BIOASSAY OF PROLACTIN

Analysis of the Pigeon Crop-sac Response to Systemic Prolactin Injection by an Improved Method of Response Quantification

By

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ABSTRACT

The response of the pigeon crop-sac to systemically acting prolactin (injected subcutaneously) was evaluated by measuring the wet weight of the responsive lateral lobes of the organ and by determining the dry weight of a 4 cm diameter disc of mucosal epithelium taken from one hemicrop. Of several different injection schedules tested, administration of prolactin in four daily injections was found to yield optimal responses. When compared with a graded series of prolactin doses, measurement of the mucosal dry weight proved to be a better method of response quantification than determination of the crop-sac wet weight with respect to both assay sensitivity and precision. The submucosal tissue of the crop-sac was estimated to constitute about 64% of the total dry weight of the unstimulated organ and it was found to be relatively unresponsive to prolactin stimulation in comparison with the mucosa. The lipid content of the mucosal epithelium was determined using unstimulated crop-sacs or tissues which showed varying degrees of prolactin-induced proliferation. The fat content of the mucosal epithelial cells increased only slightly more rapidly than the dry weight or the defatted dry weight of the mucosa. Suggestions are made for the further improvement of the systemic crop-sac assay for prolactin.

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Riddle et al. (1933) developed a method for the bioassay of prolactin which utilized the increase in weight of the crop-sac of pigeons or doves occurring in response to systemic injection of the hormone. This procedure has been used by a number of investigators for a variety of studies and is the standard method for estimating the potency of the purified prolactin preparations provided by the Endocrine Study Section of the National Institutes of Health. Bates et al. (1963) have reported a modified systemic assay procedure which improved the precision of the crop weight method. However, the sensitivity of the improved method was not greatly increased inasmuch as the minimum daily dose for a detectable response was about 1 IU per day in young pigeons and about 0.5 IU per day in adult birds. Because of their greater sensitivity, the local »micro« method of Lyons (1937), and the subsequent modifications thereof (Reece & Turner 1937; Tanabe et al. 1954; Grosvenor & Turner 1958; Kanematsu & Sawyer 1963) have been more widely employed than systemic crop weight procedures for experimental purposes despite the fact that the latter method is completely objective whereas the former suffer from variable degrees of subjectivity.

Recently, a completely objective method of quantifying crop-sac responses to local prolactin injections was reported (Nicoll 1967). This procedure measures the weight of a standardized 4 cm diameter disc of crop-sac mucosal epithelium taken from the injection site. The method measures crop-sac responses which are not grossly detectable; therefore, they cannot be measured by procedures which rely on visualization of the responses.

Weighing a standardized disc of crop-sac mucosal epithelium provides an indirect measure of the thickness of the responsive component of the organ (Nicoll 1967) and eliminates the submucosal tissues from the quantification procedure. Accordingly, it was of interest to determine if this procedure would improve the sensitivity and precision of the pigeon crop-sac response to subcutaneously injected prolactin inasmuch as the influence of variables, such as pigeon or crop size and thickness of the submucosa, can be eliminated.

**MATERIALS AND METHODS**

**Animals**

The pigeons used in these experiments were all 6 to 8 week old animals of the Silver King strain obtained from the Palmetto Pigeon Plant, Sumpter, South Carolina. Birds of both sexes were used.

**Hormones**

The ovine prolactin was a gift of the Endocrine Study Section of the National Institutes of Health. The preparation used (P-S-5) had a stated potency of 16.8 IU/mg with 95% fiducial limits of 10.5–26.9 IU/mg. For convenience, a potency of 15 IU/mg was used for the computations in this paper.
Injections

All injections were administered subcutaneously in the loose skin between the legs and lower abdomen. The prolactin was dissolved in Earle’s physiological saline (equilibrated with air to give a pH of 8.5) and administered in 1 ml per injection.

Statistical Analyses

The bioassay data were analyzed by procedures described by Finney (1964) using an I.B.M. 7094 computer at the University of California in Berkeley.

Response Quantification

All pigeons were killed by decapitation and the crops were exposed by a midline incision. The responsive lateral region of the right hemicrop was removed, rinsed under cold running tap water, blotted on moist paper towels, then weighed. The left hemicrop from each bird was processed for removal of a standardized 4 cm diameter disc of mucosal epithelium (ME) from the center of the prolactin-responsive region of the tissue. The apparatus and procedure used for removal of the 4 cm disc of ME have been described in detail previously (Nicoll 1967).

