PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 158, 524–529 (1978)

TSH and ACTH Secretion and Cyclic Adenosine 3′5′ Monophosphate Content following Stimulation with TRH or Lysine Vasopressin *in Vitro:* Suppression by Thyroxine and Dexamethasone (40239)[1]

J. C. ROSE[2] AND P. M. CONKLIN[3]

[2] *Department of Physiology and Pharmacology, Bowman Gray School of Medicine, Winston-Salem, North Carolina 27103 and* [3]*Department of Physiology, Medical College of Virginia, Richmond, Virginia 23229 (Deceased).*

Hypothalamic releasing hormone stimulation of anterior pituitary hormone release may be mediated by the andenylate cyclase–cyclic adenosine 3′5′-monophosphate system (1–3). Some, (4, 5, see 6 for additional references) but not all, investigators (7, 8) have noted synthetic releasing hormones increase pituitary cAMP levels and hormone release and studies with releasing hormone analogs possessing a broad spectrum of bioactivity suggest a close correlation between their effects on pituitary hormone release and cyclic AMP content (9). Phosphodiesterase inhibitors increase intracellular cAMP levels and pituitary hormone secretion and potentiate hormone release from the pituitary in response to TRH, LRH and vasopressin (2, 9–13). Although universal agreement does not exist (7), exogenous cAMP or its dibutyryl derivative have been reported to stimulate release of all six anterior pituitary hormones in vitro (3, 10–17). Thus extensive evidence suggests a role for cyclic AMP in the mechanisms of action of releasing hormones.

Studies with thyroxine (2, 4) and glucocorticoids (14, 18) indicate these hormones can inhibit the secretion of TSH and ACTH respectively, by direct effects on the pituitary gland. Mechanisms involved in this feedback inhibition are not known. Target gland hormone feedback effects at the pituitary may include inhibition of cAMP production and/or its subsequent effects. The following studies were done to further elucidate the dynamics of releasing hormone induced pituitary hormone secretion and cAMP accumulation and to determine if feedback hormones concomitantly affect these parameters. TSH secretion and concomitant changes in pituitary cAMP were measured during TRH stimulation and during T_4 suppression of such stimulation in vitro. Similarly, ACTH secretion and concurrent changes in pituitary cAMP were examined using lysine vasopressin (LVP) to stimulate ACTH secretion and dexamethasone to suppress such stimulation.

Material and methods. Anterior pituitaries from male, Sprague–Dawley rats (150–300 g) were placed in iced physiologic saline and sliced into either four or eight pieces of approximately equal size, depending upon the experiment. Each experimental run utilized at least two glands and four to eight beakers. Fragments from each pituitary were equally distributed among the beakers so that each contained a comparable pool of tissue. The pools of tissue were weighed and placed in 5 ml beakers containing Krebs ringer bicarbonate buffer pH 7.3 with glucose (200 mg%) and bovine serum albumin (100 mg%) and incubated at 37° in a Dubnoff incubator under an atmosphere of 95% O_2–5% CO_2. Tissue weight to medium volume ratios for experiments with TSH were 2 mg of tissue per 1 ml of incubation medium and for ACTH, 2 mg/0.5 ml. Duration of preincubation and incubation varied with experiments and will be described for each series.

I. Incubations with TRH; pituitary cAMP; medium-TSH. A. Temporal relationships. Four pituitaries divided into eights were evenly (1 fragment/pit./beaker) distributed into eight beakers, thereby allowing one control beaker and one experimental beaker at each of four times studied.

Following a 1 hr preincubation with medium changes at 30 and 60 min, TRH was added to the experimental beakers at a concentration of 10 ng TRH/mg pituitary tissue incubated. At 10, 20, 30 and 60 min following TRH, tissue and media were collected from a pair of beakers for assay of tissue cyclic

[1] Supported by USPHS Grant No. AM 14713-02 awarded to P.M. Conklin.

0037-9727/78/1584-0524$01.00/0

AMP and medium TSH.

B. Dose response studies. Tissue fragments were prepared and preincubation procedures were the same as above, except two glands and four beakers were used; one beaker received TRH diluent (isotonic saline) and 0.10, 1.0, 10.0 ng TRH/mg tissue was added to the remaining three beakers. Incubations lasted for 20 min.

