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Physico-chemical Characteristics of the Interstitial Cell Stimulating Hormone from Sheep Pituitary Glands*

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It is well known that many pure proteins have not been crystallized and conversely that crystalline proteins may not be chemically pure. Physico-chemical studies of protein preparations are hence highly important for the determination of purity.

The duality of pituitary gonadotrophins, as suggested by Fevold, *et al.*,[1] can be regarded as established. The best support of this concept would be the isolation of either gonadotropic component in chemically pure form. The isolation of pure interstitial cell stimulating hormone (ICSH) from pig[2] and sheep[3] pituitary glands has been reported. This paper is devoted to a re-examination of the purity of sheep ICSH. Some physico-chemical characteristics of the hormone are reported.

Method of Isolation

The essential steps in the preparation of pure ICSH have been published.[3] An alternative method which has recently been devised is given below; all steps were carried out at from 4 to 6°.

(a) The starting material was a dry powder prepared from a 40% ethanol extract of fresh sheep pituitaries. The 1% sodium chloride extract of this powder was precipitated by 1.74 *M* ammonium sulfate. This precipitate contained 20 rat units (R. U.) per milligram.[4] A 3% solution of this precipitate was then dialyzed against distilled water until free of salt. A precipitate formed during dialysis contained (after thorough washing) only 4 R. U. per milligram and was therefore discarded. The water soluble components contained 40 R. U. per milligram of dry protein.

(b) The dialysis supernatant, plus washings of the insoluble fraction, which contained approximately 0.5% protein and had a *p*H of about 5.3, was then brought to 1.06 *M* ammonium sulfate (McMeekin's technique).[5] The precipitate formed, which contained very little biological activity (2–4 R. U. per milligram), was discarded. The ammonium sulfate concentration of the supernatant liquid was then raised to 1.74 *M* by the same technique.

(c) The 1.74 *M* ammonium sulfate precipitate was redissolved in 1% solution (100 cc.) and step (b) was repeated twice. The final precipitate thus obtained had a potency of about 100 R. U. per milligram and was practically free from other contaminating hormones, but electrophoresis still revealed two components.

(d) The fraction obtained in step (c) was redissolved in water (1% solution) and dialyzed until salt-free.[6] The dialyzed solution was then precipitated isoelectrically at *p*H 4.0–4.1 (glass electrode) in 1.06 *M* ammonium sulfate solution. The salt was added first by the technique above. The solution should remain clear until the hydrochloric acid is added (0.1 *N* hydrochloric acid, dilutions made with 1.06 *M* ammonium sulfate). The isoelectric precipitation was repeated twice under the same conditions. The final precipitate contained 200 R. U. per milligram of ICSH (0.005 mg./R. U.) and was free of other biologically active pituitary proteins at the highest levels tested. Total doses of 3 mg. were free of follicle stimulating hormone; 2 mg. doses were free of thyrotropic hormone; 10 mg. doses were free of adrenocorticotropic, lactogenic and growth hormones. This product was subjected to the following physico-chemical examinations for purity.

Criterion of Purity

Electrophoresis.—Electrophoresis studies were conducted in a Tiselius apparatus with the Longsworth scanning method.[7] Determinations were made with 1% protein solutions. Two buffer solutions, *p*H 7.40 and 5.90 phosphate buffers of ionic strength 0.1, were used. Typical results from these experiments are shown in Figs. 1 and 2. Only one sharp peak appears indicating the homogeneity of the preparation.

In some experiments, the current was reversed after electrolysis for three hours and the phenomenon of "reversible boundary spreading" occurred, as described by Tiselius and Horsfall.[8] The phenomenon was first encountered in hemocyanin solutions and has been reported since by other investigators[9,10] for other protein solutions. It is still possible that this phenomenon indicates the heterogeneity of protein solutions but this has not been proved, as both crystalline and non-crystalline proteins have exhibited "reversible boundary spreading."[11]

Solubility.—The solubility of ICSH was studied at 25° in buffer solution of *p*H 4.0 (0.1 *N* acetate buffer containing 0.904 *M* sodium sulfate). The technique was essentially

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(1) Fevold, Hisaw and Leonard, *Am. J. Physiol.*, **97**, 291 (1931).

