CHEMICAL STUDIES ON THE PITUITARY GONADOTROPIC HORMONE

BY L. C. MAXWELL AND FRITZ BISCHOFF

(From the Chemical Laboratory, Santa Barbara Cottage Hospital, Santa Barbara)

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Fevold et al. (1) and Wallen-Lawrence (2) have reported the separation of a follicle-stimulating principle and a luteinizing principle from hypophyseal gonadotropic preparations. The latter author includes evidence for a chemical separation involving destruction of the luteinizing principle by formalin. Maxwell (3) on the other hand was able to obtain a pure follicular ovarian response simply by sufficiently distributing the dosage of unfracti- 
nated hypophyseal preparations.

In the present paper the formalin-treated hypophyseal prepara-
tions of Wallen-Lawrence were studied under various conditions of dosage. The action of various other chemically reactive com-
ounds upon gonadotropic preparations was also investigated. It was hoped by this attack that evidence for or against the dual hormone theory and information relating to the structural groups concerned with the physiological activity might be obtained.

EXPERIMENTAL

Methods Since the chemical procedures in most instances are identical with those used by various workers in similar investiga-
tions on insulin or parathyroid preparations, the detailed procedure whenever possible has been omitted and reference made to the method employed.

Freshly prepared aqueous extracts (8 mg. per cc.) of a desiccated sheep hypophyseal preparation which corresponded to the Powder A of Wallen-Lawrence (2) with the pH 8.5 and the 40 per cent ethanol-insoluble proteins removed, were employed as the starting material. Unless otherwise stated all experiments were carried out at room temperature and in the presence of an excess of the
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reagent. Control preparations were subjected to identical experimental conditions with each experiment. At the conclusion of the reaction the preparations were adjusted to pH 6.0, the protein fraction containing the active principle precipitated with 85 per cent ethanol, and taken up in aqueous solution or suspension for immediate assay. All experiments were made in duplicate or further repeated until consistent results were obtained.

Assay—21 to 23 day old female albino rats were dosed subcutaneously once daily for 4 days and sacrificed on the 6th day. With groups of eight animals, daily dosage equivalent to 1 mg., 2 mg., and 4 mg. of the powder used as the starting material gave ovaries weighing 24 ± 3 mg., 45 ± 10 mg., and 67 ± 15 mg. respectively. Due to the large individual variation in ovarian weight, it is obvious that only a semiquantitative evaluation of potency can be obtained without the use of large groups. Complete inactivation of the chemically treated preparations was considered to be accomplished when a total dose equivalent to 32 mg. of the original preparation failed to produce evidence of morphological changes in the ovaries or uterus on all of a group of three animals. As a roughly comparative evaluation of potency after chemical treatment, groups of three animals were dosed at each of the three dosage levels given above for the standard preparation.

Formaldehyde—The hypophyseal preparation was treated with varying concentrations of formaldehyde for periods up to 4 hours, the pH being maintained between 7 and 8. At the conclusion of the experiment excess formaldehyde was removed under a vacuum and the remainder destroyed by an excess of ammonia. The reaction was then adjusted to pH 6.0 and the gonadotropic fraction thrown out with 85 per cent ethanol. Typical experiments are presented in Table I. Although a marked loss in potency is observed, it will be noted that following prolonged treatment with formaldehyde a follicular ovarian response is obtained in the majority of instances when the increase in ovarian weight does not exceed 100 per cent.

Distribution of the dosage of formalin-treated preparations was investigated to determine whether the action of formalin could be attributed simply to a partial destruction of potency accompanied by a denaturation sufficient to retard absorption of the active
principle in the tissues. The experimental data (Table I) indicate that the small amount of active principle remaining in the formalin-treated preparation was so slowly absorbed in the tissues that the distribution of the dosage used was insufficient to alter the response significantly.