C. Thyroxine feedback studies. Pituitaries were prepared and preincubation times were the same as in the preceding section. Throughout the experiment, beakers 1 and 2 received thyroxine diluent (methyl ammonium hydroxide) and 3 and 4 received thyroxine (0.5 and 5.0 μg/ml tissue [10–100 μg/ml] respectively in a constant volume. After the preincubation, TRH (10 ng/mg incubated tissue) was added to beakers 2–4 and tissue and medium were collected 20 min later.

II. Incubations with LVP; pituitary cAMP, medium ACTH. Preincubation lasted 3 hr with medium changes after each hour as described by Fleisher and Vale (18) for all the following studies with ACTH. A. Temporal relationships. Pituitaries were divided and distributed as described for the TRH temporal relationship studies. At the end of the preincubation period, the media was replaced and LVP (Sandoz Pharmaceuticals) was added to the experimental beakers to achieve a concentration of 20 mU LVP/ml medium. At 10, 20, 30, and 60 min the tissue and media were harvested from one experimental and one control beaker.

B. Dose response studies. Pituitaries were divided and distributed in the same manner as in the TRH dose response studies. Incubation with LVP in various doses (10, 20, 50 mU/ml media) lasted 20 min.

C. Dexamethasone feedback studies. One pituitary was removed and divided into eights and the fragments evenly distributed between two beakers. Both beakers received LVP (20 mU/ml medium) during the 20 min incubation and experimental beakers received dexamethasone (0.01, 0.1 or 1.0 μg/ml) during preincubation and incubation periods.

III. Hormone assays. A. TSH. TSH was measured by a radioimmunoassay (19) which utilized rat TSH-RP-1, 0.22U/mg supplied by the hormone distribution officer NIAMD as a standard and bovine TSH (courtesy of Dr. J. C. Pierce) for iodination. The TSH antiserum was a gift from Dr. Seymour Reichlin. No significant cross reactions with other pituitary hormones were noted using this antisera. Biological validation of the assay was established by finding no detectable TSH in T_4 treated animals, elevated levels (1.2 ± 0.1 mU/ml, mean ± SEM) in propylthiouracil treated animals and basal levels of 0.47 ± 0.03 mU/ml in untreated animals. All unknowns were assayed in duplicate and at least two different dilutions. Parallelism between standards and unknowns was always noted. The intrassay coefficient of variation was ±5%. Interassay variability was somewhat greater, so all samples from a given experimental set were analyzed in the same assay to minimize the effects of this variability.

B. ACTH. ACTH in the incubation fluids was measured by a minor modification of the radioimmunoassay previously described (20). Purified human ACTH, a gift from Dr. C. H. Li, was used for iodination and standards. Intraassay coefficient of variation for the assay was ±8%. Interassay variability was greater, however all samples from a single experiment were again analyzed in the same assay to minimize the effects of this variability.

C. —cAMP assay. Pituitary cyclic AMP concentrations were measured by minor modification of the protein binding assay described by Gilman (21). Recovery of cAMP added to tissue homogenates averaged 95 ± 3%. Serial dilutions of samples showed parallelism with the standard curve. Tissue extracts incubated with phosphodiesterase contained no detectable cAMP; paired aliquots of the same extracts not incubated with phosphodiesterase did. Intraassay coefficient of variation was 10%. All samples from a single experiment were analyzed in the same assay.

IV. Statistical Procedures. Experiments involving dose response studies and feedback were analyzed by analysis of variance and Duncan's multiple range test.

Differences between control and experimental values (i.e., changes *between* treatment groups) in time course stimulation studies following treatment with TRH or LVP were analyzed by the paired *t* test. Changes in

control values and in experimental values at the various time intervals (i.e. changes *within* treatment groups) were analyzed by the Freeman two-way analysis of variance by rank test.

Results. Time course of pituitary cyclic AMP elevation and TSH release after TRH. TRH stimulated TSH release above paired control values and increased pituitary cyclic AMP concentrations at all times studied ($P < 0.01$ in all cases). The increase in pituitary cAMP levels was greatest at 10–20 min following addition of TRH and declined thereafter whereas TSH release continued longer. Control cyclic AMP levels did not change significantly with time, although TSH release by control pituitaries did (60 min vs. 10 min, $P < 0.02$) (Table I).

