EVALUATION OF AN in vitro TECHNIQUE FOR QUANTITATIVE ASSAY OF ADRENOCORTICAL SECRETION IN THE CALIFORNIA GROUND SQUIRREL*

Author: ADAMS, LOWELL

Source: Journal of Wildlife Diseases, 8(1) : 10-18

Published By: Wildlife Disease Association

URL: https://doi.org/10.7589/0090-3558-8.1.10

EVALUATION OF AN *in vitro* TECHNIQUE FOR QUANTITATIVE ASSAY OF ADRENOCORTICAL SECRETION IN THE CALIFORNIA GROUND SQUIRREL*

LOWELL ADAMS.

The G. W. Hooper Foundation, University of California, San Francisco, California 94122.

Abstract: Experiments to determine whether *in vitro* secretion rates of adrenocortical glands reflect pre-existing *in vivo* secretory potentials were conducted using California ground squirrels *(Spermophilus beecheyi).* When the excised glands from ACTH-injected squirrels and from controls (untreated squirrels shot in the field) were incubated in ACTH-supplemented media, cortisol production was significantly greater in the ACTH-injected squirrels.

The rate of corticosterone production in glands from ACTH-injected squirrels— in contrast to the cortisol relationships — was less than or equal to that from controls. The difference was significant in glands incubated without ACTH but not in those incubated with ACTH.

Thus *in vitro* cortisol production reflects *in vivo* production. The same may also be true for corticosterone if it can be shown that ACTH injection decreases corticosterone production.

In forthcoming studies of social behavior and adrenocortical function in California ground squirrels *(Spermohphilus beecheyi),* I plan to use the weights of adrenal glands as indicators of secretion rates. Since Munday[4] questioned the reliability of gland weight as an indicator and since Gordon *et al.*,[2] Sanzari *et al.*[6] and van der Vies[7] showed that gland weights may not correspond to secretion rates under some conditions, it is pertinent to determine whether large glands secrete more than small ones.

To test the relationship of gland weight to secretion rate, I proposed gland incubation as a useful method of gauging *in vivo* secretion rate. However, since it is not known whether this technique reflects the actual secretory potential of the glands, I undertook to test its validity in this study. I was further interested to learn whether ACTH in the incubation medium affects the relationship between *in vitro* production and *in vivo* potential. ACTH increases the yield of hormone in gland incubation, which is helpful in qualitative studies. However, hyperstimulation of the incubating glands could mask quantitative differences between high- and low-producing glands. Van der Vies[7] found that *in vitro* secretion rates are usually positively correlated with pre-existing chronic *in vivo* rates, but he did not investigate the effect of ACTH in stimulating greater hormone production *in vitro.*

This paper reports results of an experiment to determine whether adrenocortical hormone production *in vitro* can be used to discriminate between more productive and less productive glands and to compare *in vitro* production with and without ACTH.

*This study was supported by Research Grant GM 11993 from the National Institutes of Health, U.S. Public Health Service.

METHODS

Hormone analyses were made in a laboratory established for this purpose at the Hastings Natural History Reservation, a 1,600-acre area 30 miles southeast of Monterey, California. Squirrels used experimentally were shot or live-trapped on the Reservation or on adjacent ranches during the period November to March.

Adrenal glands were made hyperactive in live-trapped, caged squirrels by injecting intraperitoneally ACTH (Armour's Acthar) dissolved in a 1:20 mixture of beeswax and peanut oil. Some animals were injected daily with 5 USP units for 1, 2, 3, 5, 7, or 11 days, others with 10 units for 2, 4, or 6 days. (These injection regimens were followed to explore the effects of dosage gradients as a basis for designing future experiments. The glands from all regimens were grouped in one treatment class, "injected," in the present analysis.)

Normal glands were obtained from squirrels shot in the field. These were shot in the head for instant "decapitation" to avoid any emergency effects on adrenal secretion.