In order to determine whether a selective destruction of a luteinizing principle occurs, the action of formaldehyde upon a urine of pregnancy preparation was investigated. 250 rat units of antuitrin S (Parke, Davis and Company) in 12 cc. of 6 per cent formaldehyde were allowed to stand at room temperature at pH 7 to 8 for 4 hours. Although some loss in potency occurred, no variation in the qualitative ovarian response from that of controls dosed with the untreated preparation could be demonstrated by microscopic examination of the sectioned ovaries. Assuming the same groups to be responsible for the luteinizing properties of both pituitary and pregnancy urine preparations, the results (Table I) fail to indicate a selective destruction of a luteinizing principle by formaldehyde.

\[ \text{Table I} \]

**Effect of Formalin upon Ovarian Response to Gonadotropic Preparations**

<table>
<thead>
<tr>
<th>Preparation</th>
<th>No. of animals</th>
<th>Total dosage</th>
<th>Average weight of ovarian response</th>
<th>No. of luteinizing responses</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypophyseal, untreated</td>
<td></td>
<td></td>
<td>24 ± 3</td>
<td>7</td>
</tr>
<tr>
<td>&quot; 4% formalin 15 min.</td>
<td>8</td>
<td>4 mg.</td>
<td>52 ± 10</td>
<td>6</td>
</tr>
<tr>
<td>&quot; 10% &quot; 4 hrs.</td>
<td>6</td>
<td>4 &quot;</td>
<td>45 ± 10</td>
<td>8</td>
</tr>
<tr>
<td>&quot; 6% &quot; 4 &quot;</td>
<td>8</td>
<td>8 &quot;</td>
<td>67 ± 15</td>
<td>8</td>
</tr>
<tr>
<td>Urine of pregnancy, untreated</td>
<td></td>
<td></td>
<td>86</td>
<td>3</td>
</tr>
<tr>
<td>&quot; 6% formalin, 4 hrs.</td>
<td>3</td>
<td>16 &quot;</td>
<td>20 ± 3</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>16 &quot;</td>
<td>25 ± 4</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>8 &quot;</td>
<td>22</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>8 &quot;</td>
<td>24</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>10 rat units</td>
<td>35 ± 7</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>10 &quot;</td>
<td>20 ± 4</td>
<td>5</td>
</tr>
</tbody>
</table>

* The mean plus the standard deviation (not the standard deviation of the mean) is given. The weight is measured in mg.

\[ \text{Nitrous Acid} - \text{Exposure of the hypophyseal preparation to an} \]
excess of NaNO₂ in 30 per cent acetic acid at 37° for 3 hours resulted in complete inactivation. A control preparation exposed to the same acidity also showed a very considerable loss in potency. When the experiment was repeated at 10°, no apparent loss in potency was observed in the control, while the physiological response both quantitatively and qualitatively of the treated preparation was practically identical to that reported for the preparations exposed to 10 per cent formaldehyde. At high dosage levels luteinized ovaries were obtained. On two series of three animals single daily dosage equivalent to 2 mg. of a preparation exposed to HNO₂ at pH 4.0 for 1.5 hours at 37° produced a follicular ovarian response accompanied by uterine hyperemia and vaginal canalization, without causing a significant increase in the ovarian weight.

Substitution and Addition Reactions—Hypophyseal gonadotropic preparations were exposed to a variety of reagents known to react with amino, imino, or hydroxyl groups.

Rapid and complete inactivation occurred in alkaline solution (pH 8.0) in the presence of a moderate excess of the following reacting substances: β-napthaquinonesulfonic acid, 15 minutes in the dark at room temperature; phenyl isocyanate, exposure for 1 hour at 0° (4); diazobenzenesulfonic acid (30 minutes, 0°) (5); diazonitrobenzene (30 minutes, 0°) (5); benzoyl chloride (30 minutes, room temperature) (5); carbon disulfide (1 hour in 60 per cent alcohol).

A 50 per cent to a 75 per cent destruction of the gonadotropic principle occurred without alteration of the qualitative nature of the response when hypophysal preparations were exposed to the following reacting substances: benzoyl chloride (30 minutes, room temperature, pH 5.0); dimethyl sulfate (30 minutes, room temperature, pH 8.0); acetic anhydride (1 hour, room temperature).

Attempts to demonstrate reactivation of the acetylated and the methylated preparations by hydrolysis with N/30 NaOH at 10° were unsuccessful.

Oxidizing and Reducing Agents—Since inactivation with some of the foregoing reagents may have been due to oxidation or reduction, the effects of various oxidizing and reducing agents were investigated. The potency was found to be almost completely destroyed by 3 per cent H₂O₂ in the presence of NaHCO₃. The
addition of a 10 per cent excess of iodine (0.01 N solution) over the amount found to combine in 1 hour with the proteins present was sufficient to destroy approximately 50 per cent of the activity. One of the iodine-treated preparations at minimal dosage levels gave follicular ovaries and an estrogenic response similar to the preparations treated with formalin. The addition of one-half the amount of iodine necessary to combine with the proteins was, however, without apparent effect upon either the quantitative or the qualitative nature of the response. FeCl₃, when incompletely removed previous to dosage, gave an augmented ovarian response (3).

