ON THE ALCOHOL SOLUBILITY OF PROLACTIN

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The various methods of extracting and purifying prolactin, the lactogenie hormone of the anterior pituitary gland, are based at least in part on the observation that this hormone is soluble in 60 per cent ethanol (pH 10) and 85 per cent acetone (pH 1.5), while most of the accompanying inert proteins are not (1-4). Alcohol and acetone of higher concentration, however, will precipitate the hormone from these solutions.

In the course of experiments on the purification of prolactin the surprising observation was made that this protein hormone becomes highly soluble in 99.8 per cent methanol and 95 per cent ethanol as well as in certain other organic solvents if the pH is adjusted appropriately. In view of the fact that up to date only a few alcohol-soluble proteins have been described it was thought desirable to learn more about this unusual behavior of prolactin. In the present report the results of investigations with purified hormone preparations will be presented first, followed by a description of experiments with fresh pituitary glands.\(^1\)

EXPERIMENTAL

**Purified Prolactin and Organic Solvents**—Prolactin, though insoluble in organic solvents at its isoelectric point of pH 5.7, is highly soluble in 99.8 per cent methanol and 95 per cent ethanol in the presence of a small amount of acid. Under the same condition it is also dissolved, but to a lesser degree, by absolute ethanol and propylene glycol. The most favorable acidity apparently lies in the neighborhood of pH 3; yet the solubility of prolactin in alcohols extends over a wide range, from below pH 1 to about pH 4.7. If the acidity is increased much beyond pH 1, the hormone becomes reversibly insoluble in these solvents as it does in water. Hydrochloric, sulfuric, glacial acetic, sulfosalicylic, trichloroacetic, and probably many other acids may be used to bring prolactin into solution with methanol or 95 per cent ethanol. Sulfosalicylic and trichloroacetic acids deserve special attention, for prolactin is precipitated completely by these two protein precipitants from an aqueous solution. Other excellent solvents for prolactin are anhydrous acids such as glacial acetic and propionic acids. The following studies, however, will be confined to solutions of prolactin in methanol and ethanol.

\(^1\) For the biological assays see the following paper.
The isolation of prolactin from its solution in acid alcohols can be achieved in three different ways.

Precipitation with Ether or Acetone—Though both solvents will precipitate most of the active material when added in quantities equal to that of the methanol solution, it was found more satisfactory to use 2 volumes of technical ether or 4 volumes of acetone, because in that case the precipitation flocculates and settles immediately. By decanting off the clear supernatant and washing the precipitate two or three times with dry ether, before it is brought to dryness, white powders are obtained which are again completely soluble in methanol without any further addition of acid.

Precipitation by Raising pH to 5 or Above—The advantage of this method lies in the smaller volume used; the disadvantages are, however, that any pigment present will precipitate along with the hormone and that in order to bring such preparations back into methanol solution the pH has to be readjusted. Elimination of the pigment can be achieved by the use of charcoal (norit A, Eimer and Amend). As large amounts of it adsorb lactogenic activity, one has to add the charcoal in small portions to the acid methanol solution of prolactin until a sample upon filtration is free from pink color. In a number of experiments carried out with different starting materials and under varying conditions, it was always found satisfactory to use 0.5 to 0.6 gm. of norit A per 1 gm. of dissolved protein, regardless of whether the concentration of protein in methanol was 0.08 or 1.6 per cent.

Evaporation of Solvent in Vacuo at Low Temperature—It was found that evaporation of the solvent is possible without loss of any prolactin activity. Small amounts can even be carefully evaporated on the water bath. Since a methanol solution of prolactin may be filtered through a Seitz pad EK without any loss of potency, sterile solutions of prolactin in methanol can thus be prepared and afterwards evaporated under sterile conditions.

In order to obtain some information on the extent to which prolactin is soluble in both methanol and ethanol, a number of solubility tests were made with two highly purified prolactin preparations, one derived from sheep, the other from beef pituitary glands. A description of these two preparations follows.

