and hydrochloric acid removed *in vacuo* over sodium hydroxide. The hydrolyzate was next dissolved in water and appropriate aliquots (usually 20–50 μl) injected into the ion chromatography for analysis. A typical analysis pattern is shown in Fig. 2 for a 24-h hydrolyzate. The detector does not recover from the conductivity depression of the amino acids–water solution injected (first eight minutes, Fig. 2) in time to establish a baseline for efficient integration. The perturbance for the shorter hydrolysis times was even less predictable, presumably due to larger peptides spreading through the eluate. This problem was solved by interposing an ion exchange treatment prior to the ion chromatography.

Hydrolysis was for 24 h as above, but after removal of the hydrochloric acid the sample was redissolved in 500 μl of 0.1% acetic acid. An aliquot (usually 400 μl) was passed over the individual sample column and washed through with four

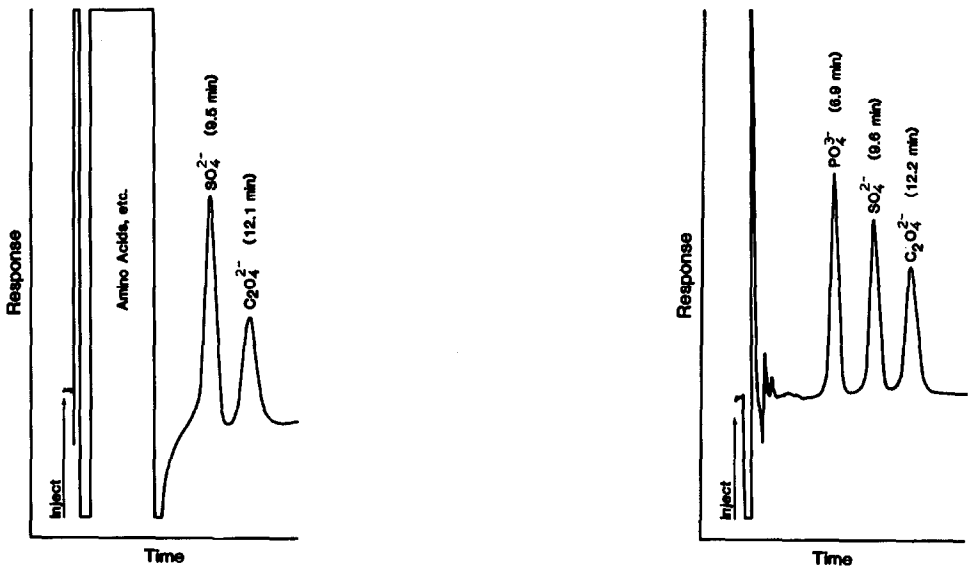

Fig. 2. Ion chromatograph pattern for a hydrolyzate (24 h) from ovine lutropin. The aliquot was taken to provide 20 nmole sulfate for analysis, dried *in vacuo*, redissolved in water and injected into the ion chromatograph. An adequate baseline was not established before the sulfate peak was eluted (9.5 min). The oxalate (10 nmole, 12.1 min) was added as an internal standard after hydrolysis.

Fig. 3. Ion chromatograph pattern for a hydrolyzate (24 h) of ovine lutropin. The aliquot was taken to provide 15 nmole sulfate for analysis and spiked with 10 nmole oxalate and 18 nmole phosphate after hydrolysis and prior to the cation-exchange treatment (see text). Note satisfactory baseline recovery prior to phosphate elution (6.9 min) and sulfate elution (9.6 min).

100-μl water washes. (It was shown that three washes were adequate to remove the sulfate or phosphate quantitatively from the resin column.) The sulfate- or phosphate-containing effluent was dried *in vacuo* and redissolved in a suitable volume of water for injection into the ion chromatograph. Fig. 3 shows a combined phosphate and sulfate sample submitted to this procedure. The oxalate in all the figures was added as an internal standard (usually 10 nmole) at the time the samples were redissolved following hydrolysis to serve as a control for mechanical losses during handling. This procedure was effective except for certain samples as will be described below.