The adrenal glands were excised immediately after each squirrel's death, weighed, minced with a razor blade, and divided into two portions: one incubated with and one without ACTH. The tissue was placed in a modified Krebs-Ringer bicarbonate solution and adjusted to pH 7.4-7.5 with an added 2 mg of glucose/1 ml of solution. Tissue not exceeding 75 mg was incubated in 3 ml of the solution under O_2-CO_2 (95:5) and shaken in a Dubnoff incubator at 37.5-38.0 C. One USP unit of ACTH/100 mg of tissue was added to the incubations with ACTH. The secretions obtained during the first 30 minutes were discarded, and the tissue was then incubated for 3 hours with a change of medium after 1½ hours. The adrenocortical hormones secreted during this 3-hour period were extracted with diethyl ether, dried under nitrogen at 40 C, and stored at 4 C before chromatography. Cortisol and corticosterone were purified and separated by thin-layer chromatography (TLC) according to the methods of Nandi and Bern[5] with certain modifications: The two unknowns were applied to one edge of the plate in a chloroform-methanol (1:1) solution. In line with the unknowns, cortisol-corticosterone standards (supplied by Upjohn and Merck, Sharp and Dohme) were spotted in concentrations of 1, 2, 4, and 8 μg. The plates were developed with chloroform-methanol-water (90 : 10 : 1), dried, and sprayed with blue tetrazolium (BT) in a solution of 1 mg BT, 0.5 ml 10% NaOH, and 0.5 ml ethanol. The amounts of cortisol and corticosterone present in the incubated sample were estimated by blind comparison of the size and color-intensity of these spots with the size and color-intensity of standard spots.

Estimates were made to the nearest standard value or midpoint between adjacent values, that is, to the nearest 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, 6.0, 8.0, or 8.0+ μg. In 22 out of 25 blind tests (with standards for "unknowns") the samples were correctly classified. The three incorrect estimates were in the upper range —6.0 to 8.0+ μg. To avoid such errors, samples expected to have large amounts of hormones were divided into two subsamples, estimated separately then summed. Also glands expected to produce amounts of hormones too small to detect individually were pooled. Most estimates were therefore made on values in the lower range where precision and accuracy were greater.

The effects of ACTH injection are presented in three ways — in terms of secretion rates, gland weights and total hormone production. Secretion rates are given as micrograms of hormone per 100 mg of tissue incubated. Gland weights are presented as milligrams per 100 mm of body length to eliminate the effects of body size. Total production is the secretion rate times the total weight of pairs of glands.

All results are presented separately by sexes since the glands are significantly larger in males than in females. Statistical significance of comparisons of means are determined by t-tests. The term, "significant" used in the text means "significant at the .05 level of probability"; "highly significant" means "significant at the .01 level."

RESULTS

Secretion Rates.

Glands from squirrels injected with ACTH produced cortisol *in vitro* at a higher rate than those from uninjected squirrels shot in the field (Fig. 1). When glands were incubated without ACTH the difference was not significant. Adding ACTH to the incubation medium caused a substantial increase in the rates of hormone production in both ACTH-injected and shot squirrels. More importantly, the addition of ACTH to the medium caused glands from the ACTH-injected squirrels to produce *significantly* more cortisol than did those from shot squirrels ($p < .01$). Clearly ACTH in the incubation medium heightens the power of the incubation technique to discriminate between levels of secretion *in vivo*.