A new method, which will be outlined in the following communication, was applied to obtain hormone preparations assaying approximately 30 I.U. per mg. They were completely soluble in alcohol but still contained some salt. They were, therefore, dialyzed in the ice box, until all of the material had precipitated isoelectrically (12 days). The precipitate was dissolved in methanol with the addition of a small amount of concentrated hydrochloric acid. 2 volumes of ether were added and the resulting precipitate was separated by centrifuging and was then dried with ether.
The dried preparations were subsequently twice redissolved in methanol and precipitated with ether in order to get rid of a small amount of material which was less soluble in methanol. No acid was used in these last steps. The two preparations thus obtained were dried in a desiccator over calcium chloride. Biological assays indicated that both preparations now contained only 20 I. U. per mg. As may be seen from the experiments discussed in this and the following publication, treatment with acid methanol does not change the potency of prolactin preparations. It is therefore suggested that this reduction in potency is due to the prolonged dialysis rather than to the treatment with acid methanol.

When 25.0 mg. of each preparation were dissolved in 10 cc. of water, the solutions showed pH values of 3.04 and 3.10 for sheep and beef prolactin, respectively. (The same values were encountered when methanol was used as solvent.) To get the isoelectric reaction (pH 5.70), 2.63 and 2.58 cc. of 0.01 M sodium hydroxide had to be added. This amounts to 1.04 milliequivalents of hydrochloric acid bound in each gm. of prolactin hydrochloride, corresponding to 1.46 per cent of basic nitrogen. Chlorine determinations, on the other hand, revealed 3.96 and 4.08 per cent chlorine, respectively, corresponding to an average of 1.58 per cent of basic nitrogen.

To 0.200 gm. of prolactin hydrochloride (sheep) 0.8 cc. of methanol (99.5 per cent, Eimer and Amend) was added and the mixture stirred well with a glass rod. An extremely viscous solution formed which became somewhat opalescent while it was being shaken at room temperature for 4 hours. It was then centrifuged for 30 minutes at 2000 R.P.M. which caused separation of a small amount of clear heavy liquid from the gelatinous remainder. The supernatant solution was weighed, brought to dryness in a vacuum desiccator over calcium chloride, and the dry weight determined. It was found that 56.2 mg. of methanol solution contained 12.3 mg. of prolactin. The concentration was, therefore, 21.9 per cent by weight (t = 24.5°).

Other tests were performed to determine the solubility of the two prolactin preparations in absolute and 95 per cent ethanol as well as in methanol saturated with sodium chloride (1.29 per cent of sodium chloride by weight). All these experiments were made by extracting 50 mg. of prolactin hydrochloride with 0.4 cc. of the solvent for 4 hours and evaporating a determined weight of the centrifuged solution to dryness. The results of these experiments are summarized in Table I. Though saturation was reached with certainty only in three experiments, others are listed as well to show the minimum solubilities. From the data in Table I, the following can be seen. (1) Prolactin hydrochloride is quite soluble even in absolute ethanol. The preparation derived from beef pituitary glands exhibited a somewhat higher solubility. (2) The solubility in 95 per cent ethanol is
at least 3 times greater than in absolute ethanol. (3) Prolactin hydrochloride is highly soluble in methanol even in the presence of sodium chloride.

**Acid Alcohol Extraction of Prolactin from Fresh Pituitary Glands**—For the following experiments, fresh undissected pituitary glands derived either from sheep or from beef were extracted with enough acidified absolute ethanol or methanol so that a final concentration of approximately 95 per cent resulted.

250 gm. of undissected pituitary glands, passed two or three times through a meat grinder, are extracted in an ice bath for 1 to 2 hours with 4 liters of methanol and 5 cc. of concentrated hydrochloric acid (pH 3.9). If a stirrer that is not powerful enough is used, it might be necessary to force the tissue through a sieve, so as to avoid the formation of lumps. The mixture is then centrifuged for 10 minutes at 2000 r.p.m. The pink