(2) Shedlovsky, Rothen, Greep, van Dyke and Chow, *Science*, **92**, 178 (1940).

(3) Li, Simpson and Evans, *Endocrinology*, **27**, 803 (1940).

(4) The R.U. for ICSH is the minimum dose which, given intraperitoneally in 3 daily doses, followed by autopsy 72 hours after the first injection, causes interstitial tissue repair in the body of hypophysectomized female rats (26 to 28 days at operation, 10 to 12 days post-operative at autopsy).

(5) McMeekin, THIS JOURNAL, **61**, 2884 (1939).

(6) When precipitation occurs during dialysis the precipitate should be removed before the addition of saturated ammonium sulfate.

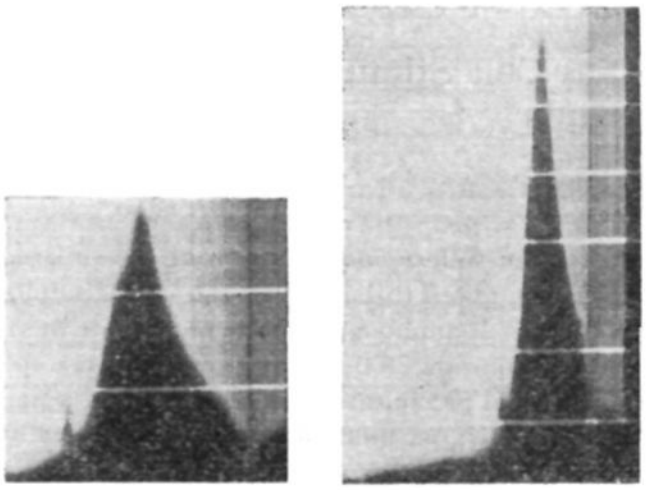
(7) Longsworth, THIS JOURNAL, **61**, 529 (1939).

(8) Tiselius and Horsfall, *Ark. Kemi. Mineral. Geol.*, **13A**, No. 18 (1939).

(9) Lauffer and Ross, THIS JOURNAL, **62**, 3296 (1940).

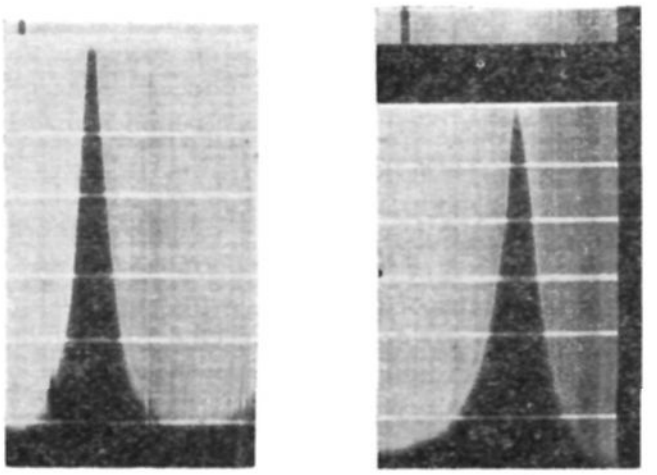
(10) Longsworth, Cannan and MacInnes, *ibid.*, **62**, 2580 (1940).

(11) L. G. Longsworth, private communication.



180 min. 90 min.

Fig. 1.—Electrophoresis patterns of sheep ICSH solution (1.0%) in a phosphate buffer of ionic strength 0.1 at *p*H 7.40 and 2°. Scanning exposures made for the ascending boundary after the solutions were electrolyzed for 90 and 180 minutes at 2.84 volts per cm.



a ← → d

Fig. 2.—Electrophoresis patterns of a 1.0% solution of sheep ICSH in a 0.1μ phosphate buffer at *p*H 5.90 and 2°. Scanning exposure made after electrophoresis for 120 minutes at 6.07 volts per cm.

the same as described previously.[12] The nitrogen content of the solutions was determined by semi-micro Kjeldahl method. As shown in Fig. 3, the results indicate that the preparation contained a single component, the solubility being constant from the first appearance of turbidity. Bioassays of material soluble and insoluble showed the same potency.