In our series of analyses the use of the oxalate internal standard indicated excellent recovery and essentially no loss of sulfate or phosphate on handling, until we came to the glycoprotein samples. Here the oxalate "recovery" proved too good. If the oxalate value was used to estimate the absolute aliquot applied to the column the sulfate values were lower than instances where the volumetric estimate of sample applied was used in the calculation. The source of this error proved to be the generation of an oxalate-like material in the hydrolysis of most (if not all) glycoproteins. (We assume this material is an oxidative by-product of the carbohydrate degradation and may indeed be oxalate.) This is demonstrated in the experiment depicted in Fig. 4. A hydrolyzate of 650 μg of oLH was made and equal aliquots of the redissolved sample in 0.1% acetic acid were placed in separate drying tubes. To one was added 20 nmole of oxalate, the other was processed without addition. Both were passed over individual ion-exchange columns, the effluent and wash collected, dried *in vacuo*, and analyzed. The analytical patterns are compared in Fig. 4. It is clear that the oxalate-like material at 12.2 min renders this type of hydrolyzate unsuitable for an oxalate internal standard. As a consequence additional care is required to insure

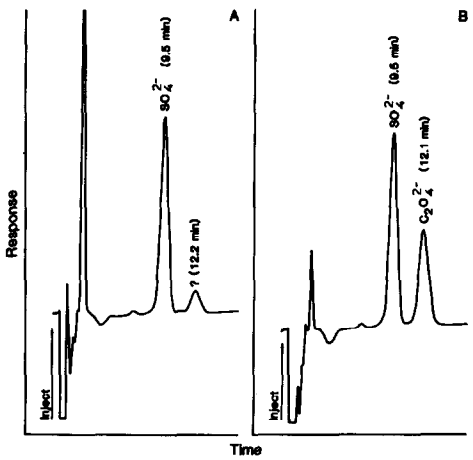

Fig. 4. (A) Ion chromatograph pattern for an aliquot of a lutropin hydrolyzate processed as in the text. The aliquot was adjusted to provide 32 nmole sulfate. (B) Ion chromatography pattern equivalent to that in A, except a 10 nmole internal standard of oxalate was added after hydrolysis and before the cation exchange treatment (see text). The high recovery of oxalate [including the oxalate (?) peak seen in A] would lead to underestimates of sulfate if the internal standard were used to "adjust" the sulfate values for manipulative losses. The oxalate (?) material appears to be a by-product of the hydrolysis of glycoproteins and is probably oxalate produced by oxidation of sugar residues.

quantitative recovery and transfer of such samples, and in practice this is easily achieved. It should be possible to find an anion with an elution time of 14 min or greater under our analysis conditions that could serve as an internal standard, but we have not identified a suitable compound at this time.

We tested the routine application of the method based on the protocol developed above. Table I summarizes the analytical values obtained with a variety of sulfate or phosphate esters. Analysis of the sulfate content in a series of glycoprotein hormones is presented in Table II. The data are presented as the replicates from individual sample hydrolyzates. Sample to sample variation may be assessed from the separate samples of the same compound (Table I). The percent of theory (last column, Table I) is satisfactory within ±3%. The analyses in Table II are notable in that whole integer values are not observed. This undoubtedly results from the presence of various isohormones containing various molar ratios of sulfate. This has been demonstrated for the isoelectric defined species[9] or chromatofocused species[10,11] of these hormones as detected by radioimmunoassay. One of the objectives of the present study was to provide a method to estimate precisely the contribution of sulfation to these isohormones as defined by their charge distribution. As shown

TABLE I

ANALYSIS OF SUGAR SULFATE OR PHOSPHATE ESTERS

Sample	*No.*	*Theory (nmole)*	*Observed* sulfate (nmole)*	*Percent of theory***
D-Glucose-6-sulfate, potassium salt	1	17.50	17.10	97.7
			17.28	98.7
			17.42	99.5
	2	20.31	20.12	99.1
			20.18	99.3
			20.17	99.3
	3	19.00	19.07	100.4
			19.18	100.9
			19.00	100.0
				Mean = 99.4 ± 0.88
			phosphate (nmole)	
D-Galactose-6-phosphate, disodium salt	1	16.60	16.13	97.2
			16.43	99.0
	2	15.00	14.94	99.6
			15.40	102.7
	3	13.20	13.18	99.5
			13.35	101.1
				Mean = 99.8 ± 1.88
Cytidine triphosphate, sodium salt	1	19.80	20.04	101.2
			19.91	100.5
			19.72	99.6
				Mean = 100.4 ± 0.80

* This series of analyses did not include a blank for inorganic phosphate or sulfate, since this would have required revision of the resin treatment step.