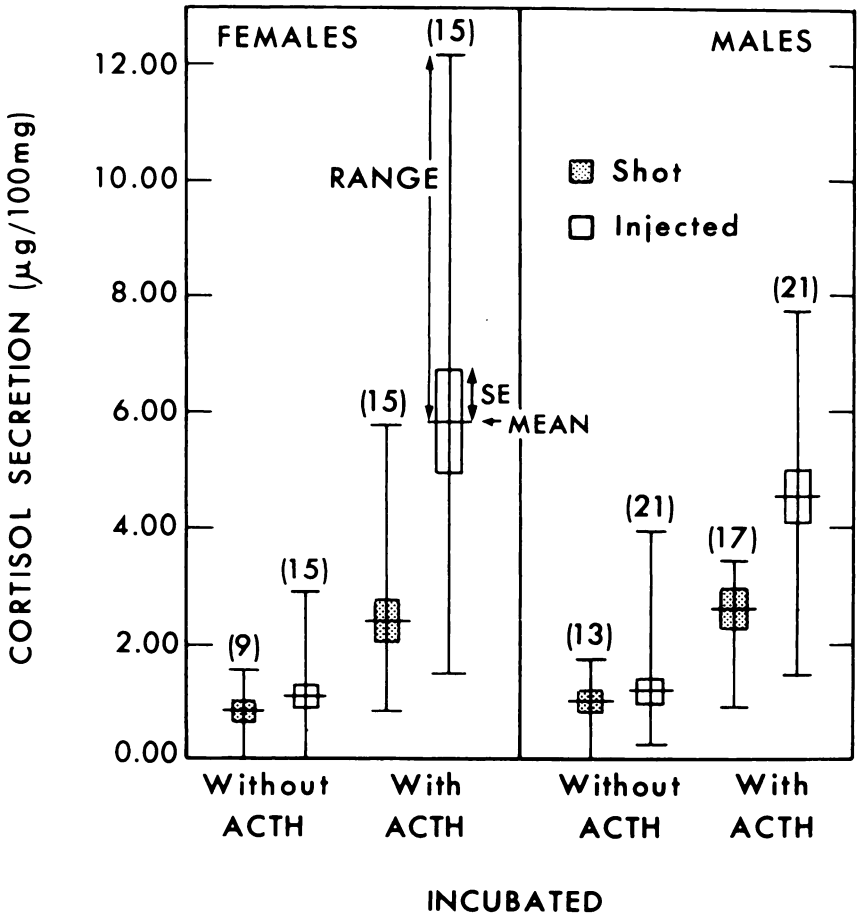

FIGURE 1. Secretion rates of cortisol, as measured **in vitro** with and without ACTH in the incubation media, in squirrels injected with ACTH or shot. Units of measurement: micrograms of hormone per 100 mg of adrenal gland.

Corticosterone production rates *in vitro* (Fig. 2) were lower in glands from injected squirrels than in those from shot squirrels. In females the differences were significant (for glands incubated without ACTH, $p < .01$; for glands incubated with ACTH, $p < .02$). In males they were not. Glands incubated in ACTH-supplemented media produced hormones at a higher rate than those in unsupplemented media.

Glands from injected squirrels produced cortisol at a higher rate than corticosterone (Table 1). The difference was significant in all categories except shot females.

Gland Weights.

In all sex-age groups glands from injected squirrels were much larger than those from uninjected ones (Fig. 3). The difference was highly significant in all cases (probably including young males although the sample was too small for statistical testing).

In shot females, glands from adults were significantly larger than those from young. In shot males, adult glands were larger than those classed as "unaged" so-called because aging criteria failed part way through the experiment. Most unaged squirrels were young ones.

Glands of injected adults weighed no more than those of the young or unaged.

