STUDIES ON PITUITARY LACTOGENIC HORMONE

VI. MOLECULAR WEIGHT OF THE PURE HORMONE*

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In recent publications experiments were described demonstrating the homogeneity of our lactogenic hormone preparations by means of both electrophoretic (1, 2) and solubility (3) criteria. Of pituitary hormones the lactogenic seems to have been the first isolated in chemically pure form. Its molecular weight, however, has not yet been reported, although for many years it has been thought to be comparatively low. In this paper, the molecular weight of the lactogenic hormone is evaluated from both osmotic pressure measurements and analytical data.

Criteria of Purity

Electrophoresis—In previous studies (1, 2) our lactogenic hormones have behaved as single substances in the Tiselius electrophoresis apparatus used with the schlieren method to examine the moving boundary. Some investigators doubted that results obtained by the simple schlieren method were satisfactory in establishing the homogeneity of proteins. An experiment was therefore performed under the observation of Dr. L. G. Longsworth by his schlieren scanning technique (4). A lactogenic hormone solution (0.5 per cent) in an acetate buffer of ionic strength 0.05 at pH 4.03 was electrolyzed for 11,000 seconds in a potential gradient of

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8.083 volts per cm. at 0.5°. The Longsworth scanning patterns, shown in Fig. 1, revealed only one moving boundary with a mobility\(^1\) of \(6.455 \times 10^{-5}\) sq. cm. per second per volt. These observations were in perfect agreement with those obtained by us in our apparatus.

**Solubility**—The application of phase rule (6) in the study of the solubility of proteins is generally accepted as the best method by which to establish their purity. In a previous paper (3) we emphasized the importance of choosing more than one solvent in which to study the solubility of protein preparations. The purity of our lactogenic preparations was therefore reexamined by this technique in another solvent.

Two preparations were used, one prepared from sheep gland and the other from ox. An 0.357 m NaCl solution, pH 2.25 (in HCl), served as the solvent. The results obtained are shown in Fig. 2. Both ox and sheep hormones gave very good solubility curves, the solubility being constant from the first appearance of

\(^1\) The mobility calculation was based on the migration of the descending boundary as suggested by Longsworth and MacInnes (5).
turbidity. Each preparation, therefore, contained but a single component.

Fig. 2 shows another interesting finding; namely, that the ox and sheep hormones exhibit different solubilities. In 0.357 M NaCl solution at pH 2.25, the sheep hormone has a solubility of 0.506 gm. in 1 liter of the solvent, whereas the solubility of the ox preparation is only 0.316 gm. This finding is consistent with our previous observations (3) that the sheep hormone is more soluble than the ox in acid solution.

**Fig. 2.** Solubility of beef and sheep lactogenic hormones in the presence of increasing quantities of solid phase.

Another experiment to differentiate the ox and sheep hormones was carried out by adding 20 mg. of a sheep preparation to 5 cc. of a saturated solution of the ox hormone. Results showed that more sheep protein was dissolved in the saturated ox solution. The difference in solubility of these two substances indicated the extreme sensitivity of the solubility methods, although we were unable to detect their differences in electrophoresis experiments (2).
Molecular Weight

Osmotic Pressure—If a protein has been proved to be a single substance, the osmotic pressure is most suitable for the determination of the molecular weight, because it is a thermodynamic property which depends only on the number of particles in solution and not on their shape. The Donnan effect, which may cause trouble in making osmotic pressure measurements, fortunately can be largely eliminated by carrying out the experiment at the isoelectric point, or not far from the isoelectric point in high salt concentration.

From the van't Hoff equation Burk and Greenberg (7) obtained the relationship of the molecular weight of proteins to the observed osmotic pressure as shown in Equation 1.

\[ M = \frac{C d R T}{100 P} \]  

in which \( C \) is the gm. of solute per 100 gm. of solvent, \( d \) the density of the solvent, \( P \) the osmotic pressure in cm. of a column of density 1.0; \( R \) and \( T \) have their usual significance.