No detectable loss of activity occurred upon exposure to nascent hydrogen, sulfur dioxide, hydrogen sulfide, ferrous sulfate, or hydrogen cyanide. Interest is attached to the qualitative nature of the assay of a preparation exposed to hydrogen sulfide, and also to one preparation treated with ammonium polysulfide. Both of these preparations gave strong estrogenic reactions at minimal dosage levels and an occasional follicular ovarian response.

Acid and Alkali—From 30 to 50 per cent of the potency of the hypophyseal preparation was lost by standing 3 hours at 37° in 0.1 N HCl. Complete inactivation resulted when the dry powder was dissolved in concentrated H₂SO₄ at 0° according to the method employed by Bischoff and Sahyun (6) with insulin. Exposure for 20 hours at room temperature to 0.73 N HCl in 75 per cent ethanol also resulted in complete inactivation. Attempts to demonstrate reactivation of this preparation by hydrolysis with 0.08 N NaOH in the cold were unsuccessful.

Complete inactivation resulted with 0.1 N NaOH in 3 hours at 37°, while N/30 NaOH under these conditions destroyed from 30 to 50 per cent of the potency. Preparations exposed to N/30 NaOH at 10° showed no appreciable loss in potency. No change in the qualitative nature of the ovarian response was observed following exposure to either acid or alkali.

Fate of Luteinizing Fraction—Fevold et al. (1) state that the luteinizing fraction when administered alone, even in excessive dosage, produces no marked increase in the ovarian weight. However, when dosage with the luteinizing fraction and the follicle-stimulating fraction are combined, an augmentation in ovarian weight is observed. The possibility that in our chemically in-
activated preparations a selective destruction of the follicle-stimulating principle had taken place was tested out experimentally. The results were negative. Combined dosage of the inactivated preparations with dosage of various preparations giving a follicular response failed to demonstrate an augmented ovarian response. In these experiments, which are not tabulated, low dosage with hypophyseal extracts containing FeCl₃ or tannic acid (3), or extracts treated with formalin, was used to promote follicle stimulation.

DISCUSSION

If the activity of the more highly purified prolan preparations may be used as a basis of comparison, the purest gonadotropic pituitary preparations would appear to be relatively crude. In view of this question and the lack of precise methods of assay, the value of a chemical investigation of the nature described in this paper may be questioned. Results of similar early studies on non-crystalline insulin (5, 6) have to date not been greatly amplified by work on the crystalline material (7, 8) and were hence an incentive to this study. The present chemical studies appear informative and of value inasmuch as they establish a stability range to various chemical environments, which will tend to define the conditions for further work on the isolation of the gonadotropic principle or principles.

Much useless discussion has arisen in the insulin work as to the exact nature of the groups attacked by the various reagents. None of the reagents studied is exactly specific for any single chemical group. The present studies which are analogous indicate the amino, imino, or hydroxyl groups or their sulfur analogues may be concerned with the physiological activity of hypophyseal gonadotropic preparations.

The most significant finding appears to be that denaturation of the protein aggregate may produce the same physiologic effect as slowed tissue resorption of the original product. The evidence is therefore against the dual hormone theory.

SUMMARY

Reagents known to react with the amino, imino, or hydroxyl groups cause a partial to a complete inactivation of hypophyseal gonadotropic preparations.
The activity is completely destroyed by strong oxidizing agents but is unaffected by mild oxidizing agents or by reducing agents. Complete inactivation occurs in 0.1 N NaOH, and partial inactivation in \( \frac{1}{30} \) NaOH in 3 hours at 37°. 0.1 N HCl in 3 hours at 37° causes partial inactivation.

Evidence has not been obtained to support the dual hormone theory of gonadotropic preparations. The formalin-treated gonadotropic pituitary preparations show no augmentation effect when given in divided doses. The nature of the ovarian response to the urine of pregnancy preparation is not changed when formalin-treated preparations are used.

**BIBLIOGRAPHY**

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L. C. Maxwell and Fritz Bischoff


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