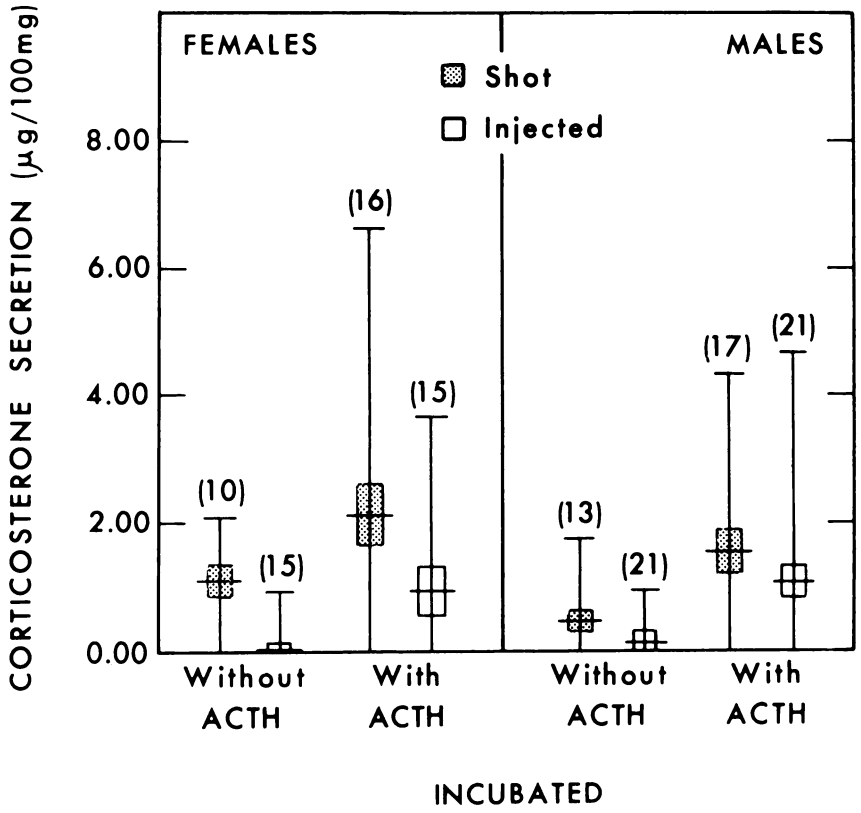

FIGURE 2. Secretion rates of corticosterone. Methods, symbols and units of measurement are as in Fig. 1.

TABLE 1. Relative production rates of cortisol and corticosterone.

Treatment	Incubation	Mean Secretion Rates (μg/100 mg)[1] Cortisol	Corticosterone	Ratio	Significance[2]
		Females			
Shot	Without ACTH	.87	1.10	.79	NS
Shot	With ACTH	2.41	2.18	1.11	NS
Injected	Without ACTH	1.13	.06	18.83	**
Injected	With ACTH	5.88	.98	6.00	**
		Males			
Shot	Without ACTH	1.03	.46	2.24	*
Shot	With ACTH	2.60	1.58	1.65	*
Injected	Without ACTH	1.23	.13	9.46	**
Injected	With ACTH	4.60	1.12	4.11	**

[1] μg of hormones per 100 mg of tissue.

[2] Significance of differences in production rates of cortisol and corticosterone.

NS— Not significant; *—$p < .05$; **—$p < .01$.

Total Hormone Production.

Cortisol production (Fig. 4) was greater in glands from injected squirrels than those from shot squirrels. The differences are highly significant in both sexes and in production from both ACTH-supplemented and unsupplemented media.

Corticosterone production (Fig. 5) was significantly less in glands from injected squirrels incubated without ACTH than from shot squirrels' glands so incubated. When incubation was ACTH - supplemented, production from shot squirrels' gland was the same as from those of injected squirrels.

DISCUSSION

Cortisol production under the experimental conditions clearly supports the hypothesis that *in vitro* production reflects *in vivo* production. ACTH injection increased cortisol production *in vitro* and it may reasonably be expected to do so *in vivo*.

Corticosterone production, on the other hand, does not support the hypothesis at the present stage of investigation. With ACTH injection corticosterone production actually decreases *in vitro*, or at best remains unchanged (Fig. 2). This can mean either that the corticosterone production does in fact diminish or remain unchanged when ACTH is administered, or that its increased production is not reflected *in vitro*. The present study does not support either alternative. However, ACTH is known to have differential effects on the production of cortisol and corticosterone.[3] Furthermore, Varon *et al.*[5] found that the production of corticosterone decreased relative to cortisol production in crowded compared with uncrowded immature house mice. The apparent absolute diminution of corticosterone in the present study could be simply a variation in the differential trends described for other species.

