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A Sensitive Bioassay for Prolactin Based on H^3-Methyl-Thymidine Uptake by the Pigeon-Crop Mucous Epithelium. (32185)

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Most of the many possible methods for prolactin detection(1-6) are of limited value because they are either semiquantitative, subjective, or highly insensitive. The pigeon-crop sac method is the most widely employed because of its simplicity and relative sensitivity. Nevertheless, it is still not completely objective and not sensitive enough to detect small quantities of prolactin. Many efforts were made in order to decrease its subjectivity and to increase its sensitivity and hence many modifications of the original method(7) were suggested, such as local administration of the prolactin over the crop gland rather than the original systemic application(8,9) or graphically measuring the area of the local crop-sac(10,11) or P^{32} uptake(12).

A considerable step to improve its objectivity was recently made by Nicoll who designed a special apparatus which permits removal of a standardized 4 cm diameter disc of the crop-sac mucosal epithelium from the local injection(13). However, the mentioned modifications still do not show significant reactions to doses as low as 1-10 mU of prolactin.

It has been shown that prolactin given either locally to the pigeon-crop or systemically, increases mitotic activity and hence induces proliferative activity of the crop sac (14,15). It has been shown also that the proliferation of the crop-sac is accompanied by increased synthesis of nucleic acids(16, 17). It is expected, therefore, that an increased uptake of labelled thymidine by the pigeon-crop should occur after prolactin administration, a matter which could be used as a new approach for prolactin assay with the pigeon crop-sac. An improvement of such a method could be achieved by a combination of three ways: 1) preventing of fast absorption of the hormone, a matter which could be achieved by dissolving the hormone in agar solution, making a gel to be injected subsequently intradermally over the crop-sac; 2) extracting only the reactive epithelial tissue of the crop-sac, and not the whole gland, for final estimation of the reaction; 3) exposing both sides of the reactive crop-sac tissue to the same amount and to an immediate supply of the radioactive material to be incorporated, *i.e.*, intravenous administration of the labelled thymidine.

A sensitive and objective method for prolactin assay that included these three procedures was developed.

Materials and methods. Fifty-four Silver King pigeons, 6-8 weeks old, were used for experimentation. The ovine prolactin used was the generous gift of the Endocrine Study Section, National Institutes of Health, batch #NIH-P-S-7, with mean potency of 24.3 IU/mg. For convenience, a potency of 24 IU/mg was employed in the calculations. The total amount of prolactin to be injected was dissolved in 0.4 ml of 0.5% agar, making a gel working solution. An injection of 0.1 ml of this solution was given intradermally upon the left side of the pigeon crop-sac and repeated three more times according to a schedule sum-

INJECTION SCHEDULE*

Day 1		Day 2		Day 3				
AM	PM	AM	PM	AM in hours 0	1	2	3	4
↑	↑	↑	↑	↑		X		
P†	P†	P†	P†	H^3-Thymidine 20 μc i.v.				

* AM and PM injections given between 9 and 10 AM and 4 and 5 PM, respectively. ↑ signs indicate injections; X sign indicates sacrifice.

† Prolactin was dissolved in 0.5% Agar and injected intradermally.

FIG. 1. Method used for prolactin bioassay using H^3-thymidine uptake by the pigeon crop epithelial tissue.

marized in Fig. 1. Concurrently, control injections of a gel solution of 0.5% agar alone were applied with a 27 gauge needle, to the right side of the crop-sac. The cutaneous injection sites were marked externally with black ink at the time of the first injection to insure that subsequent injections would be applied at the same location. On day 3 at A.M. time, 20 μc of H^3-methyl-thymidine per bird (Sp. Act. 6.0 c/m mol, Schwarz Bioresearch, Inc., Orangeburg, N. Y.) was injected into the external branch of the brachial vein and the animals were sacrificed 2-3 hours later.

After decapitation, the bird was bled as fully as possible in order to minimize the accumulation of blood in the crop-sac tissue. A median incision was made through both the skin and the crop-sac and flowing water was applied for rinsing. Each hemicrop was gently removed, cleaned of fat and connective tissue, and placed over a crop-sac holding apparatus which was designed and constructed by Nicoll (13).