**Table I**

*Solubility of Purified Prolactin in Methanol and Ethanol*

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Source</th>
<th>Solubility of prolactin, per cent by weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanol, 99.5%</td>
<td>Sheep</td>
<td>21.9</td>
</tr>
<tr>
<td>&quot; 99.5%</td>
<td>Beef</td>
<td>&gt;14.3</td>
</tr>
<tr>
<td>Ethanol, 100%</td>
<td>Sheep</td>
<td>3.5</td>
</tr>
<tr>
<td>&quot; 100%</td>
<td>Beef</td>
<td>4.4</td>
</tr>
<tr>
<td>&quot; 95%</td>
<td>&quot;</td>
<td>&gt;12.9</td>
</tr>
<tr>
<td>Methanol, 99.5%, saturated with NaCl</td>
<td>&quot;</td>
<td>&gt;13.2</td>
</tr>
</tbody>
</table>

extract may be treated in either one of two ways. (a) 2 volumes of technical ether are added, causing an immediate precipitation which settles down within a few minutes. The clear supernatant is decanted and the remainder centrifuged. The solids are then stirred up in the centrifuge cup with dry ether and again centrifuged. They are removed from the cup with dry ether, collected on a Buchner funnel, and, while still ether-wet, transferred to a mortar and ground to complete dryness. The yield is about 10 gm. (b) The extract is stirred for a few minutes with 5 gm. of norit A, centrifuged, and freed from colloidal charcoal by filtration through a Seitz GP or EK filter pad. The filtrate, which is golden in color, is neutralized with 5 M NaOH to pH 5 to 6. This precipitate is worked up in the same manner as described above. The yield is about the same as in the first alternative.

In Table II the results of five experiments are recorded. The data indicate that methanol extracts from fresh pituitary glands contain about
two-thirds of the prolactin activity in the case of sheep and three-fourths in the case of beef. The yield of active material is somewhat lower with ethanol. In Experiment 13-hs ground glands were pulverized in a mortar with the addition of dry ice until all of it went through a 40 mesh sieve, but no change in the distribution of the hormone between solution and residue was found. As can be seen from Table II, the potency of the active fractions lies between 1.6 and 3.4 I.U. per mg. Since purest prolactin assays 30 I.U. per mg., these crude fractions contain between 88.5 and 94.6 per cent impurities, including 2.6 to 3.2 per cent ash.

As can be seen from Table II, crude prolactin preparations with only 2 to 3 I.U. per mg., containing probably between 80 and 90 per cent of inert

**Table II**

*Acid Methanol Extraction of Prolactin from Fresh Pituitary Glands. Distribution of Weights and Activities between Alcohol Extracts and Residues*

All values are calculated on the basis of 1 kilo of whole pituitary glands.

<table>
<thead>
<tr>
<th>Preparation No.</th>
<th>pH of methanol extract</th>
<th>Weight of fractions</th>
<th>Prolactin activity Per mg.</th>
<th>Total activity</th>
<th>per cent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sheep pituitary glands</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4-hs-4, methanol-soluble*</td>
<td>2.5</td>
<td>43.8</td>
<td>1.85</td>
<td>81,000</td>
<td>63.9</td>
</tr>
<tr>
<td>4-hs-2, methanol-insoluble*</td>
<td>158.0</td>
<td>0.29</td>
<td>45,800</td>
<td>36.1</td>
<td></td>
</tr>
<tr>
<td>3-hs-4, ethanol-soluble*</td>
<td>2.8</td>
<td>40.9</td>
<td>1.63</td>
<td>66,700</td>
<td>50.5</td>
</tr>
<tr>
<td>3-hs-2, ethanol-insoluble*</td>
<td>152.0</td>
<td>0.43</td>
<td>65,400</td>
<td>49.5</td>
<td></td>
</tr>
<tr>
<td>13-hs-4, methanol-soluble</td>
<td>1.7</td>
<td>42.0</td>
<td>3.44</td>
<td>144,500</td>
<td>67.9</td>
</tr>
<tr>
<td>13-hs-2, methanol-insoluble</td>
<td>145.5</td>
<td>0.47</td>
<td>68,400</td>
<td>32.1</td>
<td></td>
</tr>
<tr>
<td>Beef pituitary glands</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6-hb-4, methanol-soluble†</td>
<td>3.9</td>
<td>37.0</td>
<td>2.30</td>
<td>85,100</td>
<td>74.1</td>
</tr>
<tr>
<td>6-hb-2, methanol-insoluble†</td>
<td>148.8</td>
<td>0.20</td>
<td>29,800</td>
<td>25.9</td>
<td></td>
</tr>
<tr>
<td>5-hb-4, ethanol-soluble†</td>
<td>3.2</td>
<td>32.1</td>
<td>2.60</td>
<td>83,500</td>
<td>72.1</td>
</tr>
<tr>
<td>5-hb-2, ethanol-insoluble†</td>
<td>161.6</td>
<td>0.20</td>
<td>32,300</td>
<td>27.9</td>
<td></td>
</tr>
</tbody>
</table>