Perhaps it is advisable and justifiable to omit the corticosterone determinations from estimates of secretion rates, relying only on cortisol levels. If corticosterone production did in fact decrease slightly or remain unchanged with ACTH injection, as this study seems to indicate, the decrease was slight relative to the great increase in cortisol production. The total

effect would be essentially the same as if cortisol alone were considered — the greater cortisol effects swamp the lesser corticosterone effect. Furthermore, because cortisol is the more potent glucocorticoid[3] it further swamps the effect of decreased corticosterone production. However, had the corticosterone production been higher *in vivo* and had the incubation simply failed to reflect this, then the reliance on the cortisol differential would still be a sufficient index of glucocorticoid trend and would not contradict the corticosterone indication.

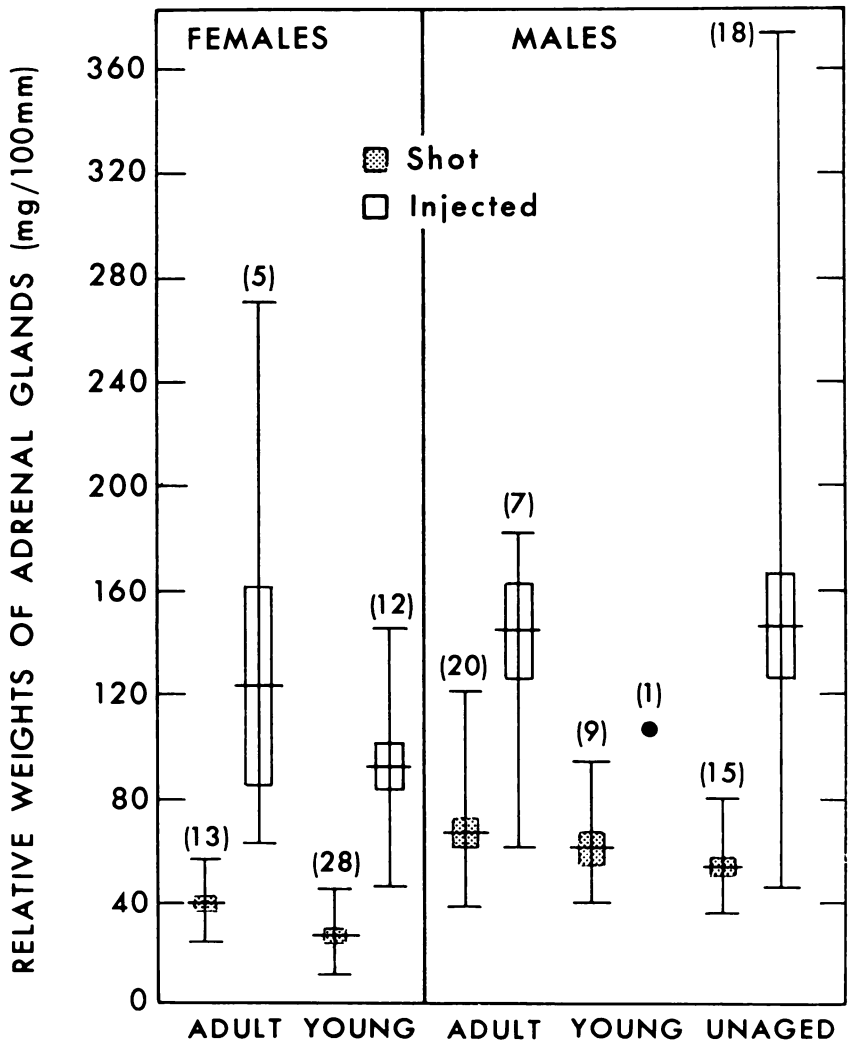

FIGURE 3. Relative weights of adrenal glands of squirrels shot in the field or kept in captivity and injected with ACTH. Units of measurement: milligrams of adrenal gland per 100 mm of body length.