A 4 cm diameter disc of the crop-sac mucosal epithelium was removed by scraping from the underlying submucosa, and placed on a small piece of aluminum foil, weighed, and put in a centrifuge tube which contained 1 ml of 0.01 N NaCl containing 100 μg of carrier DNA (Calbiochem.), highly polymerized salmon sperm DNA, 170 μg of non-isotopic thymidine, and 2.5 mg of bovine casein. Two ml of cold 10% TCA were added for precipitation. The precipitate was washed twice with 5 ml of cold 5% TCA and twice with 5 ml ether-absolute ethanol (1:3). This precipitate was finally dissolved over a 2-hour period at 60°C in hyamine, and counted in toluene-POP-POPOP in a liquid scintillation counter. The data were statistically analyzed according to T test for pairs, and also by using a "New Computer Program for the Statistical Evaluation of Biologic Assay Data" by Dr. John Cooper, formerly of Endocrinology and Metabolism Branch, NICHD, Bethesda, Md.

Results. Table I clearly shows that significant increases in H^3-thymidine uptake took place in the prolactin treated sides of the crop epithelial tissue. Doses of 1, 5, 10, 20, 50, and 100 mU of prolactin resulted in increases of radioactivity of 59, 138, 238, 361, 655, and 905% respectively. Nevertheless, no detectable visual response was obtained in the lower dose of 1 mU. Complete data of both weight and H^3-thymidine uptake responses of the same tissue to various doses of prolactin ranging from 1 mU to 100 mU are compiled in Table I, which shows a great difference between the two parameters. While the weight gain response range moves from 6.7 mg to 63.4 mg, the count average increase range is found to be between 114 c/m and 2375 c/m. A comparison between the curves of the log percentage increase of these two parameters *vs* the logarithm of the dose is illustrated in Fig. 2 which clearly indicates that the curve of H^3-thymidine percentage uptake increase is higher and more precise than the weight gain curve. By least squares line of best fit the slopes of the H^3-thymidine uptake and the weight gain are 0.606 ± 0.029; lambda = 0.196 and 0.552 ± 0.048; lambda = 0.356, respectively.

Discussion. These results show that both treated and untreated sides of the pigeon-crop epithelial tissue utilize H^3-thymidine. However, all treated sides significantly take more tritiated thymidine than the controls, and a good dose response curve was established. Furthermore, local injections of high prolactin doses of 0.5 and 1 IU resulted in H^3-thymidine uptake increase of 21 and 30 times, respectively (Ben-David, M., unpublished data), indicating that the method might have a wide range of responsiveness. The increased utilization of H^3-thymidine is

TABLE I. H^3-Thymidine Uptake* and Wet Weight Responses of 4 cm Diameter Discs of Crop-Sac Mucosal Epithelium to 6 Doses of Ovine Prolactin.†

Group	No. of pigeons	Prolactin total dose mU	Prolactin total dose μg	H^3-thymidine uptake response (C/min)‡ Control side	Injected side	Count avg increase	Count avg % increase	Wet wt response (mg)‡ Control side	Injected side	Wt avg increase	Wt avg % increase
1	10	1	.042	193 ± 16.1	308 ± 25.5	114 ± 19.4	59 ± 7.1	37 ± 3.6	44 ± 3.2	6.7 ± 1.8	17.3 ± 3.9
2	9	5	.21	191 ± 20	450 ± 49	259 ± 36	138 ± 21.3	37 ± 1.8	52 ± 3.6	15 ± 3	40.3 ± 7
3	9	10	.42	179 ± 23	588 ± 55	408 ± 35	238 ± 17.8	31 ± 2.1	51 ± 4.4	20 ± 3.4	65 ± 8.1
4	8	20	.84	156 ± 19.2	673 ± 32	517 ± 26.1	361 ± 39	31 ± 4.5	58 ± 3.9	28 ± 2.3	108 ± 20.8
5	8	50	2.1	247 ± 33.3	1875 ± 380	1620 ± 320	655 ± 60	30.0 ± 2.7	76.2 ± 7	46.1 ± 4.6	156 ± 11.2
6	10	100	4.2	267 ± 47	2642 ± 475	2375 ± 431	905 ± 56	38 ± 7	101 ± 11	63.4 ± 10	166.5 ± 24