Dose Response Relationships: TRH and TSH secretion and pituitary cAMP levels. Both TSH release and pituitary cAMP elevation increase progressively with a log dose of TRH (Fig. 1). TSH release was significantly greater than basal release at the two highest TRH doses ($P < 0.05$) but not at the lowest dose ($P > 0.05$). Similarly the lowest dose did not significantly ($P > 0.05$) increase cAMP above resting values but the two higher doses did ($P < 0.05$) (Fig. 1).

Thyroxine pretreatment and TRH stimulation: effects on pituitary TSH release and cAMP content. Thyroxine significantly inhibited TRH-stimulated TSH release ($P < 0.05$ either dose vs. TRH). Both doses of thyroxine suppressed release to the same extent but neither reduced TSH release to unstimulated control levels ($P < 0.05$) (Fig. 2).

Both doses of thyroxine also significantly ($P < 0.05$) inhibited TRH stimulated cAMP elevation and the effect varied with the dose (Fig. 2). Cyclic AMP levels after incubation with 0.5 μg T_4 per mg tissue were significantly different ($P < 0.05$) from unstimulated control levels whereas levels after incubation with 5 μg/mg were not. Pituitary cAMP levels tended to be higher after incubation with 0.5 μg T_4/mg than with 5.0 μg T_4/mg; however, this difference was not significant.

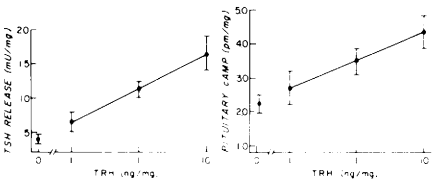

FIG. 1. Effect of increasing doses of TRH on TSH release (left panel) and cAMP content (right panel) following 20-min incubation of pituitary fragments. Points represent means (±SEM) for seven beakers.

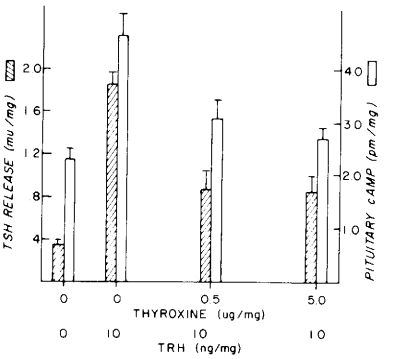

FIG. 2. Interaction between TRH and thyroxine on cAMP concentration and TSH release. Values represent means (±SEM) of seven beakers at each dose.

TABLE I. TIME COURSE OF PITUITARY TSH RELEASE AND cAMP ELEVATION DURING STIMULATION WITH TRH (10 ng/mg TISSUE).[a]

Incubation time in minutes		10	20	30	60
TSH Release (mU/mg)	Control	0.46 ± .07[b]	0.87 ± 0.19	1.53 ± 0.48	1.86 ± 0.43
	Experimental	1.86 ± .43	2.80 ± 0.80	5.40 ± 1.91	5.03 ± 1.48
	P	<0.01	<0.01	<0.01	<0.01
Pituitary cAMP (pm/mg)	Control	3.40 ± 0.43	3.02 ± 0.62	3.24 ± 0.52	2.88 ± 0.29
	Experimental	4.80 ± 0.50	4.70 ± 0.59	4.06 ± 0.65	3.47 ± 0.55
	P	<0.01	<0.01	<0.01	<0.01
	Pairs of beakers	6	6	6	6

[a] Four pituitaries were used in each series of eight beakers; each pituitary was divided into eight fragments evenly distributed among the beakers. Differences between control and experimental values at each time interval were analyzed by the paired *t* test.

[b] Mean ± SE.

TABLE II. TIME COURSE OF PITUITARY ACTH RELEASE AND cAMP ELEVATION DURING STIMULATION WITH LVP (20 mU/ml MEDIUM).[a]

Incubation time in minutes		10	20	30	60
ACTH Release (mU/mg)	Control	0.048 ± 0.004[b]	0.078 ± 0.009	0.101 ± 0.016	0.093 ± 0.006
	Experimental	0.120 ± 0.009	0.187 ± 0.012	0.253 ± 0.035	0.341 ± 0.028
	P	<0.01	<0.01	<0.01	<0.01
Pituitary cAMP (pm/mg)	Control	2.37 ± 0.29	2.14 ± 0.21	2.28 ± 0.21	2.74 ± 0.23
	Experimental	3.74 ± 0.38	3.57 ± 0.37	3.22 ± 0.33	3.21 ± 0.25
	P	<0.01	<0.01	<0.01	<0.01
	Pairs of beakers	7	7	7	7

[a] Four pituitaries were used in each series of eight beakers; each pituitary was divided into eight fragments evenly distributed among the beakers. Differences between control and experimental values at each time interval were analyzed by the paired *t* test.