While the present experiment answers the question it was designed to answer (i.e. Do large adrenals secrete more and small ones less?), a valuable extension of the inquiry would be to generate a series of glands of varied sizes by injecting various amounts of ACTH over different periods of time. This was not done in the present instance because we had no information on which to base an appropriate selection of injection regimens. The regimens we used were exploratory and were selected to find the pertinent ranges of dosage and time. We found that approximately 5 units of ACTH injected for 3 days was about the right regimen to bring the glands to distinctly enlarged condition. The propos-

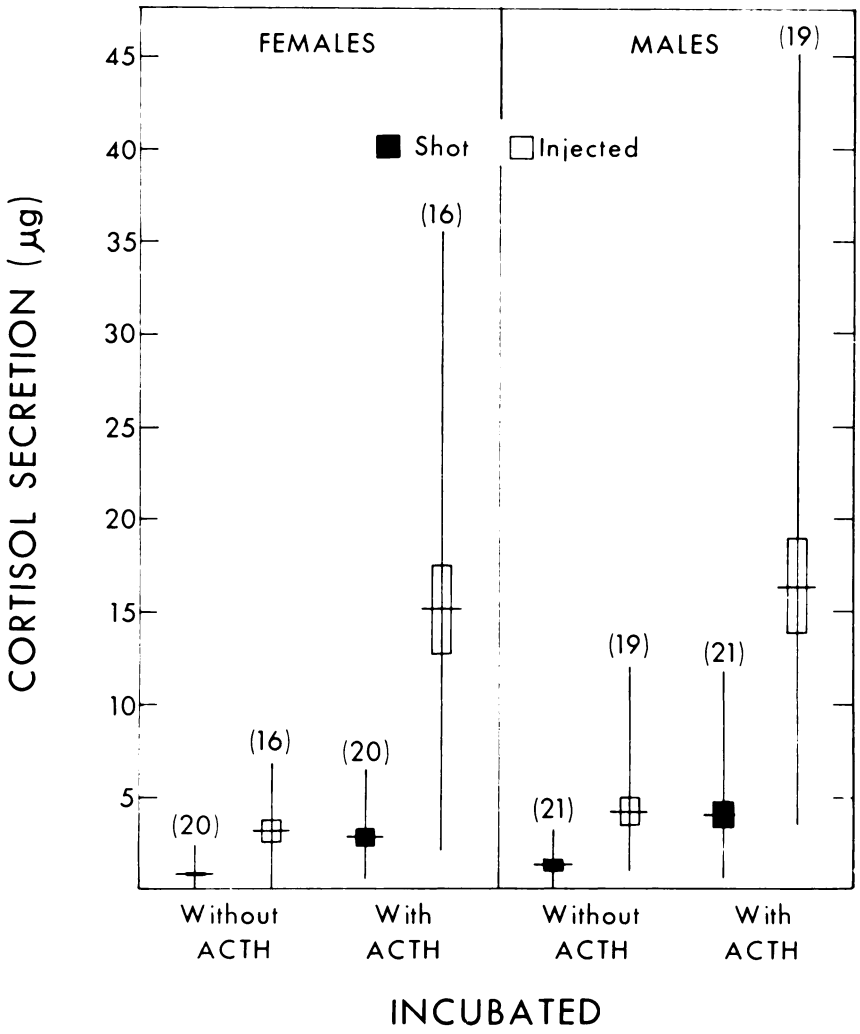

FIGURE 4. Total amounts of cortisol secreted during three hours of incubation. Totals were computed by multiplying the weights of the paired glands by their respective secretion rates.