* The same lot of sheep pituitary glands was used.
† The same lot of beef pituitary glands was used.
proteins, are easily soluble in acidified 95 per cent methanol or ethanol. It must, therefore, be concluded that the solubility in alcohols is a much more common property of proteins than had up to now been realized.

The results reported in Table II show that a considerable part of the prolactin activity remains in the acid methanol-insoluble residue. The same observation has been made with other extraction methods, as can be seen from experiments recorded in Table III. In Experiments 9-hs and 37-pr the fresh sheep pituitary glands were extracted with water at an alkaline pH. (Different lots of pituitary glands were used for the three experiments listed.)

Experiments designed to increase the extraction of prolactin activity were not successful. Even if it is assumed that the determination of the prolactin activity in the residues is falsified by the presence of inert pro-

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Description of extraction</th>
<th>Prolactin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Type</td>
<td>pH</td>
</tr>
<tr>
<td>9-hs</td>
<td>Water, 2 extractions</td>
<td>9.6</td>
</tr>
<tr>
<td>37-pr</td>
<td>&quot; 3 &quot;</td>
<td>9.7</td>
</tr>
<tr>
<td>59-pr</td>
<td>Methanol, 1 extraction</td>
<td>2.0</td>
</tr>
</tbody>
</table>

The question why part of the lactogenic potency resists extraction remains unanswered.

Since the greater portion of crude prolactin fractions is soluble in alcohols, a further treatment with these solvents cannot be expected to increase their purity. However, if a 2 per cent aqueous solution of the material is precipitated at pH 2.8 to 3.6 with 2 per cent sodium chloride and the precipitate treated again with methanol, preparations containing up to 15 I.U. per mg. are obtained.

2 gm. of the material are dissolved in 75 cc. of water. If necessary, the pH of the aqueous solution is adjusted to 2.8 to 3.6. After the mixture

2 The question whether other pituitary hormones are contained in the methanol extracts from fresh pituitary glands can be answered at this time only with regard to the follicle stimulating hormone. It was found that follicle stimulating hormone is not soluble in 95 per cent methanol under the experimental conditions described above.
has been stirred in an ice bath for 1 hour, a solution of 2 gm. of sodium chloride in 25 cc. of water is added and stirring continued for 15 minutes. It is then centrifuged. The supernatant is discarded, and the residue is extracted twice with 20 cc. of methanol to which 1 drop of concentrated hydrochloric acid is added. The methanol extractions are combined and precipitated with 80 cc. of ether. The solids from the ether precipitation are worked up in the same way as described for the crude preparations. The yield is 0.20 to 0.25 gm. of material, assaying 13 to 16 I.U. per mg.

In Table IV the distribution of weights and activities between the methanol-soluble and methanol-insoluble fractions of the precipitate obtained with 2 per cent aqueous sodium chloride is shown by two examples.

**Table IV**

**Purification of Crude Prolactin Fraction by Salt Fractionation and Methanol Extraction of Salt Precipitate**

All values are calculated on the basis of 1 kilo of whole pituitary glands.