In brief, the procedure consists of placing the hemicrops on a cylindrical device which securely holds the tissues in place while applying a uniform degree of stretch on them. The ME, lying within the perimeter of a 4 cm diameter disc of porous stainless steel or scinttered glass plate, located on the top of the holding apparatus, is scraped from the underlying submucosal tissues. After removal, the ME was frozen on dry ice, lyophilized overnight and weighed.

EXPERIMENTS AND RESULTS

1. Comparison of Injection Schedule. Previous analyses of the crop-sac response to local (intradermal) prolactin injection has shown that the number of injections and the interval between them are important variables in determining the magnitude of the tissue’s response (Nicoll 1967). It was of interest, therefore, to perform a similar analysis on the crop-sac response to subcutaneously injected prolactin.

Seventeen untreated pigeons were used for control measurements. An additional 80 birds were divided into ten groups of equal number and these received one to four injections of total prolactin doses of either 0.5 mg (7.5 IU) or 2.0 mg (30 IU). All groups were killed twenty hours after the final prolactin injection. The results of this study are shown in Table 1.

A single injection of either 7.5 IU or 30 IU of ovine prolactin significantly increased the hemicropro wet and dry weights. Increasing the number of injections and the interval between them progressively increased the magnitude of the crop-sac response as indicated by both parameters. In all cases, the mucosal dry weight (MDW) response was proportionately much greater than the increase in hemicrop wet weight. Group number five, which received four daily injections of prolactin, was the only one which showed significant dif-
Table 1.
Effects of Different Injection Schedules on the Response of the Pigeon Crop-Sac to Prolactin as Measured by Hemicrop Wet Weight and Mucosal Dry Weight.

<table>
<thead>
<tr>
<th>Group</th>
<th>Injection Schedule</th>
<th>Responses and Slopes</th>
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<tr>
<td></td>
<td></td>
<td>Hemicrop Wet Weight (gm)</td>
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<tr>
<td></td>
<td></td>
<td>Total Prolactin Dose</td>
</tr>
<tr>
<td>1</td>
<td>*</td>
<td>2.0 ± 0.1 ( + 11%)</td>
</tr>
<tr>
<td>2</td>
<td>* *</td>
<td>2.1 ± 0.2 ( + 17%)</td>
</tr>
<tr>
<td>3</td>
<td>* * *</td>
<td>2.3 ± 0.2 ( + 28%)</td>
</tr>
<tr>
<td>4</td>
<td>* * * *</td>
<td>2.5 ± 0.2 ( + 39%)</td>
</tr>
<tr>
<td>5</td>
<td>* * * * *</td>
<td>2.7 ± 0.3 ( + 50%)</td>
</tr>
</tbody>
</table>

Control (uninjected) values (N = 17): Hemicrop wet weight = 1.8 ± 0.1; Mucosal dry weight = 6.9 ± 0.4.
1. Eight pigeons per group at each dose level.
2. A.m. and p.m. injections given between 8–10 a.m. and 4–5 p.m., respectively. All groups killed 20 h after the last injection.
* indicates injection given at that time. † indicates time of killing.
3. Data presented as mean ± standard error of mean. Per cent increase over control values shown in parentheses under the means. NS = not significant.
ferences in response between the two doses of the hormone. This is reflected in the establishment of significant slopes by both indices. In all other groups, the responses to the high dose of the hormone were not significantly different from those of the low dose.

These results clearly illustrate the importance of the number of injections and the interval between them for obtaining optimal responses and discrimination between doses. Bates et al. (1963) have obtained similar results in showing that seven daily injections of prolactin are more effective than four. These findings are in complete contrast with results obtained with local injections of prolactin (Nicoll 1967). In the local assay, four closely spaced injections administered in two days were more effective than four daily injections.

In order to determine if killing the pigeons later than 20 h after the last injection had an appreciable effect on the magnitude of the response, eight birds received a total dose of 30 IU of prolactin. The hormone was administered in four injections given in two days as in group 4, but the birds were killed at 44 h after the last injection. The hemicrop wet weight and the MDW measurements were 2.5 ± 0.1 g, and 4.8 ± 1.0 mg, respectively. In comparison with the pigeons of group 4 which were identically treated but were killed 24 h earlier, it is evident that the crop-sac response had regressed substantially between the 20th and 44th h after the last injection. This is in agreement with a similar study on the response of the crop-sac to local prolactin injection (Nicoll 1967).