** The mean values for each compound are also given with the standard deviation.

TABLE II

ANALYSIS OF SULFATE IN SELECTED GLYCOPROTEIN HORMONE PREPARATIONS

Preparation analyzed	*Aliquot analyzed (μg)*	*Amount of hormone (nmole)*	*Sulfate observed (nmole)*	*nmole sulfate/ nmole hormone*
Ovine lutropin (oLH)	110	3.79	10.43	2.75
	110	3.79	10.55	2.78
	110	3.79	10.48	2.77
	88	3.03	8.31	2.74
Ovine lutropin α subunit (oLHα)	108.5	7.70	11.65	1.51
	108.5	7.70	11.59	1.51
	108.5	7.70	11.60	1.51
	108.5	7.70	11.75	1.53
Ovine lutropin β subunit (oLHβ)	109	7.26	8.98	1.24
	109	7.26	9.15	1.26
	109	7.26	9.09	1.25
	109	7.26	9.09	1.25
Human lutropin (hLH)	99.8	3.47	4.03	1.16
	99.8	3.47	4.10	1.18
	99.8	3.47	4.01	1.16
	99.8	3.47	3.99	1.15
Equine lutropin (eLH)	101	2.89	5.35	1.85
	101	2.89	5.29	1.83
	101	2.89	5.24	1.81
	80.5	2.37	4.31	1.82
Equine follitropin (eFSH)	91.2	2.72	6.47	2.38
	91.2	2.72	6.46	2.37
	91.2	2.72	6.45	2.37
	91.2	2.72	6.42	2.36
Equine chorionic gonadotropin (eCG)	122	2.03	1.89	0.93
	122	2.03	2.11	1.04
	122	2.03	2.01	0.99
Human chorionic gonadotropin (hCG)	291	8.08	0.00*	None
	233	6.47	0.00*	None
Bovine lutropin (bLH)	140	4.83	14.01	2.90
	140	4.83	14.10	2.92
	140	4.83	13.87	2.87

* This is an example of a sample with a significant free sulfate blank. These values have been corrected for a 0.53 nmole/nmole hCG blank.

in the ovine lutropin and the α and β subunits prepared from it (first three samples, Table II) the heterogeneity is attributable to both subunits. The replicates in Table II represent aliquots from a single sample hydrolysis of the preparation analyzed, thus sample to sample variance for a given preparation cannot be assessed. For the samples in Table II the inorganic (free) sulfate blank was not significant except for the human chorionic gonadotropin preparation, as indicated in the footnote.

DISCUSSION

This procedure for sulfate and phosphate analysis has been designed to eliminate interference from ampholytes (such as amino acids) that might be produced as a by-product of the hydrolysis of natural componds. For most applications there is also the implicit requirement that the material being analyzed was first purified satisfactorily. The ampholyte removal step with the resin treatment is absolutely required for suitable results with protein hydrolyzates. The hydrolysis conditions were selected to insure complete hydrolysis of the protein, and is more than adequate for the cleavage of phosphate or sulfate esters. It is also necessary to use a volatile acid (we chose 0.1% acetic acid) to wash the sulfate or phosphate from the resin, since this must be removed by drying *in vacuo* before the sample aliquot is applied to the ion chromatograph. For injection to the chromatograph we used two approaches. If we were not certain about the sulfate or phosphate content we dissolved the sample in water and injected 20 μl (or less) to get a preliminary estimate. This produces a large current drop below baseline level in the first three minutes of chromatography, but it has the advantage that the balance of the sample can be redried and the aliquot volume adjusted (usually to a more concentrated aliquot) as may be required. If the appropriate aliquot size is known the sample can be dissolved directly in the developing buffer to improve the pattern in the early-eluting area of the chromatogram.

Although the protocol for the analysis is designed to remove ampholytes or positively charged ions, which are replaced by protons after the resin step, it is apparent that some neutral substances may be accommodated since the sugars or cytosine obtained as a by-product of the hydrolyses in Table I did not interfere with the analyses. Note that the resin step would remove the sodium or potassium ions.

The foregoing comments should alert the reader that some consideration should be given to the nature of the hydrolysis by-products for new applications of this procedure.

The present method of sulfate analysis was designed specifically to study the sulfate content of the isohormones of LH and FSH[9–11]. However, it should be widely applicable to the analysis of other sulfated proteins and proteoglycans. There also are several biologically important compounds that contain both sulfate and phosphate (see, for example, refs. 12–15). The role of phosphate has long been appreciated in biological processes. Protein sulfation on carbohydrate of glycoproteins or tyrosine residues is a frequently observed posttranslational modification (see ref. 16 for a review), but the functional significance of the sulfation is only poorly understood. It has recently been reported[17] that deiodination and clearance of circulating thyroid hormones is mediated by a sulfation step, while Cozzi and Zanini[18] suggest sulfation of pituitary hormones such as LH may be involved with secretion and that there are proteins (secretogranin II, highly sulfated on tyrosine residues) presumed to be involved in the packaging of the gonadotropin for secretion.

ACKNOWLEDGEMENTS

This work was supported in part by research grants AM-09801 and HD-18210, from the National Institutes of Health, and Grant G-147 from The Robert A. Welch Foundation.

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