[b] Mean ± SE.

Time course of pituitary cAMP elevation and ACTH release after LVP. ACTH release and pituitary cAMP content were increased by 20 mU of LVP per ml incubation medium at all time intervals studied (Table II). The ACTH concentration in the media of the control beakers increased during the first 30 min of incubation ($P < 0.01$, 30 min vs. 10 min) and stabilized thereafter. Control values for pituitary cAMP did not change significantly with time (Table II). LVP-stimulated release of ACTH continued throughout the incubation period whereas pituitary cAMP concentration was greatest at 10–20 min and declined thereafter (Table II).

Dose response relationships: LVP on ACTH secretion and pituitary cAMP levels. All doses of LVP increased ACTH release ($P < 0.05$) and pituitary cAMP content ($P < 0.05$) over unstimulated levels. Increasing doses of LVP significantly increased ACTH release over each previous dose ($P < 0.05$). Although cAMP levels tended to increase, levels in stimulated pituitaries did not differ significantly as a function of dose (Fig. 3).

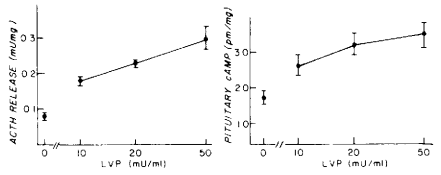

FIG. 3. Effect of increasing doses of LVP on ACTH release (left panel) and cAMP content (right panel) following 20-min incubation of pituitary fragments. Points represent means (± SEM) for eight beakers.

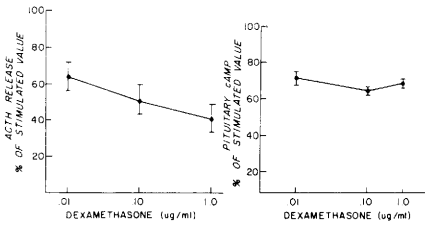

FIG. 4. Effect of increasing concentrations of dexamethasone on the LVP-stimulated release of ACTH. Results are expressed as % of paired stimulated (LVP alone) beakers. Points represent means (± SEM) of seven pairs of beakers.

Dexamethasone pretreatment and LVP stimulation on pituitary ACTH release and cAMP content. All three doses of dexamethasone used decreased ($P < 0.05$) LVP stimulated ACTH secretion. ACTH release in the presence of dexamethasone has been expressed as a percent of release of control pituitaries receiving LVP only (Fig. 4). ACTH release of 20 milliunits of LVP per ml alone averaged 0.204 ± 0.018 nm/mg in this series of experiments. There was significantly greater inhibition of ACTH release ($P < 0.05$) by the 1 μg/ml dose of dexamethasone than by the 0.01 μg/ml dose; inhibition by 0.10 μg/ml did not differ significantly from either of the other doses. Stimulated cAMP levels averaged 3.53 ± 0.23 picomoles/mg of tissue in these experiments. Pituitary cAMP levels were significantly ($P < 0.05$) reduced by dexamethasone relative to control tissue receiving LVP alone; however, the extent of suppression was the same at all doses of dexamethasone tested (Fig. 4, results expressed as a percent of beaker with LVP only).

Discussion. The purpose of these investi-

gations was to further examine relationships between hypothalamic releasing hormone stimulation of pituitary hormone secretion and cAMP levels and the influence of feedback hormones on these relationships. Evidence has been presented that releasing hormones as well as target gland hormones can influence pituitary cAMP levels, and thus, that cAMP may act as a second messenger at the pituitary level. However, a dissociation of pituitary hormone secretion and changes in cAMP levels was also demonstrated. Such a disassociation has been noted by others (7, 8) and suggests qualification of the second messenger theory may be necessary in the case of releasing hormone induced anterior pituitary hormone secretion. The changes observed in pituitary cAMP levels following TRH indicate that a transient increase in cAMP is sufficient for the induction of hormone secretion.