2. Comparison of Hemicrop Wet Weights and Mucosal Dry Weight Measurements at Several Doses of Prolactin. Five groups of 8 pigeons each were injected daily with prolactin for 4 days giving total doses ranging from 0.375 to 30 IU (25 µg to 2.0 mg). On the fifth day, the crops were removed and one half of each was used for determination of the wet weight. A 4 cm diameter disc of ME was removed from the remaining hemicrop of each bird for determination of its dry weight. The results of these bioassay data are shown in Fig. 1 and 2.

The data obtained with the hemicrop wet weights (Fig. 1) show that a linear log dose-response relationship was obtained between the doses of 1.5 and 30 IU. The two lower doses did not fall on the linear portion of the curve. The slope of the curve was highly significant (2.1 ± 0.5) but the index of precision ($\lambda = 0.36$) was poor.

Fig. 2 shows the results obtained with the MDW measurements. A linear log dose-response relationship was obtained between the doses of 0.75 and 30 IU. Only the lowest dose did not conform to the linear portion of the curve. The slope of the curve was also highly significant (26.5 ± 1.0) and the index of precision ($\lambda = 0.20$) was satisfactory and far superior to the $\lambda$ of the wet weight method of response quantification. These results demonstrate that measurement
Log dose-response relationship of the pigeon crop-sac to subcutaneous injections of prolactin. The wet weight of one half of each crop-sac was measured. Each point represents the mean ± standard error of the mean of eight hemicrops. 

\( \lambda = \text{index of precision} \)

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Log dose-response relationship of the pigeon crop-sac to subcutaneous injections of prolactin. The dry weight of a 4 cm diameter disc of crop-sac mucosal tissue from one hemicrop was determined as the index of prolactin stimulation. Each point represents the mean ± standard error of the mean of eight hemicrops. 

\( \lambda = \text{index of precision} \)

of the MDW provides a more sensitive and precise method for quantifying the response of the crop-sac to systemically injected prolactin than measurement of the tissue's wet weight.
3. **Mucosal and Submucosal Relationships.** Determining the wet weight of the crop-sac involves measurement of not only the prolactin-responsive mucosal cells, but also a considerable amount of submucosal tissue. The latter consists of muscle, connective and adipose tissues, and blood vessels; however, most of the fat can be readily removed before the crop is weighed. It was of interest, therefore, to determine what proportions of the crop-sac were comprised of mucosal and submucosal tissues. The degree of change in dry weight of the submucosa, occurring when the mucosa was highly stimulated by subcutaneous injection of prolactin, was also determined.

The right hemiceps of 24 untreated pigeons were placed on the crop-sac holding apparatus for removal of the 4 cm diameter disc of mucosal epithelium. The underlying 4 cm diameter disc of submucosa was then cut from the remaining crop membrane using a scalpel and the mucosal and submucosal tissues were dried by lyophilization. The crops of two groups of pigeons which had received daily subcutaneous injections of prolactin for four days were similarly processed. The data from this study are shown in Table 2.

On the basis of these dry weight measurements, the submucosa constitutes about 64% of the crop tissue of the untreated birds. A prolactin dose which increased the mucosal dry weight by about 300% (group A) caused an increase in the submucosal dry weight of only 13.2%. A higher dose of prolactin (group B), which produced an increase in the mucosal dry weight of almost six fold, caused only a 44% increase in the submucosal dry weight. These results demonstrate that the submucosal tissues of the crop-sac show very little change in response to prolactin in comparison with the changes occurring in the mucosa. Accordingly, measurement of the wet or dry weight of the whole crop-sac includes a substantial portion of relative unresponsive tissue.

4. **Relationship Between the Lipid Content of the Crop-Sac Mucosa and its Dry Weight:** One of the pronounced histologic changes which occurs in the crop-

### Table 2.

<table>
<thead>
<tr>
<th>Group1</th>
<th>Treatment2</th>
<th>Dry Weight of Crop Tissues (mg)³</th>
<th></th>
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<tbody>
<tr>
<td>Controls (24)</td>
<td>None</td>
<td>7.7 ± 0.4</td>
<td>13.6 ± 0.4</td>
</tr>
<tr>
<td>A (7)</td>
<td>2.5 IU prolactin</td>
<td>22.6 ± 1.7</td>
<td>15.4 ± 0.5</td>
</tr>
<tr>
<td>B (6)</td>
<td>20 IU prolactin</td>
<td>43.8 ± 5.8</td>
<td>18.0 ± 1.0</td>
</tr>
</tbody>
</table>

1. Number of pigeons per group in parentheses.
2. Total doses of prolactin given in four daily injections.
3. Data presented as mean ± standard error of mean.