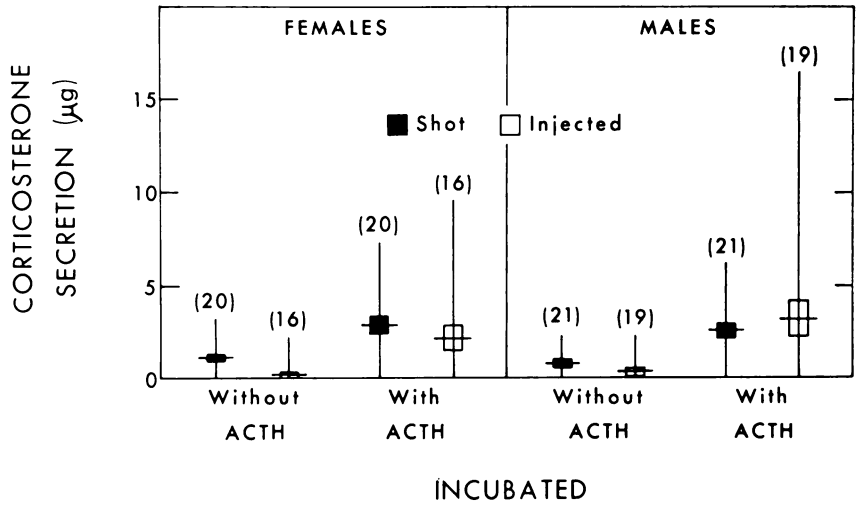

FIGURE 5. Total amounts of corticosterone secreted during three hours of incubation. Totals were computed as explained for Fig. 4.

ed experiment should therefore use a range of about 1 to 6 units over a 3-day period to study chronic or steady state conditions. If acute effects were of interest, secretion rates should be tested at intervals less than 3 days after injections begin.

In a similar sense, an experiment using graduated amounts of ACTH in the incubation media might be desirable. This point is discussed elsewhere.[1]

Since the experiments reported here, the *in vitro* method has been used to assay hormone production rates relative to adrenal size in shot squirrels.[1]

CONCLUSIONS

The *in vitro* technique quantitatively reflects *in vivo* secretion levels of cortisol in the ground squirrel's adrenal cortex and may be used to assay cortisol production levels. It may not reflect levels of corticosterone production, and it is recommended that the latter not be used in measuring glucocorticoid production. ACTH used as a supplement in incubation media enhances the discriminative capability of the assay method and should be used for that purpose.

Acknowledgements

Valuable advice and assistance was given by Dr. Howard A. Bern, Dr. Jean Nandi and Mrs. Julie Pieprzyk, Department of Zoology, University of California, Berkeley, in developing laboratory techniques and reviewing early versions of the manuscript. Dr. J. J. Christian, State University of New York at Binghamton, and Mr. Joseph Finn and Mr. Roger Wetmore also reviewed the manuscript.

LITERATURE CITED

1. ADAMS, L., and S. HANE. 1972. Adrenal gland size as an index to adrenocortical secretion rate. J. Wildl. Diseases. 8: 19-23.

2. GORDON, S., S. MAUER, W. P. CEKLENIAK, and R. PARTRIDGE. 1963. Mechanism of triparanol-induced adrenal hypertrophy and reduced adrenal function. Endocrinology 72: 643-648.
3. JONES, I. C. 1957. *The Adrenal Cortex*. Cambridge Univ. Press.
4. MUNDAY, K. A. 1961. Aspects of stress phenomena, p. 168-189. *In Mechanisms in Biological Competition*. Symposia of the Soc. for Exp. Biology, No. XV. Cambridge Univ. Press.
5. NANDI, J., and H. A. BERN. 1965. Chromatography of corticosteroids from teleost fishes. Gen. Comp. Endocrinol. 5: 1-15.
6. SANZARI, N. P., G. POSSANZA, and R. C. TROOP. 1965. Lack of correlation between body weight and cortisol secretion, and between adrenal weight and cortisol secretion in the dog. Life Sci. 4: 1345-1351.
7. VAN DER VIES, J. 1960. Corticoid production *in vitro* as a test of adrenocortical function in rats. Acta Endocrinol. 33: 59-66.
8. VARON, H. H., J. R. TOUCHSTONE, and J. J. CHRISTIAN. 1966. Biological conditions modifying quantity of 17-hydroxycorticoids in mouse adrenals. Acta Endocrinol. 51: 488-496.

Received for publication April 26, 1971