If cAMP is a second messenger involved in TSH secretion, then one may postulate that increments in the dose of TRH could be accompanied by progressive increases in both hormone secretion and pituitary cAMP. Studies limited to 20 min incubation periods showed a linear log dose response relationship between TRH and TSH secretion. The fact that a similar relationship was also obtained in pituitary cAMP levels is compatible with the second messenger hypothesis. These results using TRH are consistent with reports that it rapidly stimulates TSH secretion and increases pituitary cAMP levels (4, 5).

Studies reported here dealing with feedback confirm the ability of thyroxine to suppress TRH stimulated TSH secretion by direct action at the pituitary (2, 4, 22). Within the range of thyroxine used, suppression of TSH release was independent of dose and hormone release was never returned to nonstimulated levels. If a larger range of thyroxine concentrations had been used, possibly a greater suppression of TSH release would have been observed.

Data reported here indicate that thyroxine also inhibits the increase of pituitary cAMP following TRH. These findings are consistent with the report that triiodothyronine (in vivo) diminishes the pituitary cAMP response to TRH (*in vitro*) (4). These results fit the second messenger hypothesis as well, since physiological agents which block an increase in hormone release could act by inhibiting the increase in an intracellular mediator of this release. Somatostatin, a peptide which inhibits TSH release, is capable of reducing pituitary cAMP levels (23). This also suggests inhibition of an increase in an intracellular mediator of hormone release can diminish hormone secretion. Our observations suggest that thyroxine blocks the increase in the intracellular mediator, but do not exclude the possibility that inhibition of the actions of the intracellular mediator also occur. Such a possibility is suggested by observations *in vitro* that dibutyryl cAMP induced increases in TSH and ACTH secretion are abolished by dexamethasone and thyroxine respectively (14, 24). Perhaps inhibition of the intracellular mediator is via stimulation of enzymes which catabolize the second messenger—for example, phosphodiesterase.

At the highest dose of thyroxine used cAMP levels were not significantly different from unstimulated control values whereas TSH secretion was still significantly elevated above that of control levels. Possibly, TRH has two modes of action, only one involving cAMP. For example, TRH may change the permeability of the thyrotroph membranes resulting in uptake of calcium which may induce TSH secretion by a mechanism as yet unknown. Other reports have suggested Ca^{2+} translocation may mediate hormone secretion by the anterior pituitary and a partially purified preparation of growth hormone releasing factor has been reported to stimulate calcium uptake by the pituitary (25). The existence of another intracellular mediator which plays a role in hormone secretion but is not influenced by thyroxine is another possible explanation for the divergence noted between pituitary cAMP levels and TSH secretion in the presence of thyroxine. A prime candidate would be cyclic GMP. The dibutyryl derivative of this compound has been shown to stimulate growth hormone release from rat pituitaries incubated *in vitro*, and treatment with a crude hypothalamic extract which increased hormone secretion also elevated the pituitary concentration of cyclic GMP (26).

The second system studied employed LVP as a CRF since purified or synthetic CRF(s) are not available and LVP can act directly on

the pituitary to stimulate ACTH release (14, 18). In general, results observed using LVP are similar to those using TRH with regard to abrupt onset and duration of action.

Others have shown that dexamethasone can inhibit ACTH release stimulated by hypothalamic extracts and by LVP in vitro (14, 18). Results reported in this study confirm these findings with LVP. The fact that dexamethasone also blocked the increase in pituitary cAMP caused by LVP is of considerable interest, the more so because a similar effect was seen with TRH and thyroxine.

A divergence was noted between suppression of stimulated ACTH release and suppression of pituitary cAMP levels. The graded suppression of hormone release was not accompanied by a graded suppression of stimulated cAMP levels. cAMP was returned to levels approximately equal to those found in earlier experiments in unstimulated pituitary tissue by the lowest dose of dexamethasone whereas ACTH release remained elevated except in the presence of the highest dose of dexamethasone. Thus, increased ACTH release occurred while cAMP levels were not above stimulated values. This is similar to the results noted using TRH and thyroxine where cAMP levels returned to control levels while TSH release was elevated. The explanation of this divergence in pituitary cAMP levels and hormone secretion is presently unknown. Such a disassociation suggests the pituitary secretory response to a hypothalamic hormone is not mediated solely via an elevation in cAMP levels.