<table>
<thead>
<tr>
<th>Preparation No., 2% NaCl ppt.</th>
<th>pH of methanol extract</th>
<th>Weight of fractions</th>
<th>Prolactin activity</th>
<th>Total activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>gm. per cent i.u. i.u. per cent</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sheep prolactin derived from 42.0 gm. of Preparation 13-hs-4 (see Table II)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>67-pr-4, methanol-soluble</td>
<td>3.6 5.2 15.7 15.7 81,600 60.6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>67-pr-2, methanol-insoluble</td>
<td>28.0 84.3 1.9 53,200 39.4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>33.2 100.0 134,800 100.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Beef prolactin derived from 37.0 gm. of Preparation 6-hb-4 (see Table II)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>66-pr-4, methanol-soluble</td>
<td>2.8 3.7 13.2 12.9 47,700 53.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>66-pr-2, methanol-insoluble</td>
<td>24.4 86.8 1.7 41,600 46.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>28.1 100.0 89,200 100.0</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

These data indicate that (1) the total yields from 1 kilo of whole sheep and beef pituitary glands were 81,600 and 47,700 I.U., respectively, (2) 2 per cent aqueous sodium chloride precipitated about 79 and 76 per cent of all proteins present in the original crude prolactin fractions, (3) not more than 16 per cent of the salted-out material was soluble in methanol. These fractions, however, contained 54 to 61 per cent of the prolactin activity.

It could be shown that this low methanol solubility was not a property of the original crude prolactin fractions (Preparations 13-hs-4 and 6-hb-4), the greater part of which was methanol-soluble. The great decrease in alcohol solubility of these proteins when wet salt precipitates were extracted with methanol, but not when the dry material was taken up in the same
solvent, suggests that alcohol denaturation is favored by the presence of either salt or water, or both.

**DISCUSSION**

Solubility of a protein in a high concentration of alcohol has been considered up to now to be a very rare phenomenon. It has been shown in the present communication that alcohol solubility is, on the contrary, quite a common property among native proteins. Prolactin constitutes but a small fraction of the 20 per cent of pituitary proteins which were found to be soluble in 95 per cent alcohol.

The known alcohol-soluble proteins are of two distinctly different types:

1. Proteins which are alcohol-soluble at neutral reaction. Only a few plant proteins, the prolamin, are known to show such a property. They are best soluble in 70 per cent alcohol, but insoluble in either pure alcohol or pure water. They are believed to owe their peculiar behavior to a deficiency in charged groups and to an abundance of uncharged polar groups (low basic but high amide nitrogen).

2. Proteins which are alcohol-soluble due to salt formation. In most instances the solubility of proteins in high concentrations of alcohol has been investigated only at a limited pH range. A protein which shows the remarkable property of being soluble in slightly ammoniacal 99 per cent ethyl alcohol is carbonic anhydrase, as was recently reported by Scott and by Scott and Fisher (6). A number of other proteins are known to be soluble in 60 to 70 per cent alcohol in the presence of alkali, one of them being prolactin (1). On the other hand, proteins which are soluble in such alcohol concentrations in the presence of acid are also known. To this group belongs Osborne's alcohol-soluble casein fraction with the surprisingly high molecular weight of 375,000 = 11,000 (Svedberg and coworkers (7)) and various hormones. Though prolactin is quite unique in its high affinity towards alcohols, it is probable that it differs from the known representatives of this group more in degree than in kind. In which respect these proteins differ from other proteins which are unable to form alcohol-soluble salts is not known. Osborne and Wakeman (8) were unable to attribute the alcohol solubility of their protein to any special groups in its molecule, especially if comparison was made with the alcohol-insoluble casein; both have approximately the same proportion of amide nitrogen, and casein contains about 36 per cent more basic nitrogen than Osborne's protein. However, the observation that alcohol-soluble pituitary proteins, except prolactin, lose their solubility in methanol during the process of purifying prolactin\(^3\) suggests that the relative proportion of amino acids is not the only decisive factor which determines the solubility, but that the general structure of a protein is also of the greatest importance.

\(^3\) See also the following paper.
SUMMARY

Prolactin is shown to be highly soluble, at a pH below its isoelectric point, in 99.8 per cent methanol and 95 per cent ethanol. This peculiarity, however, is shared by a large part of the proteins from fresh pituitary glands.

A method is described for the extraction and purification of prolactin.

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