Since the lactogenic hormone is very insoluble at its isoelectric point, the osmotic pressure measurements were carried out at about pH 6.4 in phosphate buffers. The Donnan effect may be seen from the pH determinations of solutions inside and outside of the dialysis bag after the attainment of equilibrium. In every case, the difference in pH is about 0.02 unit. As shown by Adair and Adair (8), such a difference in pH hardly influences the true osmotic pressure of the solution. Furthermore, since the concentration of the protein was not more than 1.5 per cent, the effect of hydration on the value of \( C \) in Equation 1 need not be considered. Results, summarized in Table I, indicate that the molecular weight of the hormone is 26,500 and that the ox and sheep hormones have the same value.

In previous experiments (9) it has been shown that the hormone is not inactivated in 6.66 M urea solutions, and that in such solutions it reacts with iodine in the same manner as in aqueous solutions. It was of interest, therefore, to investigate the osmotic behavior of the hormone in urea solution, since some proteins (7, 10) dissociate into fragments in this solvent.

As shown in Table II, the molecular weight of the hormone deter-
mined in urea and in aqueous solutions is essentially the same. It remains to be shown whether the hormone may be denatured by urea and still have the same molecular weight, since Burk (11)

### Table I

Osmotic Pressure of Lactogenic Hormone Solutions in Aqueous Buffer Solution at 0°

<table>
<thead>
<tr>
<th>pH</th>
<th>Solvent</th>
<th>Species</th>
<th>Protein per 100 gm. solvent</th>
<th>Pressure observed</th>
<th>C:P</th>
<th>M, from Equation 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.48</td>
<td>0.04 M phosphate buffer in 1.0 M NaCl</td>
<td>Ox</td>
<td>0.450</td>
<td>4.01*</td>
<td>0.112</td>
<td>26,000</td>
</tr>
<tr>
<td>6.50</td>
<td>&quot;</td>
<td>Sheep</td>
<td>0.342</td>
<td>3.23</td>
<td>0.106</td>
<td>24,600</td>
</tr>
<tr>
<td>6.40</td>
<td>0.02 M phosphate buffer in 0.5 M NaCl</td>
<td>&quot;</td>
<td>0.916</td>
<td>7.96</td>
<td>0.116</td>
<td>27,000</td>
</tr>
<tr>
<td>6.40</td>
<td>&quot;</td>
<td>&quot;</td>
<td>0.582</td>
<td>4.75*</td>
<td>0.123</td>
<td>28,500</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>26,500</td>
</tr>
</tbody>
</table>

* The equilibrium was attained from higher pressure.

### Table II

Osmotic Pressure of Lactogenic Hormone Solutions in Urea Buffer Solution at 0°

<table>
<thead>
<tr>
<th>pH</th>
<th>Solvent</th>
<th>Species</th>
<th>Protein per 100 gm. solvent</th>
<th>Pressure observed</th>
<th>C:P</th>
<th>M, from Equation 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.21</td>
<td>0.05 M acetate buffer in 6.66 M urea solution</td>
<td>Ox</td>
<td>1.230</td>
<td>10.52</td>
<td>0.117</td>
<td>29,700</td>
</tr>
<tr>
<td>5.85</td>
<td>0.1 M acetate buffer in 6.66 M urea solution</td>
<td>&quot;</td>
<td>0.491</td>
<td>4.76</td>
<td>0.103</td>
<td>25,800</td>
</tr>
<tr>
<td>5.90</td>
<td>&quot;</td>
<td>&quot;</td>
<td>0.911</td>
<td>8.16</td>
<td>0.112</td>
<td>28,400</td>
</tr>
<tr>
<td>5.90</td>
<td>&quot;</td>
<td>Sheep</td>
<td>0.948</td>
<td>9.40</td>
<td>0.101</td>
<td>25,600</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>27,400</td>
</tr>
</tbody>
</table>

has shown that gliadin is denatured in urea even though its osmotic pressure remains unchanged. In the case of some proteins denaturation may be accompanied by the appearance of the —SH group in the molecule, but in iodination experiments (9) this group was
not detectable in urea solutions. Together with the fact that the hormone shows no change in biological activity nor in molecular weight, this indicates that it probably remains "native" in urea.

