STUDIES ON PITUITARY LACTOGENIC HORMONE

III. Solubilities of Sheep and Beef Hormones*

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INTRODUCTION

Northrop and coworkers (1–3) believe that solubility studies constitute the most sensitive and reliable test for determining the homogeneity of proteins and have used them successfully as a test for purity of their crystalline enzyme preparations. Herriott, Desreux, and Northrop (4) were able to show that a number of pepsin solutions that appeared to be homogeneous in electrophoresis experiments contained several protein components as indicated by solubility studies. Steinhardt (5) has recently shown that in the case of some proteins difficulties may attend the application of the test.

Cohn and coworkers (6, 7) have carried out protein solubility studies using the salting-out effect of electrolytes in buffer solutions. If proteins are pure, a plot of the logarithm of solubility against ionic strength may appear to be a straight line. Slopes and intersects to the ordinate are used in characterizing the protein.

The solubility method is not only useful in determining the purity of a preparation but is a sensitive method for distinguishing species specificity of proteins (8). Our preparations of pituitary lactogenic hormone have been demonstrated recently to act as a homogeneous substance in the Tiselius apparatus (9), and preparations of beef and sheep hormone were indistinguishable (10) electrophoretically. Bischoff and Lyons (11) were not able to differentiate the hormone prepared from beef or sheep pituitary by certain immunological methods. It seemed worth while, therefore, to investigate the solubility behavior of our beef and sheep lactogenic hormones in order to determine not only whether they were pure but

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also whether they were species specific. The beef and sheep lactogenic hormones used in this work were prepared in essentially the same manner as previously described (12).

A. Solubility in NaCl-HCl Solution

Preparation and Solvent.—L299S1 contained approximately 30 International Units per mg. The isoelectric precipitate (about 300 mg.) was washed twice with 40–50 cc. of solvent. The washed precipitate was used for the solubility experiments. The solvent was made with 0.302 mM NaCl in 0.01 N HCl solution and contained 2 per cent butanol. The pH of the solution was 2.022 (glass electrode).

Methods.—The precipitate which had been washed twice with the solvent was broken into a fine suspension by agitation for 15 hours in a closed test tube (12 X 100 mm. Pyrex).

Varying amounts of this suspension were distributed into test tubes and each tube was then filled with the solvent. The technique to displace the remaining air bubbles was essentially the same as that described by Kunitz and Northrop (13). The tubes were then agitated on the wheel for 2 or 3 days. The solutions were filtered through a Whatman filter paper (No. 42) and the filtrate analyzed for nitrogen. Nitrogen was determined by a micro-Kjeldahl method and the results represent averages of two or more determinations.

The amount of protein dissolved was sometimes checked by Folin's phenol reagent (14) and carried out in the following manner. To 1 cc. of the filtrate, diluted to 10 cc., were added 2 cc. of 1 M NaOH and 3 cc. 1:3 Folin's reagent. The mixture was kept in an oven at 40°C. for 15 minutes. The color produced was measured in the Cenco photometer using the red filter. The protein content was then read from a standard curve which had been constructed by using known quantities of protein in the same manner. The amount of nitrogen was obtained by assuming that the hormone contained 14.52 per cent nitrogen.

Results.—Results are given in Fig. 1. The curve shows that the preparation obeys strictly the requirements of the phase rule for a single substance. It may be noted that the initial slope of the curve is 1, indicating that the solutions were perfectly clear before the appearance of the solid phase. This

1 Throughout this paper S indicates sheep and B, beef origin of the pituitary lactogenic preparations.

2 In this connection, the stability of lactogenic hormone in acid solutions was studied. 10 mg. L299S were dissolved in 5 cc. 0.1 M HCl. The mixtures were kept at room temperature (20–21°C.) for 1, 2, and 3 days in the presence of 2 per cent butanol as preservative. Before being assayed solutions were neutralized and it was found that there was no difference in potency in the 1 and 2 day samples, whereas the 3 day sample showed a loss in potency of about 50 per cent.

3 The test tubes were clamped on a revolving wheel; two glass beads were placed in each tube to agitate the material.

4 This value is low because corrections were not made for ash and moisture.
fact as well as our determination that the biological potency of the soluble protein was the same as that of the insoluble protein in saturated solutions presents further evidence for homogeneity of the hormone.

B. Solubility in Water

Water is well known to be the most favorable solvent for solubility studies because interactions which sometimes take place between salts and their saturating body are eliminated. As pointed out by Cohn (16), the solubility in water of a pure protein may be considered as a fundamental, physicochemical constant which may be used in identifying and in classifying proteins. However, owing to the difficulty in removing the last trace of salt, acid, and alkali from isoelectric proteins there are very few pure proteins which have been investigated as to their homogeneity, by the method of solubility in water.

L294B was an isoelectric preparation obtained from beef pituitaries. Approximately 1 gm. of wet isoelectric precipitate was triturated three times with 50 cc. water. Two solubility experiments were carried out using different quantities of the washed precipi-
tate in the same volume of water. The test tubes (as in experiments of section A) were agitated on the wheel for 2 days at room temperature; the suspensions were then rocked in a cold room at a temperature of 7–8°C. for another 2 days. The solubility of isoelectric beef lactogenic hormone in water (at 7–8°C.) was found to be 0.102 gm. per liter in the tube containing the smallest amount of solid, while in the tube containing seven times that amount of saturating body, the solubility was practically the same (0.104 gm. per liter). This indicates that the preparation behaved as a single substance. The low solubility of the protein suggests that it has a low dissociation constant and possibly that it has a small number of free polar groupings in the molecule.