Ultracentrifuge.—We are indebted to Dr. M. A. Lauffer of the Rockefeller Institute for Medical Research, Princeton, New Jersey, for analysis of the sheep ICSH in the ultracentrifuge. These experiments were carried out in an ultracentrifuge of Bauer and Pickels' type. The solvent used was a 0.172 *M* sodium chloride solution. The hormone appeared to be homogeneous and it had a sedimentation constant[13] 3.6×10^{-13}.

Some Physico-chemical Properties

Isoelectric Point.—The electrophoretic mobilities of the hormone in acetate and phosphate buffers of 0.1 ionic strength at 1.5° were determined and results summarized in Table I. The mobilities were calculated from the descending boundaries as suggested by Longsworth and MacInnes.[14] A plot of *p*H against the mobility reveals the isoelectric point of ICSH to be 4.6.

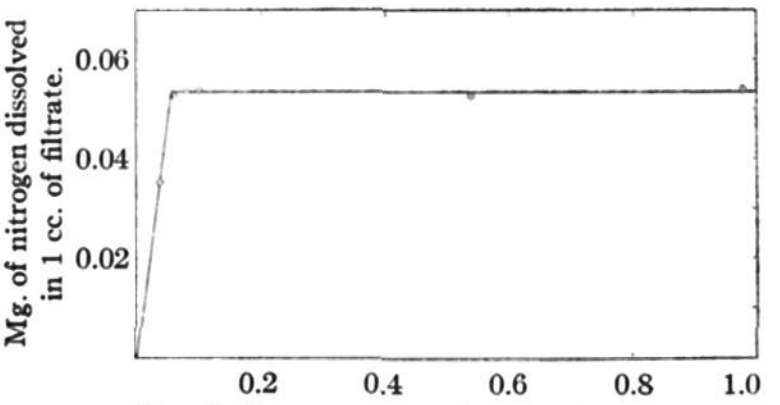


Fig. 3.—Solubility of sheep ICSH in 12.8% sodium sulfate of *p*H 4.0, 0.1 *N* acetate buffer solution at 25°.

TABLE I

THE ELECTROPHORETIC MOBILITY OF ICSH IN BUFFERS OF DIFFERENT *p*H

*p*H	Buffer	Mobilities, 10^5
7.40	Phosphate	−6.0
6.52	Phosphate	−3.2
5.40	Acetate	−2.4
4.00	Acetate	+2.0
3.00	Citrate	+3.4

Molecular Weight.—Osmotic pressure measurements have been used extensively for the determination of the molecular weight of proteins. Since the osmotic pressure is a thermodynamic property which depends only on the number of particles in solution and not on their shape, it gives the *true* molecular weight of the substance. There is only one disadvantage to this method as compared with the ultracentrifugal determination, namely, it does not indicate the homogeneity but gives the average particle weight of the solution. If the substance has been proved to be a single component, the osmotic pressure does, however, offer *a suitable and convenient method for the molecular weight* determination.

From the van't Hoff equation, Burk and Greenberg[15] obtained the relationship of the molecular weight of proteins to the observed osmotic pressure as shown in Equation 1:

$$M = CdRT/100P \qquad (1)$$

in which C is the grams of solute per 100 g. of solvent, d the density of the solvent, P the osmotic pressure in centimeters of a column of density 1.0; R and T have their usual significance.

The osmotic pressure measurements were carried out in an osmometer of Burk and Greenberg's type. The experimental procedure has been described in a previous paper.[12] The solvent used was 1 *N* acetate buffer of *p*H 4.60. Results, shown in Table II, give the molecular weight of sheep ICSH an average value of 40,000.

(12) Li, Lyons and Evans, *J. Biol. Chem.*, **140**, 43 (1941).

(13) A quantitative analysis of the results will be reported separately by Dr. Lauffer.

(14) Longsworth and MacInnes, THIS JOURNAL, **62**, 705 (1940).

(15) Burk and Greenberg, *J. Biol. Chem.*, **87**, 197 (1930).

TABLE II

OSMOTIC PRESSURE OF ICSH SOLUTIONS AT 0°

pH	Protein concn./100 g. of buffer C, g.	Water pressure, p, cm.	C/P	Mol. wt.
4.60	0.990	5.55	0.178	41,000
4.58	1.090	6.35	0.172	39,800
4.60	0.925	6.10	0.168	39,000
			Average	40,000

It is of interest to note that the molecular weight of the hormone, as calculated from the analytical results previously[16] reported, is also approximately 40,000. It is assumed that the hormone consisted of two molecules of tryptophan and 10 molecules of tyrosine.