Summary. The studies reported here demonstrate temporally related changes in pituitary hormone release and levels of cAMP following stimulation with either TRH or LVP. They thus strengthen the postulation that cAMP is a second messenger in the action of releasing hormones on the pituitary. However, a divergence between hormone release and pituitary cAMP levels was noted in several instances. Therefore, a definitive role for cAMP as mediator of anterior pituitary hormone release was not established. These studies also show target gland hormone feedback in vitro can inhibit releasing factor produced elevations of pituitary cAMP levels and suggest such an action may be a method by which target gland hormones inhibit tropic hormone release.

1. Zor, U., Kaneko, T., Schneider, H. P. G., McCann, S. M., Lowe, I. P., Bloom, G., Borland, B., and Field, J. B., P.N.A.S. **63,** 918 (1969).
2. Steiner, A. L., Peake, G. T., Uteger, R. D., Karl, I. E., and Kipnis, D. M., Endocrinology **86,** 1354 (1970).
3. Zor, U., Kaneko, T., Schneider, H. P. G., McCann, S. M., and Field, J. B., J. B. C. **245,** 2883 (1970).
4. Bowers, C. Y., Ann. N.Y. Acad. Sci. **185,** 263 (1971).
5. Dannies, P. S., Gautuik, K. M., and Tashjain, A. H., Endocrinology **98,** 1147 (1976).
6. Labrie, F., DeLean, A., Barden, N., Ferland, L., Drown, J., Borgeat, P., Beaulieu, M., and Morin, O., *in* "Current Topics in Molecular Endocrinology" (F. Labrie, J. Meites, and G. Pelletier, eds.), Vol. 3, p. 147. Plenum Press, New York (1976).
7. Sundberg, D. K., Fawcett, C. P., and McCann, S. M., Proc. Soc. Exp. Biol. Med. **151,** 149 (1976).
8. Eto, S., and Fleischer, N., Endocrinology **98,** 114 (1976).
9. Borgeat, P., Labrie, F., Cote, J., Ruel, F., Scally, A. V., Coy, D. H., Coy, E. J., and Yanaihara, N., J. Mol. Cel. Endocrinology **1,** 7 (1974).
10. Scofield, G. J., Nature (London) **215,** 1382 (1967).
11. Hertelendy, F., Todd, H., Peake, G. T., Machlin, L. J., Johnston, G., and Pounds, G., Endocrinology **89,** 1256 (1971).
12. Ratner, A., Life Sci. **9,** 1221 (1970).
13. Wakabayashi, W., and Tomoki, B., Endocrinology **92,** 698 (1973).
14. Fleischer, N., Donald, R. A., and Butcher, R. W., Amer. J. Physiol. **217,** 1287 (1969).
15. Tang, L., and Spies, H. G., Proc. Soc. Exp. Biol. Med. **151,** 189 (1976).
16. MacLeod, R. M., and Lehmeyer, J. E., P.N.A.S. **67,** 1172 (1970).
17. Jutisz, M., and de la Llosa, M. P., Endocrinology **86,** 761 (1970).
18. Fleischer, N., and Vale, W., Endocrinology **83,** 1232 (1968).
19. Reichlin, S., Martin, J. B., Boshans, R. L., Schalach, D. S., Pierce, J. G., and Bollinger, J., Endocrinology **87,** 1022 (1970).
20. Newsome, H. H., and Rose, J. C., J. Clin. Endocrinology **33,** 481 (1971).
21. Gilman, A. G., P.N.S.A. **67,** 305 (1970).
22. Schally, A., and Redding, T., Proc. Soc. Exp. Biol. Med. **126,** 320 (1967).
23. Borgeat, P., Labrie, F., Drouin, J., Belanger, A., Immer, H., Sestanj, K., Nelson, V., Gotz, M., Schally, A., Coy, D., and Coy, E., B.B.R.C. **56,** 1052 (1974).
24. Wilber, J. F., Peake, G. T., and Uteger, R. D., Endocrinology **84,** 758 (1969).
25. Milligan, J. V., Kraicer, J., Fawcett, C. P., and Illner, P., Can. J. Physiol. Pharmacol. **50,** 613 (1972).
26. Peake, G. T., Steiner, A. L., and Daughaday, W. H., Endocrinology **90,** 212 (1972).

Received January 1, 1978. P.S.E.B.M. 1978., Vol. 158.