* 20 μc/pigeon of H^3-thymidine was i.v. injected 2-3 hr before sacrifice.
† Prolactin was dissolved in 0.5% agar and injected intradermally over the left side of the crop-sac; the control right side received agar solution alone.
‡ Data presented as mean ± standard error of the mean ($P < .001$ for H^3-thymidine uptake response; $P < .01$ for wet weight response).

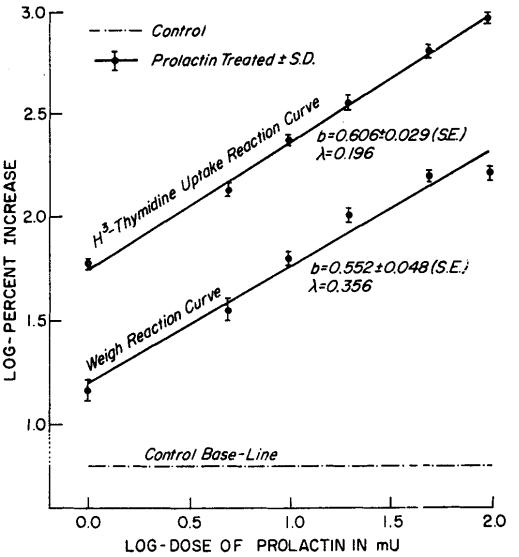

Fig. 2. Comparison of percentage increase between weight gain and H^3-thymidine uptake by the pigeon crop epithelial tissue after intradermal injections of ovine prolactin over one side of the crop. The other side used as a control. Regression of log percent increase on log dose of prolactin in mU calculated by $y' = a + bx'$.

most likely brought about due to the increase in DNA synthesis(16,17) and mitotic activity(14,15) which takes place after local prolactin administration.

The use of 0.5% agar solution as a solvent, which is easily injected, considerably improved the local effect of the prolactin by preventing fast absorption of the hormone. This advantage permits an assay of very small amounts of prolactin which could not be previously detected by other methods. Although no visual reaction was seen at the lower dose of 1 mU prolactin, the H^3-thymidine uptake was significantly increased by 59% ($P < 0.001$). It seems that this method might be useful for direct estimation of prolactin in the blood.

It was noticed that the control sides of different pigeons of the same experimental group utilize various amounts of H^3-thymidine. In order to eliminate the influence of these biological differences, the control and the prolactin injections were always given to the same animal and only percentage increases in H^3-thymidine uptake of the treated sides compared with the controls were taken into account for final interpretation. Both the

described injection procedure and extraction of the mucosal epithelium alone for radioactivity measurement eliminate any false positive results which could be achieved by nonspecific inflammatory reactions which usually involve the submucosal tissue of the crop-sac after the local injections(18). The present method includes 3 modifications of the original widely employed Lyon's local pigeon crop method(8). These modifications, which highly improved the sensitivity and objectivity of Lyon's method, are: a) H^3-thymidine uptake instead of weight gain; b) only the reactive epithelium (and not the whole gland) is involved; and c) using 0.5% Agar solution as a solvent for prolactin. However, its specificity and its application for detection of prolactin from various sources, especially from animal and human blood, are now under further examination.

Summary. A simple, sensitive, and entirely objective bioassay for prolactin is described, using H^3-thymidine uptake by the pigeon crop-epithelial tissue only. Doses as low as 1-100 mU of highly purified ovine prolactin (NIH-P-S-7, 24.3 IU/mg), dissolved in 0.5% agar solution and intradermally injected as a gel over one side of the pigeon crop resulted in highly significant increase in H^3-thymidine uptake ranging between 59% and 905% as compared to the control sides. A good dose response curve was established. The sensitivity and the objectivity of the method are discussed.

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