C. **Salting Out Effect of NaCl in Acid Solution**

The influence of salts on the solubility of proteins has been used not only for separation and purification purposes but for the characterization of proteins. Cohn (17) has shown that solubility is defined by an equation of the form

\[ \log S = \beta - K_s \mu \]

in which \( K_s \) is a salting-out constant characteristic of the salt employed, \( \beta \) an intercept constant characteristic of the saturating substance, \( \mu \) the ionic strength per 1000 gm. of water, and \( S \) the solubility of the protein in gm. per liter. Using this technique, Green, Cohn, and Blanchard (18) were able to show the species specificity of horse and human carboxyhemoglobins. Since species specificity has not hitherto been shown to exist in the case of lactogenic hormone, it seemed interesting to attempt with the aid of this method a differentiation between our beef and sheep lactogenic preparations.

**Experimental.**—L287B and L288S were acetone-dried isoelectric precipitates. They both contained approximately 30 I.U. per mg. 20 mg. of L287B or L288S were dissolved in 2 cc. of 0.01 N HCl and to this were added 3 cc. of 0.01 N HCl containing different amounts of NaCl.\(^5\) The test tube was then shaken slowly for about 2 hours at room temperature. The suspension was filtered and the filtrate was analyzed for nitrogen as described in section A.

**Results.**—The behavior of these two lactogenic hormones is graphically represented in Fig. 2. These results show that the solubility of both L287B and L288S followed the rule represented by the equation previously given. Furthermore, sheep lactogenic hormone was found to be more soluble than the beef hormone in these acid solutions.\(^6\) It is of interest to note that the

\(^5\) Since solubility is a function not only of the concentration of the salt, but of the pH and the temperature, it is very important to make up the solvent with extreme accuracy. This has not been attempted in the present experiments since the results were intended primarily for the purpose of comparing sheep and beef hormones rather than establishing characteristic absolute values for the solubility.

\(^6\) In experiments using citrate buffer (0.1 M, pH = 6.36) beef lactogenic hormone has been found to be more soluble than sheep.
slopes (K) of the two curves are almost the same whereas the intercept constants (β) exhibit differences.

It may also be seen that NaCl proved to be a very effective precipitant for the hormone dissolved in dilute HCl. We have always observed that the lactogenic hormone is very soluble in this solvent, but its solubility is greatly decreased in the presence of salts. The solubility in alkaline solu-

The addition of HCl to a strength of 0.5 M causes the precipitation of lactogenic hormone.
tion is, however, not so much affected by the presence of NaCl. Thus, the precipitation of lactogenic hormone began only when 32 gm. of NaCl were added to 100 cc. 0.01 n NaOH solution; on the other hand, the hormone began to precipitate out in 0.01 n acid solution when 1.5 gm. of NaCl were added. Solubility is a measure of the interaction between protein dipoles and the ions of a salt and the difference in behavior of the protein in acid and basic solutions may be attributed, in part, to the fact that the dissociation constants of certain groups are much higher above than below the isoelectric point.

D. DISCUSSION

It is generally agreed that crystalline proteins are not always homogeneous with respect to sedimentation, electric charge, and solubility, but ultracentrifugation, electrophoresis, and solubility studies also have limitations. Since proteins fall into molecular weight classes (19), and so many proteins have molecular weights ranging from 34,000 to 42,000, the ultracentrifuge has often proved unsatisfactory in characterizing the purity of a protein. Furthermore, a single boundary in the Tiselius apparatus does not necessarily signify a pure substance. Solubility studies constitute the best available method for determining homogeneity of a protein, especially if they are carried out with a variety of solvents, and due regard is given to the possibility that solubility anomalies may occur under certain circumstances (5, 20, 21).

Although we have not yet been able to secure the pituitary lactogenic hormone in uniform crystalline state, our preparations behave in electrophoresis experiments (9) as a single substance. The solubility studies herein reported also failed to disclose more than a single component. Preliminary work on ultracentrifugation of the lactogenic hormone has so far shown it to be homogeneous, but further data have yet to be collected.

The species specificity of pituitary lactogenic hormone as demonstrated in this paper is particularly interesting. In our previous work we (10) were not able to distinguish the sheep pituitary hormone from that of beef pituitary in electrophoresis experiments. Bischoff and Lyons (11) were also unable to differentiate them through the use of precipitin, anaphylaxis, or the Dale and Arthus reactions. An analogous situation has been found to be the case with pepsin (22).

SUMMARY

The solubility of sheep pituitary lactogenic hormone in 0.302 m NaCl at pH 2.02 (solution in HCl) has been determined at room temperature.
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It showed a constant solubility in the presence of a considerable excess of the solid phase, an indication that the preparation contained but one component.

Beef lactogenic hormone showed a constant solubility in distilled H₂O at 7–8°C in the presence of excess of the solid phase.

The salting-out effect of NaCl in acid solution of both beef and sheep hormones has been studied at room temperature. In these studies both preparations behaved as pure substances, but they exhibited differences in solubility, thus indicating a species specificity.

BIBLIOGRAPHY