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Effect of Volume Used for Injection in Micro-Assay of Prolactin.

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The local crop-sac method, or micro-method, of testing for prolactin (intracutaneous injection over the pigeon's crop) introduced by Lyons and Page[1] involves a subjective element and is open to still other sources of error; it has, however, the advantage of extreme sensitivity. Bates and Riddle[2] reported that subcutaneous injections over the crop-sac area, though they place the prolactin nearer to the responding tissue, are markedly less effective than similar intracutaneous injections. Since the micro-method is currently used in some laboratories for quantitative assay of prolactin, particularly in blood and urine where the concurrent use of 0.1 and 1.0 ml fluid was once advised,[1] it is desirable to learn the sources of error in the use of this method. The present study is concerned with the effect of injecting equal quantities of hormone in unequal volumes of fluid.

In the local crop-sac method the intracutaneously injected prolactin stimulates that area of the crop tissues which lies immediately beneath the site of injection. This fact suggests a direct diffusion from the injection site. If uncomplicated diffusion is involved one would expect the response to be directly proportional to the concentration of prolactin in the solution injected. The crop epithelium is the tissue upon which prolactin acts (by causing cell proliferation) and in passing from the site of injection to this epithelium the hormone must traverse the following very thin structures: lower dermis; a layer of loose connective tissue and fat lying between the crop and the skin; the serosa; and two thin layers of muscle in the crop wall. The crop epithelium is in actual contact with ingested food and the proliferation induced by prolactin occurs in its basal cells.

When 0.05 ml of liquid is injected intracutaneously over the crop it forms a small disk-shaped blister or bleb of fluid, roughly 5 mm in diameter and 2 mm in thickness. Similarly 0.5 ml of liquid forms a blister 16 mm in diameter and 2 mm in thickness. The relative areas are as 1 : 10, but they have essentially the same thickness. Hence

1 Lyons, W. R., and Page, E., PROC. SOC. EXP. BIOL. AND MED., 1935, **32**, 1049.
2 Bates, R. W., and Riddle, O., PROC. SOC. EXP. BIOL. AND MED., 1936, **34**, 847.

the concentration per unit area may be considered independent of the volume of fluid injected. It is our experience that when stretched for examination the area of crop epithelium stimulated is 5 to 10 mm larger in diameter than the area of the skin displaced by the injected fluid, and that this area is located directly beneath the site of injection. If the mode of transfer of prolactin is by diffusion alone a prolactin solution which produces minimum stimulation when a volume of 0.5 ml is injected should also show minimum stimulation (over a smaller area) when a volume of 0.05 ml of the same solution is injected. The following experiments were designed to test this theory along with a determination of effects of unequal volumes on micro-assay.

Experimental. In tests made with 2 daily injections into the same site 7 groups of 4 birds each were used. Each bird was injected intracutaneously over both crop-sacs with the same amount of prolactin but on one side the volume was 10 times that on the other (0.5 and 0.05 ml); thus the concentration was 10 times greater in the smaller volume of solution. The right crop-sac of all birds of 4 of the 7 groups received the larger volume (Series A, Table 1). The left crop-sac received the larger volume in all birds of 3 other groups (Series B, Table I). The 2 sides of the crop were thus shown to be equally responsive. Each bird was

TABLE I.

Effect of Injection Volume on Response of Crop-sacs to Local Intradermal Injections of Prolactin. Both Sides of Crop (R and L) Injected Twice at 24-hour Interval with Autopsy 48 Hours After First Injection.

Total dose prolactin No. 437	No. of birds	Daily volume, (ml)	Concentration, γ/ml	Series	Side of crop used (R or L)	Results	
						No. positive	Sum of plus values
2.0γ	4	.5	2	A	R	4	+12
2.0γ	4	.05	20	A	L	4	+ 6
1.0γ	4	.5	1	A	R	4	+10
1.0γ	4	.5	1	B	L	4	+ 6
1.0γ	4	.05	10	A	L	2	+ 2
1.0γ	4	.05	10	B	R	2½	+ 2½
.5γ	4	.5	0.5	A	R	2½	+ 4½
.5γ	4	.5	0.5	B	L	3½	+ 3½
.5γ	4	.05	5.0	A	L	1½	+ 1½
.5γ	4	.05	5.0	B	R	0	+ 0
.25γ	4	.5	0.25	A	R	3½	+ 6
.25γ	4	.5	0.25	B	L	1½	+ 1½
.25γ	4	.05	2.5	A	L	1½	+ 1½
.25γ	4	.05	2.5	B	R	0	+ 0

injected twice in the same spot and killed 48 hours after first injection. The presence of stimulation was estimated from examination of the stretched excised crop by transmitted and reflected light. Several of the birds were also given colchicine 6 to 8 hours before killing and the presence or absence of accelerated cell division was determined histologically. In all tests the gross degree of stimulation was estimated as plus 4, 3, 2 and 1; ½ was used for cases in which stimulation was doubtful. Degree of stimulation is significant (though arbitrarily determined) and the sum of "plus" values for the 4 birds of each group is tabulated. The value assigned to each individual case was probably affected by the larger area of stimulation which results from the larger volume; we tried, however, to consider thickness only.

Crop-sacs stimulated by 0.5 ml containing 0.125 γ in each of 2 injections (total, 0.25 γ) responded as much on the average as crop-sacs stimulated by 0.05 ml containing 0.5 γ (total of 1.0 γ). The ratio of concentrations in these 2 cases is 40 : 1. Thus one obtains the wholly unexpected result that a dilution of 10 times increased the effectiveness of the injected prolactin by a factor of 4.