Comparison with Pig ICSH

Recently Shedlovsky, *et al.*,[2] isolated ICSH in chemically pure form from pig pituitary. The physical properties described by them differ considerably from those found for sheep ICSH. The pig ICSH is said to have a molecular weight of about 90,000 while that of sheep as described in this paper is 40,000. The isoelectric point of pig ICSH is stated to be 7.45 whereas that of sheep is 4.6.

The ICSH isolated from pig and from sheep pituitary glands also appear to differ considerably chemically. A pure pig ICSH which was kindly supplied by Dr. van Dyke was analyzed for carbohydrate and tryptophan content. It was found to contain approximately 2.0% carbohydrate and 3.8% tryptophan using the orcinol[17] and glyoxalic acid[18] methods, respectively. The sheep ICSH on the other hand contains 4.5% carbohydrate and 1.0% tryptophan. It is not unreasonable that hormones like other proteins isolated from different species may not be identical.

(16) Li, Simpson and Evans, *Science*, **92**, 355 (1940).

Summary

1. A modification of the method for isolation of sheep interstitial cell stimulating hormones (ICSH) is described.

2. The preparation behaves as a single substance in electrophoresis and in ultracentrifuge and solubility studies.

3. The isoelectric point of the sheep ICSH has been shown to be *p*H 4.6. The molecular weight has been determined to be 40,000, both from osmotic pressure studies and analytical data.

4. A comparison between sheep and pig ICSH has been made.

(17) Sørensen and Haugaard, *Biochem. Z.*, **260**, 247 (1933).

(18) Shaw and MacFarlane, *Canad. J. Research*, Sect. B., **16**, 351 (1938).

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The Influence of Structural Changes in the Aglucons on the Enzymic Hydrolysis of Alkyl β-D-Glucosides[1]

BY WILLIAM WARD PIGMAN AND NELSON K. RICHTMYER

A study of the effect of alterations in the sugar portions of glycosides has shown that "almond emulsin does not hydrolyze all glycosides but instead only the glycosides of naturally occurring sugars or of sugars which may be considered to be derived from these sugars by a simple substitution outside the pyranose ring."[2] This type of specificity may be termed "sugar specificity." Although slight structural or configurational changes in the sugar portion of hydrolyzable glycosides may inhibit completely the enzymic hydrolysis, considerable alterations may be made in the structures of the aglycons of hydrolyzable glycosides without preventing completely the enzymic hydrolysis. This type of specificity might be designated as "aglycon specificity." Quantitative studies of the "aglycon specificity" have been limited principally to the hydrolysis of β-glucosides by almond emulsin. Helferich and his co-workers,[3] in particular, have studied the effects of substitution in the aromatic nucleus of phenyl β-glucoside, while Veibel and his associates[4] have reported in considerable detail, although under somewhat different conditions, on the results of structural alterations in the aglucon groups of the alkyl β-glucosides. In the present investigation we have made a quantitative study of the action of the β-glucosidase of sweet almond emulsin on the *n*-alkyl β-D-glucosides[5] from the *n*-amyl to the *n*-nonyl glucoside, and on several

(1) Publication authorized by the Director of the National Bureau of Standards, and by the Surgeon General, U. S. Public Health Service (not copyrighted). Presented before the Division of Sugar Chemistry and Technology at the Atlantic City meeting of the American Chemical Society, September 8–12, 1941.

(2) W. W. Pigman, *J. Research Natl. Bur. Standards*, **26**, 197 (1941); THIS JOURNAL, **62**, 1371 (1940).

(3) B. Helferich, H. Scheiber, R. Streeck and F. Vorsatz, *Ann.*, **518**, 211 (1935); B. Helferich and C. P. Burt, *ibid.*, **520**, 156 (1935).

(4) S. Veibel and H. Lillelund, *Kgl. Danske Videnskab. Selskab, Math. fys. Medd.*, **17**, no. 6 (1940); *Z. physiol. Chem.*, **253**, 55 (1938).

(5) We are indebted to Dr. C. R. Noller of Stanford University for supplying us with samples of a number of the *n*-alkyl glucosides which have been used in this study.