With regard to this problem, we have examined the biological activity of the hormone after the treatment of trichloroacetic acid because this acid is also known to denature proteins. To 4 cc. of a 0.5 per cent hormone solution 1 cc. of 20 per cent trichloroacetic acid was added at room temperature. The hormone was completely precipitated by the acid. After the removal of the precipitate by centrifugation, it was dissolved in a dilute alkaline solution and was then dialyzed. The dialyzed material was assayed and no loss of activity was found.

It may be concluded, therefore, either that the hormone is a very stable protein and remains "native" in urea and trichloroacetic acid solutions, or that if the hormone is denatured by urea or trichloroacetic acid the process must be reversible.

Analytical Data—An accurate determination of a constituent of a protein is often proved to be very useful in calculating the true molecular weight. If a protein contains $X$ per cent of $A$ atom or $B$ molecule which has an atomic or molecular weight $m$, the minimal molecular weight will be $100m/X$. It follows, therefore, that the true molecular weight of the protein is

$$100nm/X$$

where $n$ is the number of $A$ atoms or $B$ molecules. The usefulness of this method has been emphasized many times by Cohn (13).

Tyrosine and Tryptophane Content—In a recent paper (14) we reported that ox pituitary lactogenic hormone contained 5.7 per cent tyrosine, whereas the hormone prepared in an identical manner from sheep pituitaries contained 4.5 per cent. This difference has been confirmed by Dr. C. A. Knight of The Rockefeller Institute for Medical Research, Princeton, New Jersey, with two dif-

2 Unfortunately, the hormone cannot be assayed in 40 per cent urea solution. The biological activity was tested after the removal of urea by dialysis. It is possible that the protein, if it is denatured in urea, becomes native again after the urea has been dialyzed.

3 The same result is also observed with another pituitary protein, interstitial cell-stimulating hormone (12).
ferent preparations by different methods of analysis. He found 5.42 per cent tyrosine in ox hormone and 4.7 per cent in sheep preparations.

The hormone derived from these two sources did not differ in tryptophane content. The Lugg method (15) employed involved alkaline digestion, and the possibility of destruction of some of the tryptophane had to be borne in mind. At the suggestion of Dr. Knight we have redetermined the tryptophane content of ox and sheep lactogenic preparations, using a method (16) that does not require digestion of the protein.

The results summarized in Table III show that no difference was found in the tryptophane content of ox and sheep hormones. They both contained about 2.5 per cent tryptophane and not 1.3 per cent as previously reported.

Arginine Content—The Thomas modification (17) of Sakaguchi's method was used for the estimation of arginine content. 30 mg. of a protein sample were dissolved in 1 cc. of 6 M hydrochloric acid in a small Pyrex test-tube. The tube was then sealed and put into a steam bath for 24 hours. After hydrolysis, the hydrolysates were transferred to a volumetric flask. An aliquot of this dilute hydrolysate was used for arginine determinations. The method of Thomas et al. was followed as closely as possible.

Results summarized in Table IV show that both sheep and ox lactogenic hormones have practically the same arginine content and a mean of thirty-five analytical values from five preparations gives 8.31 per cent.

Sulfur and Cystine Content—The total sulfur content as determined by Carius' method was found to be practically the same in both ox and sheep hormones. They contain 1.79 per cent sulfur from an average value of five determinations (1.84 per cent, sheep; 1.79, sheep; 1.75, ox; 1.78, ox; 1.80, ox), which approximates the figure (1.77 per cent) obtained by White et al. (18).

The cystine content of the hormone is 3.0 per cent, which accounts for about 45 per cent of the total sulfur. Since we have already shown the absence of cysteine in the hormone (9), the rest

4 We are indebted to Miss E. Burtner for her assistance in making the arginine determinations.

6 The unpublished results of H. Fraenkel-Conrat.
of the sulfur must be due to methionine or some other sulfur-containing amino acids.