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sac mucosal epithelial cells in response to prolactin stimulation is the accumulation of cytoplasmic lipid granules (Beams & Meyer 1931; Patel 1936; Leblond & Noble 1937; Webber 1962; Dumont 1965; Chadwick 1966).

In order to obtain quantitative data on the relationship between lipid content and degree of mucosal thickening, the crop-sacs of forty-four pigeons were used. Some of these birds were untreated controls and the remainder showed varying degrees of mucosal stimulation in response to different doses of prolactin. The dried 4 cm diameter discs of mucosal epithelium from these birds were defatted by immersion in three changes of a large excess of a methanol, chloroform, ether mixture (1:1:1). The tissues were exposed to the fat solvent mixture for two successive four-hour periods then finally overnight. The fat solvents were removed under a vacuum and the fat content of the tissue was determined by the difference between the dry and defatted dry weights. The results are shown in Fig. 3. The increase in fat content which occurred in response to a prolactin injection was found to be proportionately only slightly greater than the increase in mucosal dry weight (i.e., mucosal thickness). The relationship was described by the exponential equation \( Y = 0.11X^{1.2} \) where \( Y \) equals fat content and \( X \) equals mucosal dry weight, both in mg. When the increase in fat content was related to the change in defatted dry weight of the mucosa, the relationship was \( Y = 0.12X^{1.3} \).

**DISCUSSION**

The results of these experiments indicate that measurement of the dry weight of a standardized disc of crop-sac mucosal epithelium provides a more sensitive
and precise method of response quantification for the systemic assay of prolactin than simply measuring the wet weight of the responsive lateral lobes of the organ. The reason for the increased sensitivity and precision becomes apparent with the disclosure that the submucosal tissues are relatively unreactive to prolactin. Thus, the wet weight measurements include a substantial proportion of rather unresponsive tissue. Accordingly, elimination of the submucosal tissue from the procedure of response quantification reduces the baseline values and allows more precise measurement of the response. The slight increases in dry weight of the submucosa which did occur in response to prolactin injection may reflect the pronounced increase in vascularity which occurs in this tissue when the mucosa is stimulated. Kurcz (1966) has reported that the pattern of vascularization of the prolactin-stimulated crop-sac is a specific one.

It is of interest that administering prolactin in four daily injections gave better responses than when the hormone was given according to other injection schedules, including four injections in two days. This is in contrast with results obtained by the local intradermal route of prolactin injection (Nicoll 1967) where four injections given in two days were found to be better than four daily injections. The reason for this difference is not known, but could reflect a difference in the half-life of the prolactin in the circulation after subcutaneous injection at a site distant from the crop-sac, as compared with its half-life in the cutaneous tissues after intradermal injection over the crop-sac.

Bates et al. (1963) have reported that increasing the number of daily injections from four to seven greatly increased the slope and precision of the systemic crop-sac assay in both juvenile and adult pigeons. The effect was particularly pronounced in the adult birds. Thus, my results are in good agreement with those of Bates et al. (1963) with regard to the influence of the frequency of injection in increasing the response of the tissue. It seems likely that combining the procedures of measuring the dry weight of a 4 cm diameter disc of crop-sac mucosa, and that of giving up to 7 daily prolactin injections in adult pigeons, would further improve the precision and utility of the systemic crop-sac assay for prolactin.

The results of determining the relationship between accumulation of lipid in the mucosal tissue and its increase in dry weight in response to prolactin stimulation indicated that the accretion of fat occurs only slightly more rapidly than the combined hypertrophic and hyperplastic response. This finding is surprising in view of the histologic evidence which indicated that accumulation of lipid granules in the crop-sac mucosal cells is a striking aspect of the tissue's response to prolactin. These results indicate that the histologic observations may reflect aggregation of intracellular lipid into granules more than uptake or synthesis of fat by the cells. Chadwick (1968, personal communication) has also observed that the increase in lipid content of the crop-sac mucosa is not as
pronounced as one would expect on the basis of the histologic appearance of the tissue.

Although the procedure of response quantification for the systemic assay of prolactin described in this paper does improve the sensitivity and precision of the assay method, the procedure is still about fifty to one hundred times less sensitive than the local intradermal crop-sac assay procedures (cf. Nicoll 1967). The improvement of the systemic assay method as described herein, in conjunction with the use of a longer period of daily prolactin injections (i.e., greater than four days) using adult pigeons, may provide an even more useful and precise assay procedure. Such a method may be of utility for assaying preparations which cannot be tested by the local intradermal methods because of volume, inflammatory characteristics or other reasons.

REFERENCES


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