In a third series of tests (Table II) 4 groups of 3 pigeons each were treated as in the preceding tests except that the total dose was given in a single injection (and killed after 48 hours). Identical results on the relation of volume to response were obtained although 4 to 8 times as much prolactin was required to produce any particular grade of response with a single injection. One γ in 0.5 ml produced as much stimulation as 4 γ in 0.05 ml; 2 γ in 0.5 ml produced as much stimulation as 8 γ in 0.05 ml. Comparison of "once"

TABLE II.
Effect of Injection Volume on Response of Crop-sacs to a Single Local Injection of Prolactin with Autopsy 48 Hours Later.

Total dose prolactin No. 437	No. of birds	Volume, (ml)	Concentration, γ/ml	Side of crop used (R or L)	Results	
					No. positive	Sum of plus values
8.0γ	3	.5	16	R	3	+ 8
8.0γ	3	.05	160	L	2½	+ 4½
4.0γ	3	.5	8	R	2½	+ 4½
4.0γ	3	.05	80	L	1	+ 1
2.0γ	3	.5	4	R	2½	+ 4½
2.0γ	3	.05	40	L	½	+ ½
1.0γ	3	.5	2	R	2	+ 3
1.0γ	3	.05	20	L	0	+ 0

injected groups with "twice" injected groups indicates a quadrupling of effectiveness in the latter case.

Discussion. Our observations do not support the assumption that local stimulation of the crop-sac is due to uncomplicated diffusion of prolactin. The volume of fluid injected seems to have an influence or control on the sensitivity of the reaction and this influence is far greater than that which is expected. This may indicate that, in response to the irritation produced by the injection, some substance released in the skin serves to augment the effect of the prolactin. This assumption is supported by the low effectiveness of prolactin injected subcutaneously over crop-sacs, and by other current observations made in this laboratory which show that substances which irritate or insult the skin at the site of injection will cause some cellular proliferation (confirmed histologically) of the crop epithelium. To date none of those irritating substances induce such proliferation when injected systemically.

Calculations of the relative sensitivity of the local and the systemic methods are of interest. Systemic assays of prolactin No. 437, the preparation used in all our tests, showed it to contain 5 Riddle-Bates (or 5 International) units per mg. This is equivalent to about 1.5 to 2.0 systemic minimum stimulating doses (M.S.D.; 50% positive) per mg in a 450 g pigeon (*i.e.*, 500 to 700 $\gamma = 1$ M.S.D.). Using 2 injections, each of 0.5 ml volume, over the crop-sac the M.S.D. of No. 437 was found to be not more than 0.25 γ; when a single injection was employed 4 times as much prolactin (1 γ) was required. These values thus indicate an increase in sensitivity of at least 500 and 2,000 times for single and double injections, respectively. The magnitude of these increases agrees well with Lyons'[3] value of 1,000 for a single injection; but it differs greatly from results of Bergman, Meites and Turner[4] who report an increase of only 178 although they used the still more sensitive 4-day test (of Lyons). From such 4-day tests Lyons[5] reported an increased sensitivity of 10,000 times.

The importance of volume of fluid injected is not appreciable in systemic injections by the intramuscular route.

Summary. In 2 types (single and double injection) of 48-hour micro-methods for assaying prolactin, in which the injection volume was 0.05 ml, 4 times as much prolactin was required for minimum stimulation as when the volume was 10 times larger, 0.5 ml. A

[3] Lyons, W. R., PROC. SOC. EXP. BIOL. AND MED., 1937, **35**, 645.

[4] Bergman, A. J., Meites, J., and Turner, C. W., *Endocrinol.*, 1940, **26**, 716.

[5] Lyons, W. R., *Cold Spring Harbor Symposia on Quant. Biol.*, 1937, **5**, 198.

minimum stimulation dose of prolactin in 0.05 ml thus has its apparent effectiveness increased 4 times by a dilution of 10 times. No simple explanation of this result is apparent. Micro-assays of prolactin by intracutaneous injections over crop-sacs must utilize a constant volume of fluid to be of much quantitative value.

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Use of Illuminating Gas to Check Metabolism Apparatus.

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Combustion of alcohol, ether or acetone is standard procedure to check the operation of metabolism apparatus (Carpenter, *et al.*[1]). We have found it impossible, however, to devise a burner by which the combustion of any of these would proceed evenly for metabolically significant periods at rates comparable to the respiratory metabolism of the rat. On the other hand, small validity would seem to attach to a test several times more severe than planned capacity; failure would be no indication of inability of the apparatus to do what it was designed to do; nor would success be any guarantee that it could perform the more delicate task for which it was made. A micro-balance is not checked with kilogram weights.

As a result, recourse has been had to combustion of gas which can be successfully controlled at almost any desired rate. It was originally intended to use a pure, commercial preparation of one of the lower hydrocarbons in order to eliminate the necessity of control determinations. Preliminary work with ordinary illuminating gas from the city mains was so satisfactory, however, that it has been adhered to; especially since equipment was at hand for the necessary control determinations which involve only slightly additional work.

Since the only difficulty in the application of this principle is accurate measurement of the small volume of gas burned, description of an apparatus which has been found accurate and simple to operate and is easily assembled from odds and ends about any laboratory may be of interest.

This apparatus is shown diagrammatically in Fig. 1.

[1] Carpenter, T. M., Fox, E. L., and Sereque, A. F., *J. Biol. Chem.*, 1929, **82**, 335.