All of the analytical results are summarized in Table V. If we assume that the hormone consists of 3 molecules of tryptophane, 8 molecules of tyrosine for ox preparations, 6 molecules of tyrosine for sheep, 3 molecules of cystine, 12 molecules of arginine, and 14 atoms of sulfur, the average molecular weight as calculated by Equation 2 will be 24,900. This value is in good agreement with that obtained from the osmotic pressure measurements.

* The qualitative analysis of a preparation shows the presence of methionine. The sulfur distribution in the hormone is under investigation.
Methods

Solubility Experiments—A 2 per cent solution (approximate) of the hormone was prepared with the aid of a minimal amount of dilute NaOH and HCl. The pH of this stock solution was about 7.0. Different aliquots of the solution, with a volume of not more than 2 cc., were distributed in seven or nine test-tubes (12 x 100 mm. Pyrex). Distilled water was then pipetted into each tube to make up a volume of 2 cc. To each tube 5 cc. of 0.50 m NaCl at pH 2.05 (in HCl) were added and two glass beads were used for agitation. The tubes were then closed with a small rubber stopper and inserted into a large Pyrex test-tube and rotated in a 25° thermostatically controlled water bath for 3 days. The solutions were filtered through Whatman filter paper (No. 42) and the filtrate analyzed for nitrogen. Nitrogen determinations were made by semimicro-Kjeldahl analysis and sometimes checked by the Nessler method (19). The pH of each filtrate was also determined by a glass electrode and found in each case to be 2.25 ± 0.03.

The solubility in the case of high protein concentration was determined by the technique described previously (3). 7 The biological activities of the filtrate and the residue were always assayed in two or three tubes of each run. They indicated in each instance that it has no changes of potency.

### TABLE V

**Minimal Molecular Weight of Pituitary Lactogenic Hormone**

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Weight containing 1 molecule or 1 atom</th>
<th>Assumed No. of molecules or atoms</th>
<th>Estimated mol. wt.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptophane</td>
<td>2.5</td>
<td>8200</td>
<td>3</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>5.7 (Beef)</td>
<td>3200</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>4.5 (Sheep)</td>
<td>4000</td>
<td>6</td>
</tr>
<tr>
<td>Cystine</td>
<td>3.0</td>
<td>8200</td>
<td>3</td>
</tr>
<tr>
<td>Arginine</td>
<td>8.31</td>
<td>2100</td>
<td>12</td>
</tr>
<tr>
<td>Sulfur</td>
<td>1.79</td>
<td>1800</td>
<td>14</td>
</tr>
</tbody>
</table>

Mean.................................................. 24,900
Osmotic Pressure Measurements—The technique used for osmotic pressure measurements was essentially the same as that described by Burk and Greenberg (7). The collodion bags, which were made with a solution kindly supplied by Dr. D. M. Greenberg, were tested for small holes or other defects by the apparatus described by Northrop and Kunitz (20). The volume of the bag was about 10 cc., while the volume of the outer solution was about 500 cc.

The experiments were carried out in a cold room at 3-4°. The osmometers were rocked slowly in a mechanical arrangement. The equilibrium was usually reached in 2 or 3 days if it started from lower pressure, while it took about 7 days from higher pressure. After the attainment of equilibrium, the osmometer was then put into a big bath of ice and water until the reading no longer changed (1 or 2 days).

The concentration of the protein was calculated from the nitrogen determinations with the factor 100/15.8. When urea was used as the solvent, the protein content was determined by Folin's reagent (21) as described previously (3). The pH determinations were made with a glass electrode.

**Summary**

1. The purity of our lactogenic preparations has been reaffirmed in electrophoresis and solubility experiments.

2. The molecular weight of the pure hormone has been shown by osmotic pressure measurements to be approximately 26,500. The fact that the pure hormone does not dissociate into fragments in urea solution has been demonstrated.

3. The cystine, arginine, tyrosine, tryptophane, and sulfur contents of the pure hormone have been reported. From these data the molecular weight of the lactogenic hormone was estimated to be approximately 25,000.

**BIBLIOGRAPHY**

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